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Characterisation of Pharmaceutical Grade Horse Chestnut Waste

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Abstract

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Horse chestnut residue was made available by a local pharmaceutical company. The residue was subjected to compositional analysis and studied to determine potential for residual biological activity, including prebiotic, antimicrobial, anti-hyaluronidase and anti-elastase effects.

The residue had 52 % total solids, including 2.97% ash, 6.93% protein, 1.89% crude fat. Oleic acid (74.7%) was the main fatty acid components in the crude lipid component. The major ash components were potassium, phosphorus, calcium and magnesium.

Prebiotic potential of aqueous extract (HCE) from horse chestnut residue was confirmed. A new formula for calculation of prebiotic index (PI) was developed. PI for probiotics (*Lactobacillus* and *Bifidobacterium* species) grown with HCE as main nutrient were determined and compared to PI of the commercial prebiotics FOS, GOS and inulin. The experiments confirmed horse chestnut extract as a potential prebiotic. The *Lactobacillus* and *Bifidobacterium* strains are able to metabolize the extract, the extent of which varied with individual strains. All probiotics grown with the horse chestnut extract exhibited positive prebiotic index (PI). *B. breve* obtained the highest PI (4.64±0.45). While 1% (w/v) crude horse chestnut residue was added to the media, only 0.16% was soluble. Commercial prebiotics (1% (w/v)) were 100% soluble. Results obtained for PI values for individual strains e.g. *L. plantarum* on GOS (1.03), FOS (0.82), inulin (-0.58) and HCE (0.52) would indicate that HCE has significant prebiotic potential at much lower concentrations that commercial prebiotics.

Probiotic strains grown with HCE produced lactic acid and/or acetic acid. For *B. breve* the pH was 5.90±0.02 after 24 hours which correlated to 6.09mM lactic and 20.05mM acetic acid. Similar results were obtained for other strains tested.

A novel microplate assay was developed to allow for high throughput screening for prebiotic potential and the prebiotic activity of lyophilised horse chestnut aqueous extract (lHCE) was tested under different processing treatments (exposure to low pH at ambient and high temperature and Maillard reaction).

The lHCE contained fructose, glucose, sucrose as well as oligosaccharides (determined by HPLC: Cation Exchange Chromatography). A new format of modified Dubois assay was developed for high throughput screening of carbohydrate content in FPLC fractions. Size exclusion chromatography of lHCE was used to establish an oligosaccharide of average molecular mass of 2735 Da.

Additional activities were also detected. The lHCE also had anti-hyaluronidase (IC_{50}= 4.8mg/ml) and anti-elastase (IC_{50}=50mg/ml) activity. Organic extracts from the residue demonstrated significant antimicrobial activity against *E. coli* ATCC 25922, *E. aerogenes* ATCC 13048 and *S. aureus* ATCC 9144. MIC of methanolic extract (MetHCE) was determined as 53mg/ml for *E. coli*.

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Abbreviations

Å	angstrom
AA	acetic acid
AAS	atomic absorption spectrophotometry
ABS	absorbance
AcHCE	acetone extract of horse chestnut residue
AEC	anion exchange chromatography
AO	antioxidant
ARDS	adult respiratory distress syndrome
B[a]P	benzo[a]pyrene
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CA-MRSA	community associated methicillin resistant *Staphylococcus aureus*
CAT	catalase
CBB	coomassie brilliant blue
CD	Crohn's diseases
CD	cyclodextrins
CEC	cation exchange chromatography
ChHCE	chloroform extract of horse chestnut residue
CLA	conjugated linoleic acid
CLSI	Clinical Laboratory Standard Institute
CM	chloroform:methanol
Cmol	molar concentration
CNA	nonadecadienoic acid
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
Cp	percentage concentration
CRP	c-relative protein
CV	column volume
CVI	chronic venous insufficiency
Da	Dalton
DiHCE	dichloromethanolic extract of horse chestnut residue
DMAB	p-dimethylaminobenzaldehyde

DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic
DP	degree of polymerization
DPPH	α,-α-diphenyl-β-picrylhydrazyl
EC_{50}	half maximal effective concentration
EH	ethyl acetate:hexane
EM	escin mixture
EMW	ethyl acetate:methanol:water
EtHCE	ethanolic extract of horse chestnut residue
FAME	fatty acid methyl ester
FC	Folin-Ciocalteau
FOS	fructooligosaccharides
FPLC	fast protein liquid chromatography
FPP	farnesyl pyrophosphate
FRAP	ferric reducing/antioxidant power assay
GC	gas chromatography
GFPP	geranylfarnesyl pyrophosphate
GGPP	geranlgeranyl pyrophosphate
GIT	gastrointestinal tract
GOS	galactooligosaccharides
GPP	geranyl pyrophosphate
GSH	glutathione
GSSG	glutathione disulfide
HA	hyaluronic acid
HA-ase	hyaluronidase
HCE	horse chestnut aqueous extract
HDL-C	high density lipoprotein
HFD	high-fat diet
HPLC	high pressure liquid chromatography
IBS	irritable bowel syndrome
IMO	isomaltooligosaccharides
INT	p-iodonitrotetrazolium violet
IPP	isopentenyl pyrophosphate

IR	infrared spectroscopy
K_{av}	partition coefficient
LA	lactic acid
LAB	lactic acid producing bacteria
LC	liquid chomatography
LDL-C	low density lipoprotein
lHCE	lyophilised horse chestnut aqueous extract
Ln	natural logarithm
LS	lactosucrose
MBC	minimal bactericidal concentration
MetHCE	methanolic extract of horse chestnut residue
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	minimal inhibitory concentration
MM	minimal medium
MPa	megapascal
MRS	deMan, Rogosa and Sharpe medium
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
MTT	3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
MW	molecular weight
NDO	non-digestible oligosaccharides
NMR	nuclear magnetic resonance
OD	optical density
ODMA	o-desmethylangolensis
PAs	proanthocyanidins
PHa	phenolic acids
PI	prebiotic index
PUFA	polyunsaturated fatty acid
RA	rheumatoid arthritis
R_f	retention factor
RF	rheumatoid factor
RI	refractive index
RNS	reactive nitrogen species

ROS	reactive oxygen species
SCFA	short chain fatty acid
SD	standard deviation
SEC	size exclusion chromatography
SMs	secondary metabolites
SOD	super oxide dismutase
SOS	soybean oligosaccharides
TLC	thin layer chromatography
TNF	tumour necrosis factor
TOS	trans-galactooligosaccharides
t_R	retention time
TSB	Tryptic Soy Broth
U	unit
Vc	bed column volume
Ve	elution volume
Vo	void volume
WHO	World Health Organisation
XO	xylooligosaccharides

1 Introduction

1.1 Aesculus hippocastanum

1.1.1 Features of Aesculus hippocastanum

Aesculus hippocastanum is a large, deciduous tree that can grow up to 30 meters (Figure 1.1). The horse chestnut's trunk is erect and columnar, and grows up to 1.2m in diameter. Trees grow rapidly to a significant height with widely spreading branches giving a round crown shape with high branch density, as shown in Figure 1.1.
The first illustration of this species was published in 1563 by Pietro Andrea Mattioli (1501-1578) in his book "New Krëuterbuch". He also included a woodcut illustration and inserted an "excellent" description of the tree (Lack, 2000).

Figure 1.1: Horse Chestnut tree.
Source: (Michels, 2008).

The trunk is covered by dark, exfoliating bark. In young trees the bark is light and smooth, and subsequently becomes darker with rough ridges and irregular scale (Figure 1.2) Small branch points are in the shape of minute horse-shoes. This is probably the characteristic from which the tree gets its name (Grieve, 2009).

Figure 1.2: Horse Chestnut bark.
Source: (Michels, 2008).

The flowers are described as creamy-white with a reddish tinge, 30cm long in upright cluster shape (Figure 1.3a) There are two varieties, red and yellow (Grieve, 2009). Flowering time is in May.

Leaves are of opposite palmate leaf type; green and divided with 5-7 leaflets, 10-20 cm long (Figure 1.3b). There is an obovate leaflet shape. In autumn leaves turn a rusty-yellow colour.

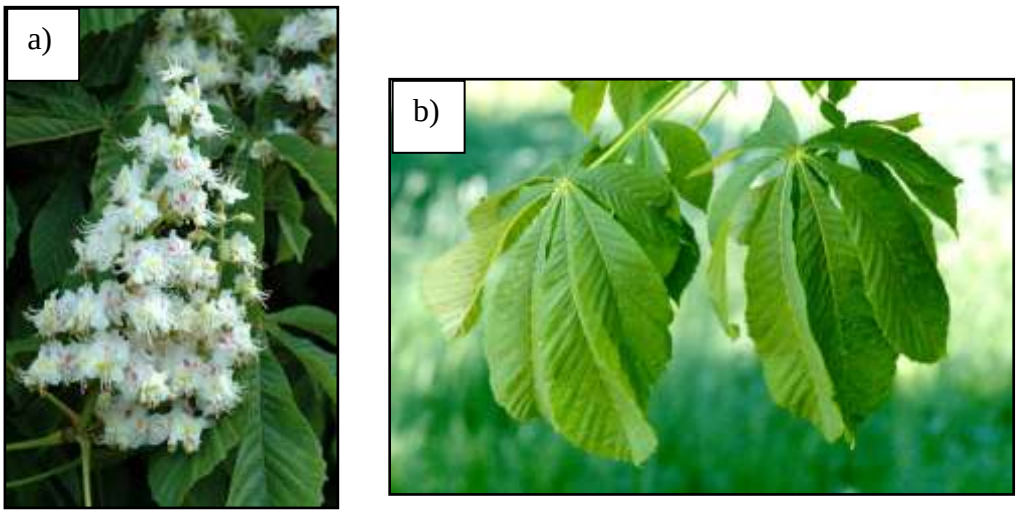


Figure 1.3: Horse Chestnut (a) flowers and (b) leaves.
Source: (Lindsey, 2005).

Fruits (Figure 1.4) are glossy brown nuts in a spiny husk (Figure 1.4b) and typically 2-5 cm in diameter. They are dry and hard, and easy to remove by splitting from the capsule during autumn (Sept.-Oct.). Nuts have white “buck eye” (Figure 1.4a).

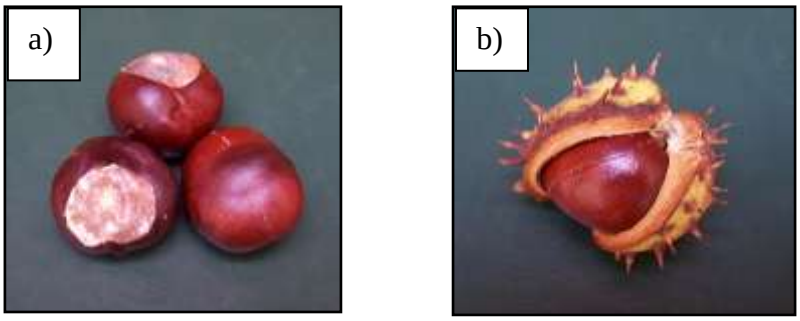


Figure 1.4: Nuts (a) and mature fruit (b) of Horse Chestnut.
Source: (Michels, 2008).

1.1.2 Origin of *Aesculus hippocastanum*

In the past most botanists supposed that horse chestnut tree had its origins in Asia; moreover in 1753 Linnaeus mentioned that this tree grows mostly in the northern regions of Asia. For many years there was acceptance of the Asian origin of *Aesculus hippocastanum* until 1800s when English explorers published a report about the natural habitat of horse chestnut tree being the Pindus Mountains in Greece. This fact was rejected for many years but eventually the Balkan origin of the tree is now generally accepted (Lack, 2000).

The Europe horse chestnut tree originated from Istanbul (Avtzis *et al.*, 2007), Albania, Central and North Greece and the former Yougoslavia (Greuter *et al.*, 1986). Cultivation has spread over the whole of Europe, and the horse chestnut tree is now widely planted in gardens, parks and squares. In some regions of USA and other countries this species is often cultivated (Fralish and Franklin, 2002).

1.1.3 Species of the *Aesculus* genus

The genus of Aesculus comprises 19 species (Table 1.1). Six are native to North America and are referred to as “Buckeyes” whereas in Europe and Asia there are 13 trees known as White Chestnut, and Red Chestnut or more generally “Horse Chestnut”

(Table 1.1). There are also nine hybrids of the *Aesculus* genus (Brown, 2009). Other common names used for *Aesculus hippocastanum* are conker or bongay tree, but apparently this latter species does not correspond to true chestnut; being *Castanea sativia* (Janick and Paull, 2008). Table 1.2 shows a nomenclature of *Aesculus hippocastanum L.*

Table 1.1: Species of *Aesculus* genus

Scientific Names	Common Names
Aesculus arguta	Texas buckeye
Aesculus californica	California buckeye
Aesculus chinensis	Chinese Horse Chestnut
Aesculus chinensis var. wilsonii	Wilson's Horse Chestnut
Aesculus flava (A. octandra)	Yellow buckeye
Aesculus glabra	Ohio buckeye
Aesculus glabra var. arguta	Ohio buckeye
Aesculus glabra var. glabra	Ohio buckeye
Aesculus hippocastanum	Horse chestnut Common
Aesculus indica	Indian Horse Chestnut
Aesculus neglecta	Dwarf Buckeye
Aesculus parviflora	Bottlebrush buckeye
Aesculus pavia	Red buckeye
Aesculus pavia var. flavescens	Red buckeye
Aesculus pavia var. pavia	Red buckeye
Aesculus sylvatica	Painted buckeye
Aesculus turbinata	Japanese Horse Chestnut
Aesculus turbinate	Japanese horse chestnut
Aesculus wangii syn. Aesculus assamica	-

Source: (Brown, 2009).

Table 1.2: Nomenclature of *Aesculus hippocastanum*

Nomenclature position	Latin Name	Ordinary Name
Kingdom	*Plantae*	Plants
Subkingdom	*Tracheobionta*	Vascular plants
Superdivision	*Spermatophyta*	Seed plants
Division	*Magnoliophyta*	Flowering plants
Class	*Magnoliopsida*	Dicotyledons
Subclass	*Rosidae*	...
Order	*Sapindales*	...
Family	*Hippocastanaceae*	Horse-chestnut
Family Genus	*Aesculus L.*	buckeye
Species	*Aesculus hippocastanum L.*	horse chestnut

Source: (Kartesz, 2009).

1.1.4 Compositional analysis of seeds from *Aesculus hippocastanum*

A study has shown that different constituents can be isolated from horse chestnuts. The chemical composition of *Aesculus hippocastanum* seeds is presented in Table 1.3 as conducted on pure species of *Aesculus hippocastanum* with white flowers (Baraldi *et al.*, 2007).

Table 1.3: Chemical composition of *Aesculus hippocastanum* seeds.

Compositional analysis	Aesculus hippocastanum
Moisture	50.80
Total solids	49.20
Proteins	2.64
Lipids	4.13
Glucids (glucose and fructose)	15.20
Ash	2.51
Cold water solubility	53.90
Total inorganic soluble salts	2.18

Source: (Baraldi *et al.*, 2007)
Data expressed as percentage of the dry mass

Horse chestnut seeds consist of a complex mixture of different molecules of which glucids are major components. Protein and lipids are minor constituents of horse chestnuts.

The fatty acid composition has also been reported (Table 1.4). Horse chestnuts contain predominantly oleic acid (43.2%), linoleic acid (35.2%), and others (11.6%).

Table 1.4: Fatty acids composition of *Aesculus hippocastanum* seeds.

Fatty acid	***Aesculus hippocastanum***
Myristic	0.6
Myristoleic	0.2
Pentadecanoic	0.1
Pentadecenoic	0.1
Palmitic	7.1
Palmitoleic	0.7
Heptadecanoic	0.1
Heptadecenoic	0.1
Stearic	0.8
Oleic	43.2
Linoleic	35.2
Linolelaidic	2.2
Linolenic	5.9
Arachidic	0.2
Behenic	0.1
Erucic	2.9

Source: (Baraldi *et al.*, 2007)
Data expressed as percentage

1.1.5 Applications of *Aesculus hippocastanum*

Horse Chestnut nuts have been used as a popular children's game known as Conkers in Britain and Ireland. Nuts can also prevent spider infestation in the home (Edwards, 2009). In Eastern countries nuts are used for feeding animals (mainly young calves, sheep and pigs). Nuts are crushed in cold water and left overnight (soaking process). The mixture can be boiled for 30 minutes and the water decanted. Finally the horse chestnut seeds are dried and partially husked to form meal. Nuts have a bitter taste. The horse chestnut meal is a rich source of starch and in a concentrated animal food product is used in strictly measured doses (Grieve, 2009).

During the World Wars horse chestnuts were used for acetone production. *Clostridium acetobutylicum* as a fermentative organism produced acetone from horse chestnuts' starch. Acetone was used in production of explosives.

In France and Switzerland seeds were used for whitening some fabrics and fulling of cloths. Here, nuts were collected for a soapy juice which was extracted in cold, soft water. The mixture was used for linen washing giving it a blue-sky colour; additionally fabrics were resistant for damages (AbsoluteAstronomy.com, 2010a).

The horse chestnut tree is very resistant to environmental conditions and so is commonly planted in areas, such as streets and parks, by municipal authorities. In Bavaria (Germany) this tree is traditionally used in beer gardens to provide shade. The tree prefers mostly sunny exposure and is widely planted for shade.

1.1.6 Pharmaceutical applications of Horse Chestnut (*Aesculus hippocastanum*)

The horse chestnut has been reported to contain a whole variety of compounds with biological activity. The main bioactive components of horse chestnut seeds are:

1) Terpenes

Oleane-type saponins (13% in the fresh seeds) in which escin as triterpene saponins (3-6%)

2) Flavonoids

<u>flavonols and their glycosides</u>

- quercetin
- kaempherol and their derivatives:
  - astrogalin (kaempherol 3-*O*-glucoside)
  - izoquercetin (quercetin 3-rutinoside)
  - leucocyanin (3,3',4,4',5,7-hexohydroxy flavone)
  - rutin (quercetin 3-rutinoside)
  - tamarixetin (Kapusta *et al.*, 2007, Wilkinson and Brown, 1999).

<u>flavan 3-ols</u>

- epicatechin

<u>condensed tannin</u>

- proanthocyanidin A_2

<u>coumarins</u>:

- esculetin
- fraxin
- scopoletin (Bombarelli and Morazzoni, 1996)

3) Phytosterols

- sistosterol

- stigmasterol
- campesterol

4) Oils
    - oleic acid (65-85%)
    - linoleic
    - linolenic
    - palmitic
    - stearic (Wilkinson and Brown, 1999)
5) Sugars
    - starch (30-60%)
    - cellulose (3%)
    - sucrose (12%)
    - glucose (5-6%)
6) Proteins (10%)

    aminoacids (adenosine, adenine, guanine)
7) Mineral salts: Mg, Ca, Cu, P, Mn, Na, Cl, S (Kapoor *et al.*, 2009, Burlando *et al.*, 2010, McLellan, 2000)
8) Other
    - allantoin
    - choline
    - citric acid
    - uric acid (Brown, 2003)

From the early 18th century, therapeutic properties of the horse chestnut have been reported. In Turkish folk medicine crushed horse chestnut seeds were used for preparing a tea which helped passing kidney stones, relieved stomach ache and swallowed whole horse chestnuts treated symptoms of hemorrhoids (Küçükkurt *et al.*, 2010). Antipyretic and anti-haemorrhoidal, anti-edematous, anti-inflammatory, venotonic properties due to horse chestnut extract were also reported (Sirtori, 2001). Almost every part of this tree contains bio-active components which can be applied for treating different disorders. Horse chestnut extracts have one of the highest "active-oxygen" scavenging abilities of 65 different plant extracts examined (Pathak, 2009) and have more antioxidant activity than Vitamin E (Kapoor *et al.*, 2009).

Seeds have been harvested for extracting many active ingredients. Chief among these is escin glycosides. Escin glycosides (Figure 1.5) typically exist as a saponin mixture (triterpene glycosides) and their presence in horse chestnuts is estimated at less than 3% (ESCOP, 2003). Extracted from horse chestnuts, escin exists in 3 forms: Beta-escin (barely soluble in water), Alpha-escin (fairly water soluble; O'Neil *et al.*, 2006), Krypto-escin (very soluble in water; Patri *et al.*, 2006). These three forms of escin are distinguished by melting point, optical rotation, hemolytic index and as mentioned above, solubility in water Sirtori, 2001). Beta-escin is a major active component and consists a mixture of more than 30 different glycosides derived from protoescigenin and barringtogenol C (ESCOP, 2003). Alpha-escin contains the same glycosides, additionally other triterpene aglycones, escigenin and barringtogenol D, are also present (Patri *et al.*, 2006).

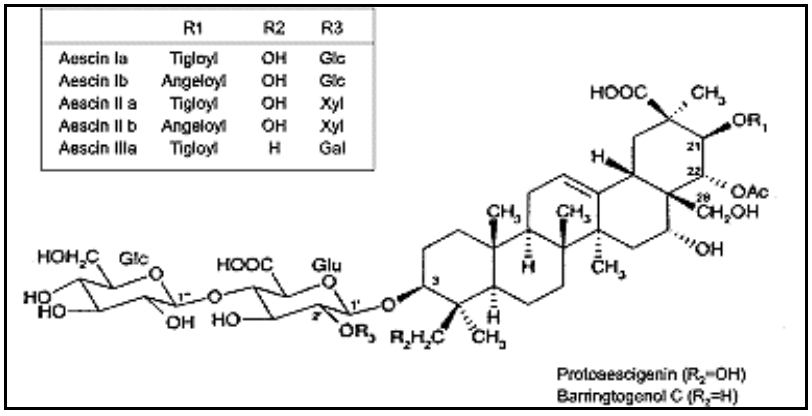


Figure 1.5: Escin molecule.
Source: (Loew *et al.*, 2000).

Escin preparations have widely used pharmaceutical properties. It is an important active component used for treatment of disorders such as chronic venous insufficiency (CVI), cancer, obesity, haemorrhoids and skin problems.

1.1.6.1 Treatment of chronic venous insufficiency

Chronic venous insufficiency (CVI) is a significant condition, affecting 20-25% of women and 10-15% of men (Piechal *et al.*, 2005). This condition typically affects the legs. In this case leg veins become unable to effectively support the flow of blood

(Topol and Califf, 2007), which is driven by residual arterial pressure, skeletal muscle action, elasticity of the vein wall and the presence of valves within the main veins. In CVI, blood may pool in veins leading to poor venous return, destruction of valve structures, increased risk of clot formation and compromised lymph drainage.

Risk factors for CVI include obesity, smoking, prolonged sitting or standing on a regular basis, pregnancy, gender, age and general phenotype (Ballard and Bergan, 2000). People suffering from CVI report painful, heavy and aching legs which tire easily. Potential complications of CVI include deep vein thrombosis (Topol and Califf, 2007) and leg ulcers (Meissner *et al.*, 2007). Clinical trials have shown that horse chestnut-derived escin has a potential therapeutic effect on CVI, with anti-oedematous, venotonic and anti-inflammatory actions (Sirtori, 2001).

a) Anti-oedematous activity of escin

The anti-oedematous effect of escin has been indicated experimentally. Escin inhibits an increase in vascular permeability in rodent paws induced by different agents (acetic acid, carrageenin; Matsuda *et al.*, 1997). Escin prevents fluid exudation into tissue which inhibits swelling. There are two mechanisms by which horse chestnut extract may prevent swelling. One is related to the ability of escin to inhibit of elastase and hyaluronidase. These two enzymes are involved in degradation of important components of capillary endothelium. Prevention by escin of leakage of capillary walls also prevents swelling of tissue (Sirtori, 2001). The second mechanism of escin action is through sensitization of smooth muscle to Ca^{2+} ions and in consequence small capillary vessels are protected from excessive permeability to water (Piechal *et al.*, 2005).

b) Anti-inflammatory effect of escin

The anti-histamine and anti-serotonin activity of escin has also been observed. Oral intake of this horse chestnut glycoside can lead to decreases in histamine and serotonin levels which reduces itch, a symptom of inflammation (Matsuda *et al.*, 1997). Escin effectively decreases up to 50% the number of leukocytes (Piechal *et al.*, 2005), has suppressive effect on leukocyte migration in the pleural cavity and also reduces neutrophil adhesion (Sirtori, 2001).

c) Venotonic properties of escin

Studies appear to confirm that escin has a venotonic activity on the human saphenous vein. Following escin treatment, Annoni *et al*. (1979) reported that veins exhibited an increase in venous tone in a similar manner to serotonin and dihydroergotamine treatment, and with an even greater response than acetocholine and vasopressin. The effect of escin was constant and stable for the same duration (Annoni *et al.*, 1979). Other experiments on animal veins have also confirmed venotonic properties of escin, for example dog veins which were exposed to escin had significantly greater venous tone (Sirtori, 2001).

1.1.6.2 Antiproliferative and anti-cancer properties of escin

Escin may also have potential applications in cancer treatment. Administration of β-escin sodium in vitro causes inhibition of the growth of tumour cell lines (such as in human KB cells, mice H22 liver cancer cells and S180 mice sarcoma cell line; (Guo *et al.*, 2003). Beta-escin has an antiproliferative effect on human chronic myeloid leukaemia cells. In this case β-escin may be good candidate to prevent leukaemia diseases (Niu *et al.*, 2008). Many clinical studies have shown the influence of β-escin on treatment of cancer therefore in the Western World this product has been investigated as a anti-cancer agent (Efferth *et al.*, 2007).

1.1.6.3 Anti-angiogenic activity of escin

The efficacy of escin on suppression of angiogenesis has also been reported. This process is probably related to the CVI application (Yang *et al.*, 2006). Angiogenesis is a process of formation of new capillaries from pre-existing vessels, and naturally occurs during the remodeling of organs. In pathological situations, angiogenesis can support cancer development (Harper and Moses, 2006). Sodium escin inhibits endothelial cell proliferation and protects against migration and apoptosis of human vein endothelial cells (Wang *et al.*, 2008).

1.1.6.4 Antioxidant properties of escin

The antioxidant effect of escin mixture (EM) from *Aesculus hippocastanum* has been investigated in mice model (Küçükkurt *et al.*, 2010). Animals fed high-fat diets (HFD) experience significantly greater oxidative stress. HFD induced dyslipidemia can directly influence the development of diseases such as cardiovascular disease, obesity, cholestasis (Küçükkurt *et al.*, 2010). Lipid peroxidation (example of oxidative stress) can occur as a result of HFD. Many natural antioxidants are induced in response to oxidative stress e.g. super oxide dismutase (SOD), catalase (CAT; Baraldi *et al.*) and glutathione (GSH; Carmiel-Haggai *et al.*, 2005). GSH is a major cellular antioxidant and its level decreases in liver, kidney and heart during HFD because GSH is converted to its oxidative form GSSG (glutathione disulfide) after reaction with oxygen species Therefore the amount of GSH is subsequently reduced. Free radicals are also reduced by SOD and CAT. Treatment with escin mixture (EM) obtained from horse chestnuts restores normal antioxidant activity by increasing GSH levels in liver, kidney and blood. In mice fed on high fat diets, EM also inhibits increasing lipid peroxidation in liver induced by this diet (Küçükkurt *et al.*, 2010).

1.1.6.5 Obesity treatment

Escin may prevent obesity. An experiment on mice showed that escin decreases the level of some hormones (leptin, FT_4) whose significantly high levels were a result of a high fat diet. Other biochemical parameters (HDL-C and LDL-C) have also improved after administration of an escin-rich diet (Avci *et al.*, 2010).

Matsuda and co-workers investigated the effects of an escin fraction on the gastrointestinal tract (GIT) in an *in vivo* model. The study showed that escin has an inhibitory effect on gastric emptying in mice (Matsuda *et al.*, 1999) and observed that the gastric emptying process increases in diabetic patients and animals. This abnormality can lead to rapid absorption of food in the gastrointestinal tract and in consequence a rapid increase in blood glucose (Chang *et al.*, 1996). Escin can have a beneficial effect in prevention and treatment of diabetes and obesity with accelerated gastric emptying.

1.1.6.6 Treatment of haemorrhoids

Haemorrhoids are related to the dysfunction of blood vessels present in anal canal. Symptoms of haemorrhoids are pain, swelling, irritation, bleeding from the anus. Many people suffer from this significant condition. Therapeutic effect of escin has been observed in patients suffering with haemorrhoid symptoms. An escin product increases the resistance of the veins that supply the anal canal. Endoscopic examination in patients treated with escin showed significant improvement with reduced bleeding and swelling (Sirtori, 2001).

1.1.6.7 Treatment of skin problems

Extracts of horse chestnut seeds are found in many cosmetic products (creams, lotions, massage oils) for treatment of swelling and water retention in legs (Dinsdale, 2000). Many cosmetic products which contain escin can relieve symptoms of CVI (swelling, pain, itching and heaviness of legs) and also treat local oedema and haematoma (EMEA, 2009). Another property of escin relates to the treatment and prevention of cellulite (Oleszek and Marston, 2000). It has been reported that escin used in ointment significantly improved the tissue microcirculation of adipose and skin tissue, increasing the flow of blood in skin capillaries and decreasing oedema (McKenna *et al.*, 2002).

Extract of horse chestnut (*Aesculus hippocastanum*) is widely used in skin care products as an anti-aging factor (Kapoor *et al.*, 2009). The claimed benefits of horse chestnut skin products include improvement of blood flow in skin capillaries, prevention of leaking of fluid from capillary beds into surrounding tissue (preventing oedema), inhibition of enzymes responsible for degradation of skin structures and an anti-inflammation effect. These activities in horse chestnut extracts are claimed to make skin healthier and younger looking (Dermaxime, 2010). These extracts are also rich in a number of flavonoids. Powdered horse chestnut contain about 0.88% of flavonoids in dry mass (Kapusta *et al.*, 2007). Among the flavonoids, quercetin and kaempferol are major seed components and with proanthocyanidin A_2 are strong anti-oxidants, which may have a protective effect on blood vessels (Wilkinson and Brown, 1999).

1.1.6.8 Antimicrobial, worm control and insect repellent effects

Powdered horse chestnut seeds can also be used as an anti-worm formula and insect repellent. Guarrera showed that powdered horse chestnuts macerated in water and added to flowerpots can eliminate insects and worms (Guarrera, 1999). Antimicrobial properties of horse chestnut have been also reported. The horse chestnut protein Ah-AMP1 belongs to the subfamily A_2 of plant defensins. The molecular structure has been established by NMR techniques (Fant *et al.*, 1999). This horse chestnut plant component, defensin, inhibits infection by fungal species and represents one of the peptide classes with antimicrobial properties (Broekaert *et al.*, 1995). Their activity may be useful as a natural product for treatment of microbial infection (Baraldi *et al.*, 2007).

1.1.7 Horse Chestnut bark

The bark of the horse chestnut contains quercetin which has venotonic proprieties (Patri *et al.*, 2006). Esculin and fraxin, also bioactive components, are coumarin glycosides. Esculin, whose presence was established as 0.7-4% (w/w) has a natural UV-B protective property and is also one of the plant-derived constituents widely used in the treatment of various peripheral vascular disorders (Stanic *et al.*, 1999). Antiflogistic, cytostatic and antimutagenic properties of esculin have been found in recent years, and so esculin is also used in cosmetic products as an agent to ameliorate ageing skin (Wilkinson and Brown, 1999). Spasmolytic and diuretic activity of bark extract are due to the glycoside fraxin (Stanic *et al.*, 1999).

1.1.8 Horse Chestnut leaves

Leaves of horse chestnut can be used as a biomonitors of metal pollution in urban areas (Tomaševič *et al.*, 2008). Metals such as Pb, Cu, Zn and Cd were determined using leaves from the same leaf level. The Tomasevic study has shown increasing concentration of harmful trace metal in leaves due to metal pollution in air. It also observed that young and old leaves had visible injuries due to this pollution.

1.2 Probiotic

1.2.1 Ecosystem of the gastro-intestinal tract

The gastrointestinal tract (GIT) is a tube about 8 metres long consisting of the mouth, oesophagus, stomach, small intestine, large intestine (also called the colon), rectum and anus (Figure 1.6). There are also other important accessories which play a role in the digestive process; salivary glands in the mouth, the liver, gallbladder and pancreas. The major role of the GIT is digestion and absorption of nutrients derived from foods. During this process, enzymatic digestion occurs in the stomach and small intestine, while absorption and assimilation of nutrients takes place in the small and large intestine. The remaining undigested part of the food is pushed through the colon where it becomes faeces and is removed through the anus.

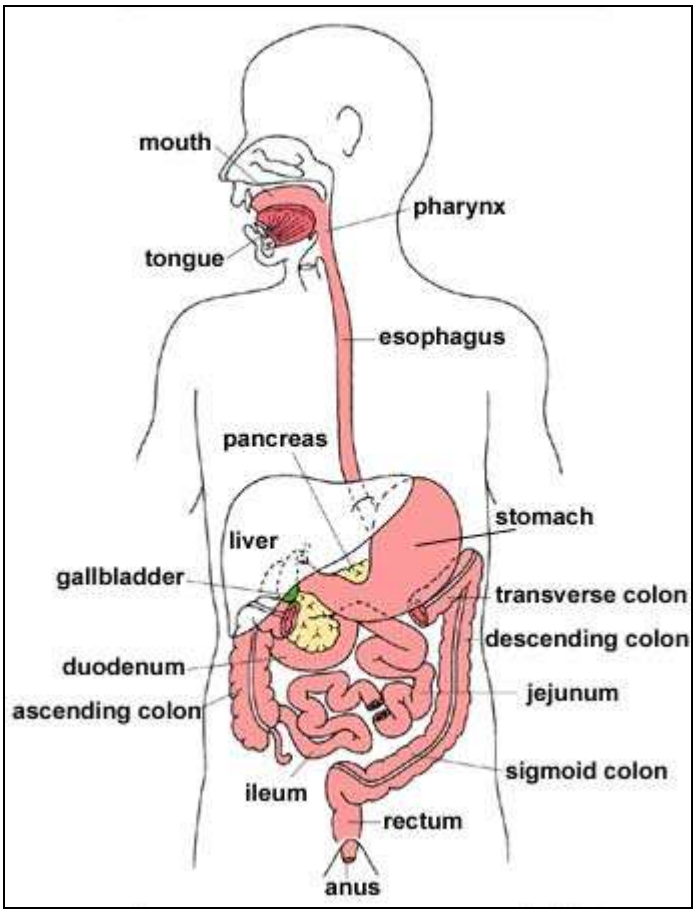


Figure 1.6: Digestive system in human.
Source: (UMMC, 2008).

The lining of the GIT is coated in mucus which plays an important role in moving digested food through the gut as well as protecting from pathogens and digestive enzymes. Mucus is produced by epithelial cells. Intestinal epithelium is a physical barrier between the lumen of the intestine and the rest of the body. The mucosal surface of epithelium has a few important properties. It provides a barrier against luminal pathogens (microbial and worms) and epithelium cells produce immune molecules in response to pathogen infections. The mucus also provides an area for adhesion of microbial commensals in the gut.

1.2.2 Microflora of the GIT

Hundreds (400-500) different species of microorganisms have been identified in the human colon. They total approximately 10^{14} cfu which is highest record for any microbial habitat (Farnworth, 2008). Figure 1.7 shows the biodiversity of microorganisms present in the GIT in healthy humans. The colon in new born babies is germ-free and colonization occurs after birth. Bacteria present in birth canal (vagina) are the first microbes which colonize the baby. There are several factors that directly influence microbial colonization:

- type of delivery (caesarean or natural)
- type of feeding
- antibiotic intake
- environment/sanitary conditions (O'Toole and Claesson, 2010)

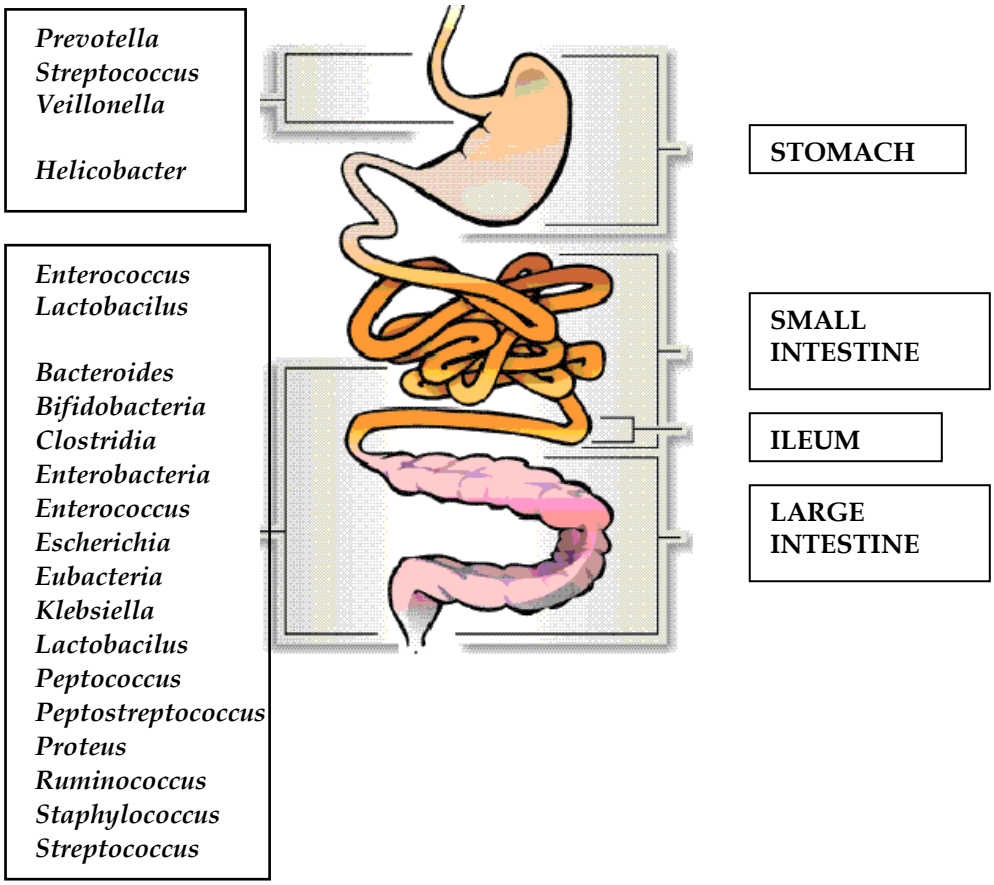


Figure 1.7: Nonpathogenic microorganisms in healthy human body.
Source: (Kiani, 2009).

While the gastrointestinal tract (GIT) contains both facultative anaerobic and obligate anaerobic microorganisms, most intestinal strains are strictly anaerobic. Table 1.5 presents the anaerobic intestinal strains found in a normal healthy intestine:

Table 1.5: Anaerobic bacteria in the human gut.

Microbal genera	**Number of cells (g dry wt $^{-1}$)**
Bacteroides	9.2-13.5
Eubacteria	5.0-13.3
Bifidobacteria	4.9-13.4
Clostridia	3.3-13.1
Lactobacilli	3.6-12.5
Ruminococci	4.6-12.8
Peptostreptococci	3.8-12.6
Peptococci	5.1-12.9
Streptococci (anaerobic)	7.0-12.3
Methanobrevibacter	7.0-10.3
Desulfovibrios	5.2-10.9

Source: (Mussatto and Mancilha, 2007)

The numbers and diversity of bacterial species can be very variable and depend on the age, genetic background and biological condition of the host of all the intestinal bacteria, the genera bifidobacteria and lactobacilli are the best studied because of their beneficial effects on human health (Bielecka *et al.*, 2002). *Bifidobacterium* and *Lactobacillus* genera may be unique for each human host in terms their genetic diversity (Holzapfel and Schillinger, 2002).

1.2.3 Definition of probiotic

Probiotic - (from Greek-for life), refers to a microorganism that confers beneficial effects on the host organism. This term was first introduced in 1954 by Vergio. In his manuscript "Anti- und Probiotica" he described the effects of antibiotics and other substances with antimicrobial properties on intestinal microbes (Holzapfel and Schillinger, 2002). The term "probiotic" is still largely used in human nutrition. Dr. Elie Metchnikoff (1845-1916), Russian microbiologist and Nobel Prize Winner made a significant contribution to the development of research on probiotics. In 1907 he observed that Bulgarian peasants who regularly drink soured milk live much longer than other Europeans (even over 100 years old) and enjoyed good health. After this discovery, he named the fermented milk drink Bulgarian yogurt and also named the strain which causes souring of milk- *Lactobacillus bulgaricum* (Farnworth, 2008). Metchnifoff published his observations in his book "The Prolongation of Life".

1.2.4 Probiotic genera

Currently probiotic bacterial strains contain the genera: *Lactobacillus, Bifidobacterium, Streptococcus, Enterococcus, Lactococcus* (Liong, 2008), all of which are lactic acid producing bacteria (LAB). In addition the yeast *Saccharomyces bouldardii* has been used as a probiotic for treating diarrhoea caused by *Clostridium difficile* (Chow, 2002).
The criteria for selection of probiotics are as follows:

- They should be resistant to the low pH in the gastrointestinal tract and to be able to survive and grow in acidic environments, as well as tolerate high salt concentration derived from bile;

- They must be able to survive in the presence of the different digestive enzymes produced by the stomach and pancreas;
- They should easily adhere to intestinal surfaces and consequently colonize them (Kaplan and Hutkins, 2000).

1.2.4.1 Genera of Lactobacilli

Lactobacillus species (Figure 1.8) are gram positive, non-spore forming strains widely found in the human oral cavity, vagina and intestine. The ability to carry out fermentation processes is a characteristic attribute of this species. Fermentation refers to the metabolic process in which carbohydrates (or related products) are oxidized releasing energy in the absence of aerobic electron acceptors (Jay, 1996). Most species of *Lactobacillus* are homofermentative organisms (*L. delebruecki, L. acidophilus, L. casei, L. plantarum, L. salivarius*) and are able to produce more than 85% lactic acid whereas in heretofermentative forms (*L. brevis*) bacteria generate lactic acid, CO_2 and ethanol/acetic acid (Wood and Holzapfel, 1995). This pattern of division of lactobacilli is based on their ability to metabolise glucose. Homofermentants can extract twice as much energy from glucose as heterofermentants (Jay, 1996). These non-pathogenic bacteria are widely used in the food industry (cheese, yogurt, pickles as well as being involved in silage production). *Lactobacillus* species are also spoilage organisms and can cause spoilage of meat (Dart, 1996).

Lactobacillus species grow at very low pH (from lactic acid production) and are anaerobic. However they may grow in the presence of oxygen, so they are called aerotolerant anaerobes (Madigan *et al.*, 2000).

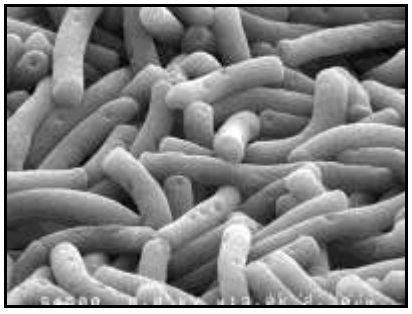


Figure 1.8: *Lactoctobacillus sp*.
Source: (Danone).

In humans *Lactobacillus* species are located along the length of the GIT but especially in the small intestine. Table 1.6 presents *Lactobacillus* species. All these strains belong to the LAB group.

Table 1.6: Human probiotic bacteria of *Lactobacillus* genera.

Lactobacillus probiotic spiecies
Lactobacillus acidophilus
Lactobacillus casei
Lactobacillus crispatus
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus paracasei
Lactobacillus reuteri
Lactobacillus rhamnosus
Lactobacillus delbruecki
L.actobacillus plantarum
Lactobacillus salivarius
Lactobacillus GG

Source: (Chow, 2002, Holzapfel and Schillinger, 2002).

1.2.4.2 Genera of Bifidobacteria

Bifidobacterium species (Figure 1.9) are gram-positive, non-gas producing anaerobic bacteria. They are predominantly found in the large intestine. Similar to *Lactobacillus* this bacterial genus produces lactic acid (Jay, 1996) . They degrade hexoses in a specific a manner called *bifidus-shunt* (Tannock, 2005). *Bifidobacterium* are present in human faeces, the vagina and in dental caries.

Figure 1.9: *Bifidobacterium sp.*
Source: (Danone).

Bifidobacteria are generally strictly anaerobic but some strains are able to grow in the presence of oxygen (Salminen *et al.*, 2004). The major *Bifidobacterium* species are presented in Table 1.7.

Table 1.7: Human probiotic bacteria of *Bifidobacterium* genera.

Bifidobacterium probiotic species
Bifidobacterium adolescentis
Bifidobacterium animalis
Bifidobacterium bifidum
Bifidobacterium breve
Bifidobacterium infantis
Bifidobacterium lactis
Bifidobacterium longum

Source: (Holzapfel and Schillinger, 2002).

1.2.5 Industrial use of probiotics

Probiotic lactic acid producing bacteria (LAB) are widely used in production of milk products e.g. cheese and yogurt. *Lactobacillus bulgaricum* and *Streptococcus thermophilus* are used as starter cultures in yogurt production (Kiani, 2009). LAB are also important organisms for production of exopolysaccharides (thickening agents) and because of their autolytic activity, they are used for enhancing the flavour of cheese (Koch *et al.*, 2008). For example *Lactobacillus delbruckii ssp*. produces enzymes (peptidases and proteinases) which are secreted to digest casein (milk protein), thus improving cheese flavour (Koch *et al.*, 2008).

1.2.6 Effect of probiotics on human health

Probiotic organisms are also widely used for prevention and treatment of different pathological conditions.

1.2.6.1 Prevention of colon cancer

There are many factors which might lead to the development of cancer. Among these factors spontaneous or induced genetic mutation of DNA cells is mostly likely. Mutagenic agents can be divided into two groups those: derived from outside the body and those occurring inside the body. There are a number of natural antimutagenic

substances which inhibit or prevent mutation (Pei-Ren *et al.*, 2002). Some intestinal *Bifidobacterium* strains have antimutagenic properties against pyrene derivatives. Benzo[a]pyrene- B[a]P is considered a mutagenic/carcinogenic compound that originates from food preparation using treatment high temperatures. Specific *Bifidobacterium* species (especially *B. adolescentis, B. bifidum, B. breve, B. infantis, B. lactis, B. longum*) show positive inhibition (48-87% of inhibition) of B[a]P, what suggests that these strains have strong antimutagenic activity (Pei-Ren *et al.*, 2002).

Some intestinal bacteria can produce different anti-carcinogenic agents. Probiotic bacteria produce short chain fatty acids (SCFA), such as butyrate, by fermentation. That, in turn, decreases the pH in the colon. An acidic environment inhibits the growth of *Escherichia coli* and *Clostridium* species and significantly improves bowel functions (Liong, 2008). Butyric acid produced mostly by *Lactobacillus* and *Bifidobacterium* as 5% of total short chain fatty acids is considered to be a major contributor to the reduction of cell proliferation in the colon and in clinical trials it has been used as a treatment for ulcerative colitis (Liong, 2008). Some genera of bacteria, *Lactobacillus acidophilus* and *Bifidobacterium longum* for example, can inhibit development of tumours induced by carcinogens produced by other intestinal microbes (*Streptococcus bovis, Bacteroides, Clostridium, Helicobacter pylori*). The properties of prebiotic strains have been shown to depend on dietary components (Davis and Milner, 2009).

1.2.6.2 Antimicrobial activity

Antimicrobial properties appear to be due to the production of a variety of compounds such as nitric oxide, hydrogen peroxide and short chain fatty acids. Some probiotic strains produce bacteriocins which are antimicrobial peptides (O'Flaherty and Klaenhammer, 2010). Bacteriocins inhibit the growth of pathogenic and undesirable intestinal bacteria such as *Listeria monocytogenes*. In an experiment using mice as subjects, the addition of *Lactobacillus salivarius* had a significant beneficial effect on prevention of disease when the mice were exposed to foodborne *Listeria monocytogenes* (Corr *et al.*, 2007). In the case of infection by pathogenic *Salmonella*, intake of probiotic *Lactobacillus/Pedicoccus* mixture significantly reduces the symptoms of salmonellosis. Moreover, it has been observed that probiotic strains more

easily adhere to the epithelial cells in the intestine than pathogenic strains (Ross *et al.*, 2010).

1.2.6.3 Treatment of diarrhoea

Diarrhoea is the condition of increased frequency and liquidity of stools (3 or more). This disorder can be caused by bacteria or rotavirus infections. Probiotic strains help to prevent diarrhoea occurring by inhibition of adhesion and proliferation of enteropathogenic bacteria (*Salmonella, Shigella, E. coli, Vibrio, Cholereae*). Production of organic acids and bacteriocins by probiotic strains also significantly decreases the number of these pathogenic microorganisms (Vrese and Marteau, 2007).

1.2.6.4 Positive effect on intestinal tight junction

Tight junctions are composed of protein complexes (major claudins and occludins) which connect membranes of two cells together. Epithelial tight junctions hold epithelial cells together and are responsible for selective transport (passive and active) of different molecules and ions through the space between cells. Disability of intestinal tight junction caused by injury has an impact on the development of different bowel diseases such as Crohn's disease and irritable bowel syndrome (IBS). Breaks in tight junctions and disintegration of their protein complexes led to "epithelial leaks" and apoptosis of cells. These processes lead to intestinal inflammation (Schulzke *et al.*, 2009). Probiotic bacteria have a positive effect on the epithelial barrier by increasing transepithelial resistance (O'Flaherty and Klaenhammer, 2010). The integrity of tight junctions in epithelial cells can be compromised by phosphorylation of proteins caused by enzymes from *E. coli* (Resta-Lenert and Barrett, 2003). In vitro studies have shown that the presence of *Lactobacillus acidophilus* significantly reduces this phosphorylation.

It has been proved that epithelial cells of the intestine are able to work in a similar way to immune cells during nematode infection. They cause expression and distribution of IL-4/Il-13/IL-25 receptors and expression of STAT6-dependent genes that play significant roles in the treatment and prevention of diseases such as diabetes and inflammatory bowel disease (Shea-Donohue *et al.*, 2010).

1.2.6.5 Protection against pathogen infection

Competitive exclusion is a natural process that occurs in the gut. Probiotic anaerobic bacteria present in the gut compete for nutrients with pathogenic microflora, limiting their concentration. The ability of beneficial bacteria to produce antimicrobial substances and to block specific adhesion sites are some of the main ways used for protection of host organism from pathogen invasion (Chow, 2002). These processes allow the maintenance of gut homeostasis (O'Flaherty and Klaenhammer, 2010).

Competitive exclusion was first described in the 1970s in an *in vivo* model where mixtures of human intestinal bacteria were administered to newly hatched chicks. The result was a significant increase in the resistance of the chicks to pathogenic *Salmonella* strains (Chow, 2002). The presence of *Lactobacillus* and *Bifidobacterium* species and their adhesion onto the mucus layer of the intestine has been shown to be very important in prevention of enteropathogenic infection caused by *Escherichia coli, Yersina pseudotuberculosis* and S*almonella typhimurium* (Bernet *et al.*, 1993).

1.2.6.6 Nutrient production

Beneficial intestinal bacteria produce vitamins from the B group (B1,B2,B6,B12) as well as nicotinic and folic acids (Mussatto and Mancilha, 2007). It has been found that strains such as *Bifidobacterium* sps. (*B. longum, B. infantis, B. breve*), *Lactobacillus* sps. (*L. acidophilus, L. delbrueckii subsp. bulgaricus, L. plantarum)* and *Lactococcus lactis subsp. lactis* are producers of folate (salts of folic acid) in an *in vivo* model. A large number of bifidobacterial species produce vitamin B12; they are: *B. adolescentis, B. breve, B. bifidum, B. infantis, B. longum* (Tamime, 2005). Vitamin K is also produced by LAB strains (Ross *et al.*, 2010). The species from the LAB genera: *Lactobacillus, Bifidobaterium, Lactococcus, Streptococcus, Enterococcus* are able to produce menaquinone which is one of the two forms of vitamin K (Tamime, 2005).

1.2.6.7 Stimulation of absorption of minerals

Probiotic activity is also related to the absorption of several different minerals (calcium, iron and magnesium). Bacteria that produce of SCFA during fermentation contribute to

acidification in the colon. Minerals can form mineral salts in reaction with SCFA e.g. calcium and magnesium salts. In this way the osmotic balance in the gut is altered and minerals can be absorbed easily. There is also the influence of non-digestible oligosaccharides (NDO) on mineral absorption. By binding to NDO, minerals are transported to the colon where they are released from NDO and absorbed (Mussatto and Mancilha, 2007). The role of the NDO will be described in another part of this thesis (section 1.3).

1.2.6.8 Production of conjugated linoleic acid

Conjugated linoleic acid (CLA) is one of the isomers of linoleic acid. There are 28 different CLA isomers. Natural CLA consists of 9-cis,11-trans-octadecadienoic acid which is found in ruminant animals (Sebedio *et al.*, 2003), and the 10-trans,12-cis isomer. These are the most studied molecules in terms of their biological activity (Ross *et al.*, 2010). Microbial production of CLA is related to the conversion of linoleic acid by polyunsaturated fatty acid (PUFA) which is produced by intestinal bacteria such as *Butyriovibrio fibrisolvens*. This species is a normal inhabitant of ruminants but also is found in human faeces (Hobson and Stewart, 1997). A number of other intestinal bacteria also produce CLA namely, lactobacilli, bifidobacteria, propionibacteria, pedicocci, enterococci, streptococci and lactococci (Ross *et al.*, 2010). Among the bifidobacteria genera, *Bifidobacterium breve* and *Bifidobacterium dentum* are the most effective CLA producers where up to 65% of linoleic acid added into growing medium is converted into 9-cis,11-trans CLA (Coakley *et al.*, 2003).

CLA has useful biological activities. It has been used for treating rheumatoid arthritis (RA) in humans. Intake of CLA significantly reduces pain and morning stiffness, while other factors which are characteristic of clinical manifestation of RA are improved, e.g. modulation of white cells, C-relative protein (CRP) and rheumatoid factor-RF (Aryaeian *et al.*, 2009). Thus CLA has anti-inflammatory activity. It reduces the expression of different inflammatory mediators (TNF and cyclooxygenase COX-2) which are found in those suffering from RA (Butz *et al.*, 2007).

Other important beneficial effects of CLA include enhancement of calcium absorption and thereby improving bone formation (Hur and Park, 2007), decreasing fat mass associated with obesity (Hur *et al.*, 2009) and a derivative of CLA called

nonadecadienoic acid (CNA) has been shown to have a greater effect on reduction of body fat than CLA (Park, 2009).

1.2.6.9 Production of equol

Gut microbes are able to produce equol and O-desmethylangolensin (ODMA) from daidzein which is a phytoestrogen. These two metabolites have stronger estrogenic activity than daidzein (Wildman, 2007). Equol is a non-steroidal estrogen and may have beneficial effects in inhibiting of some kinds of cancer. An investigation by Akaza and co-workers has demonstrated that the ability to degrade daidzein into equol may lead to the suppression of prostate cancer (Akaza *et al.*, 2004). Daidzein is an isoflavone (phytoestrogen) which is particularly rich in soyabeans. A diet rich in soya may be useful in preventing this particular male disorder. Equol and ODMA produced by intestinal bacteria, are secreted in urine and their concentration in urine can be used as a marker of bacterial profiles. About 30-50% of bacteria are able to produce equol, whereas 80-90% are capable of production of ODMA (Frankenfeld *et al.*, 2005).

1.2.6.10 Modulation of mucosal immune responses

The human intestine is the largest "immune organ". In the small intestine about 80% of all cells produce immunoglobulin (Holzapfel and Schillinger, 2002). This "organ" is also very well adapted to the presence of beneficial bacteria. In germ-free mice, intestinal immune function is not developed properly: Peyers layer, a region of intestinal epithelium in these mice, is hypoplastic with reduced number of IgA and CD4+T cells. Also other immune organs had poor structure. Spleen and lymph B and T-cells remained insufficiently formed (Macpherson and Harris, 2004). It has been found that introduction of probiotic bacteria into germ-free mice had a beneficial effect on immune system. All organs responsible for immunity typically showed enhanced activity after probiotic intake for few weeks (Mueller and Macpherson, 2006). The presence of probiotic intestinal bacteria also has an important effect on maintenance of the appropriate nutrition of mucosa (Holzapfel and Schillinger, 2002).

1.2.6.11 Prevention of allergies

Allergies are a disorder of the immune system's response to different agents called allergens. Allergens can activate many immune response pathways e.g. TH immune response (called T-helper response). T-helper cells are group lymphocytes which are responsible for maximizing the capabilities of the immune system (Folkerts *et al.*, 2000). One of the postulated roles of probiotic in the treatment of allergies is the reduction of TH2 cytokine responses (Morais and Jacob, 2006). It has been found that some probiotic strains, such as *Lactobacillus GG*, have a positive effect on the prevention of atopic disease in children with high risk of atopic eczema (Kalliomäki *et al.*, 2001). A diet rich in probiotics during pregnancy reduces atopic eczema in infants.

1.2.6.12 Reducing lactose intolerance symptoms

Lactose is a disaccharide naturally present in milk. Normally lactose is digested by the enzyme lactase produced in small intestine. This converts lactose to two simple sugars (glucose and galactose). Lactose intolerance occurs where intestinal cells do not produce sufficient amount of lactase enzyme (Duyff, 2006). Probiotic bacteria are, however, able to ferment lactose and in this way some symptoms of lactose intolerance (nausea, diarrhoea, bloating, cramping) can be relieved. For example some *Lactobacillus* strains contain the enzyme β-galactosidase or lactase which helps digest lactose derived from food in the GIT (Levri *et al.*, 2005).

1.3 Prebiotic

1.3.1 Term prebiotic

The term of prebiotic according to Gibson and Roberfroid refers to "food ingredients that beneficially effect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health" (Gibson and Roberfroid, 1995). In other words, dietary prebiotics (such as non-digestible oligosaccharides – NDO) pass through the gastrointestinal tract (GIT) intact to the colon, where they are metabolized by probiotic strains selectively improving their growth rather than other intestinal flora. This specific bacterial stimulation leads to change of microflora composition in favour of preferred organisms such as *Lactobacillus* and *Bifidobacterium* species (Gibson and Fuller, 2000).

1.3.2 Properties of prebiotics

NDO are not digestible by mouth, stomach and small intestine enzymes and reach the colon intact. In the colon prebiotics are subsequently utilized by intestinal bacteria in process called fermentation. Products of fermentation include short-chain fatty acids (SCFA; acetate, propionate, butyrate and lactate), gases (hydrogen, carbon dioxide and methane) and heat (Cho and Finocchiaro, 2010). Gases are an undesirable side effect of prebiotic fermentation. The final fermentation product (SCFA or gases) and their amount depends on the type of NDO digested by microorganisms in colon (Mussatto and Mancilha, 2007). NDO effectively stimulate growth of certain bacterial species present in GIT. *Lactobacillus* and *Bifidobacterium* belong to those intestinal genera that most utilize NDO (Bielecka *et al.*, 2002).

More than 70% of energy derived from carbohydrate fermentation is used for production of SCFA and other products whereas 30% is utilized by colonic microflora to support their growth. SCFA are the final product of bacterial fermentation. Acetate, propionate, butyrate acids constitute 83% of the SCFA producing during fermentation, produced in the ratio 60:20:20 (Dumitriu, 2005). Some SCFA (90-95%) are absorbed by large intestine in the caecum and in the ascending part of the colon (Cho *et al.*, 1999) and stimulate growth of bowel epithelium cells (especially butyrate, a major energy

substrate for colonocyte). SCFA also contribute to water and salt absorption thereby improving peristaltic action of the colon (Mussatto and Mancilha, 2007). Other SCFA take part in regulation of liver metabolism. Acetate (20-25% of total acetate production) reach muscle tissue via liver circulation providing an energy substrate (Cho *et al.*, 1999).

SCFA, in decreasing pH level in the colon, inhibit the growth of pathogenic, undesirable bacteria and at the same time stimulate growth of prebiotic strains. Increasing levels of SCFA has a beneficial effect on human health, increasing mineral absorption, regulating proliferation and differentiation of cells, along with antiproliferative and anticancer properties, regulating of cholesterol synthesis and lowering blood ammonia level (Cho and Finocchiaro, 2010).

1.3.3 Criteria of prebiotic

Potential prebiotic product should meet the following criteria in order to be classified as functional food ingredients:

a) Should not to be digested or absorbed in upper part of GIT (mouth, stomach and small intestine; Gibson, 2004).
b) Should be fermented by intestinal bacteria present in lower part of GIT (Gibson, 2004).
c) Should have beneficial effect on prebiotic strains present in the colon by selective stimulation of growth only of beneficial intestinal bacteria (Fooks *et al.*, 1999).
d) Should be chemical stable (B-linkages oligosaccharides are more stable than L-derivatives; Wang, 2009).
e) Should be stable to food processing treatment (Wang, 2009).

1.3.4 Prebiotic activity assay

Lactobacillus and *Bifidobacterium* species have been added to food as probiotic cultures. In many cases probiotic strains are added to food with prebiotics as a "synbiotic" mixture. A synbiotic mixture improves the survival and viability of

microbes from dietary supplements in GIT (Harish and Varghese, 2006), therefore it is very important to choose a specific prebiotic for selective stimulation of particularly chosen probiotic strain. Huebner and co-workers (2007) described quantitative determination of prebiotic activity in an *in vitro* assay and their work describes which prebiotic (e.g. FOS, GOS or inulin) selectively supports growth of lactobacilli and bifidobacteria. Their proposed prebiotic activity score is based on measurement of growth of probiotic strains (*Lactobacillus* and *Bifidobacterium* species) on different prebiotics relative to growth of non-probiotic enteric strain e.g. *Escherichia coli* and relative to growth on non-prebiotic substrate e.g. glucose under the same condition (Huebner *et al.*, 2007). According to this definition, potential prebiotic products with a high activity score significantly improve growth of the probiotic bacteria and are selectively metabolized by these probiotics but not by other bacteria present in the colon.

The prebiotic activity score (Huebner *et al.*, 2008) is calculated as follows:

$$\textbf{Prebiotic activity score} = \left[\frac{\textbf{(probiotic log O.D. on the prebiotic at 24h - probiotic log O.D. on the prebiotic at 0h)}}{\textbf{(probiotic log O.D. on glucose at 24h- probiotic log O.D. on glucose at 0h)}} \right]$$

$$\textbf{minus}$$

$$\left[\frac{\textbf{(enteric log O.D. on the prebiotic at 24h - enteric log O.D. on the prebiotic at 0h)}}{\textbf{(enteric log O.D. on glucose at 24h - enteric log O.D. on glucose at 0h)}} \right]$$

The prebiotic activity score of different prebiotics depends on the probiotic strain tested and type of prebiotic used. The highest prebiotic activity score identifies prebiotics which selectively support growth of a strictly defined probiotic strain. This information is very useful for preparation of synbiotic product in dietary food or others products.

Processing conditions of prepared food or dietary supplements can change the prebiotic effect therefore prebiotic stability is very important because prebiotics should reach the colon intact, where they can be selectively fermented only by those bacteria which are desirable in the colon.

Prebiotic activity can also be affected by its stability under different processing conditions (heat, pH). One of the documented ways to determine stability is through

determination of prebiotic activity score. The method is based on change in growth of probiotic strains when grown on prebiotic relative to that of non-probiotic enteric strains (e.g. *Escherichia coli*) and in the presence of three different parameters of food processing such as: low pH, heat and Maillard reaction (Huebner *et al.*, 2008).

Prebiotics should be chemically stable in heat, low pH and Maillard reaction treatment. These criteria have important significance for choosing potential prebiotic products as functional food ingredients. If prebiotic activity before and after processing condition are similar (or even increase) then potential prebiotic product may be a suitable functional food candidate. Heat and low pH treatment have huge importance in preparing prebiotic product and together with cold treatment have great importance in storage of prebiotic products. These specific conditions should not decrease prebiotic activity.

Maillard reaction causes the non-enzymatic production of Maillard products (brown in colour) during reaction of aldehyde (or ketose) with amino group during heating. Every oligosaccharide with reducing sugar content will react with amino groups and will form brown Maillard products (Dumitriu, 2005). If an oligosaccharide is indigestible it will not undergo Maillard reaction. Table 1.8 presents NDO and the chemical properties (in terms of reducing sugars present).

Table 1.8: Oligosaccharides and their reduction properties.

Oligosaccharide	Source/Origin	Reducing sugar
Short-chain fructooligosaccharides	sucrose	no
Inulin	chicory	no
Hydrolized inulin	inulin	yes
Xylooligosaccharides	xylan	yes
Soybean oligosaccharides	soybeans	no
Galactooligosaccharides	lactose	yes
Lactulose	lactose	yes
Transgalactoologasaccharides	lactose	yes

Source: (Dumitriu, 2005).

1.3.5 Functionality of prebiotics

Prebiotic oligosaccharides have many characteristic properties which are critical as ingredients in food products:

a) Water solubility is for most prebiotics the characteristic most relevant for food application.

b) Sweetness of prebiotic which depends on the chemical structure and polymerization. Low sweetness can be used in food preparation where the flavour is more important. Very sweet prebiotic products could be alternatives for sweeteners (aspartame, sucralose). Prebiotics are safe for diabetes because of their low-caloric properties. Prebiotics typically have 1.5-2.0 kcal/g or 40-50% of sucrose (Mussatto and Mancilha, 2007).

c) Chemical and biological stability; resistant to heat, cold and low pH treatment giving oligosaccharide prebiotics persistence of nutritional and physicochemical properties (Voragen, 1998).

d) High moisture-retaining capacity what prevents drying of prebiotic (Mussatto and Mancilha, 2007).

1.3.6 Non-digestible oligosaccharides

Non-digestible oligosaccharides (NDO) are complex carbohydrates referred to as "resistant oligosaccharides" because they are resistant to digestion by enzymes in GIT. As a results NDO can reach the colon and be digested to small oligomers and monomers that in turn can be metabolized by intestinal anaerobic bacteria (Cho *et al.*, 1999). NDO are naturally present in vegetables, fruits, cereals and legumes. Non-digestible oligosaccharides (NDO) differ in terms of:

a) Carbohydrate chain length which is related to their molecular weight,

b) Composition of monosaccharides (primarily built from glucose, galactose, fructose and xylose),

c) Degree of polymerisation (DP) and glycosidic linkages, which is an important factor in determination of selectivity of fermentation and digestibility in the small intestine (Tannock, 2002),

d) Purity.

NDO comprise major prebiotic dietary carbohydrate and are widely used as food ingredients (Mussatto and Mancilha, 2007). Table 1.9 shows NDO with prebiotic activity. NDO are used in functional foods but in Europe three of them in particular are

widely used and are regarded as safe: fructooligosaccharides, inulin and galactooligosaccharides.

Table 1.9: Oligosaccharides with potential probiotic properties.

Prebiotic	References
Cyclodextrins (CD)	(Singh *et al.*, 2002)
Fructooligosaccharides (FOS)	(Chow, 2002)
Galactooligosaccharides (GOS)	(Macfarlane *et al.*, 2008)
Gentioologosaccharides	(Rycroft *et al.*, 2001)
Glucoologasaccharides	(Fooks *et al.*, 1999)
Glycosylsucrose	(Nakakuki, 2002)
Isomaltooligosaccharides (IMO)	(Li *et al.*, 2009)
Isomaltulose	(Kawaguti and Sato, 2007)
Lactosucrose (LS)	(Li *et al.*, 2008)
Lactulase	(Aider and Halleux, 2007)
Maltoologosaccharides	(Crittenden and Playne, 1996)
Raffinose	(Tortuero *et al.*, 1997)
saybean oligosaccharides (SOS)	(Hayakawa *et al.*, 1990)
trans-galactooligosaccharides (TOS)	(Fooks *et al.*, 1999)
Xylooligosaccharides (XO)	(Vázquez *et al.*, 2006)
Inulin	(López-Molina *et al.*, 2005)
Oligofructose	(Cho *et al.*, 1999)

1.3.6.1 Fructooligosaccharides

Fructooligosaccharides (FOS) belong to molecules of short and medium length chains of β-D fructans with β-2,1 glicosidic linkages. FOS naturally occur in fruit (banana), vegetables (onion, garlic, asparagus, leek) also in cereals (wheat, rye), honey and Jerusalem artichoke (Khan, 1993). FOS can also be produced commercially from long-chain fructans (e.g. inulin isolated from chicory roots) by hydrolysis to smaller molecules of FOS, contained oligofructose. This is known as Raftilose (Chow, 2002).
FOS can also be produced from sucrose. Using enzyme from *Aspergillus niger* sucrose is hydrolyzed to FOS. Obtained in this way FOS, called Neosugar, has following commercial names: Nutraflora® , Meioligo®, Actilight® (Dumitriu, 2005). In FOS there are β (1,2) linked fructosyl units attached to a terminal α-D glucose residue (Shetty, 2006). Fructosyl units which build the FOS consist of oligosaccharides such as 1-kestose (GF2), nystose (GF3) and 1-β fructofuranosyl (Figure 1.10).

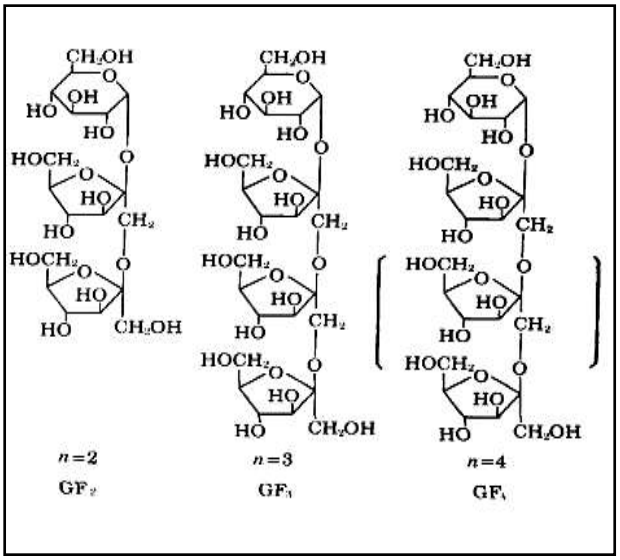


Figure 1.10: Structure of FOS.
Source: (lfbiotec, 2010).

FOS has a sweet taste similar to sucrose. The purified form reaches 30% of the sweetness of sucrose. Fructooligosaccharides are non-reducing sugars easily soluble in water and do not undergo Maillard reaction. They are also stable in low (pH≤3) acidity up to 70°C or up to 140°C in neutral medium (Khan, 1993). These oligosaccharides are the best documented prebiotics in term beneficial effect on intestinal bifidobacteria (Gibson, 2004). They are resistant to digestive enzymes present in stomach and small intestine. FOS is selectively fermented in the colon by intestinal bacteria, only supporting growth of desirable probiotic strains (Rycroft *et al.*, 2001). Products of FOS fermentation are SCFA, gas and heat (Chow, 2002).

FOS have been used as a food ingredient in food formula (powdered infant milk), cereals, sweets and can be added to food products as a mixture with prebiotic strains. Additionally FOS are low-calorie molecules (2kcal/g), so can be used by diabetic patients (Chow, 2002). FOS can also be used for treating chronic constipation, its action being similar to other laxative substances such as lactulose and sugar alcohols (Clausen *et al.*, 1998).

1.3.6.2 Inulin

Inulin is a long dispersed chain of β-2,1 fructan. The fructose units are each linked by β-2,1 bonds. At the end of each fructose chain there is a glucose molecule, linked by α-1,2

bond (Figure 1.11). The β-2,1 bonds give the inulin non reducing properties (López-Molina *et al.*, 2005).

Inulin is present in many plants. Fruits (banana), vegetables (chicory, dandelion, Jerusalem artichoke, salsify, globe Artichoke, onion, leek, garlic) and cereals (wheat and barley) are rich in inulin. Inulin varies in terms of quantity as well as degree of polymerization (Roberfroid, 2005).

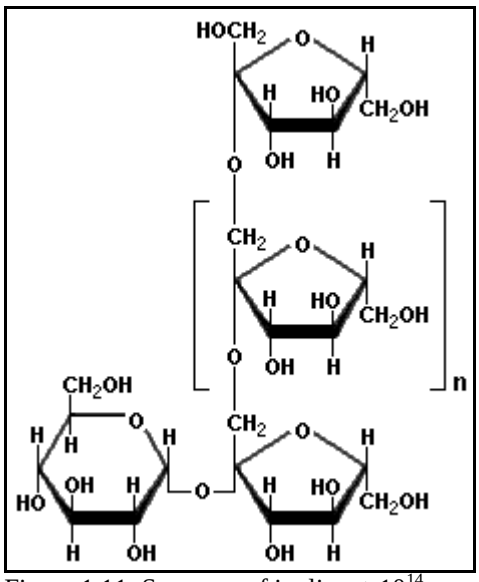


Figure 1.11: Structure of inulin: $n>10^{14}$.
Source: (Zamora, 2010).

Production of commercial inulin is from chicory (15-20g inulin/100g). Inulin structure always contains glucose, fructose, sucrose and small oligosaccharides (Roberfroid, 2005). Chicory inulin is white, of neutral taste and is slightly sweet (10% of the sweetness of the sucrose). It is readily soluble in water- 10% at room temperature (Franck, 2002). Inulin is resistant to digestive enzymes in the mouth, stomach and small intestine, and therefore is classified as a non-digestible oligosaccharide. The chicory inulin is selective utilized by bifidobacteria species in the colon and similarly to FOS has been shown to have a potent prebiotic activity (Gibson, 2004). This prebiotic is widely used in food, milk products, breakfast cereals. Due to its low-calorie index, inulin can also be found in low-calorie sweets, used as a sugar replacement and to provide additional dietary fibre (Roberfroid, 2005). Because the molecular structure is dispersed and resistant to hydrolysis, inulin has gelling properties and gels in the GIT aiding gut microflora with fermentation by increasing surface area available to enzymatic hydrolysis (Gibson, 2004). Gelling properties can also be useful for

preparation of capsules for various medications that have to reach the colon intact (de Vos *et al.*, 2010).

1.3.6.3 Galactoooligosaccharides

Galactooligosaccharides (GOS) are oligosaccharides containing galactose which form Glu α1-4[β Gal 1-6]$_n$ where n=2-5 (Gibson, 2004). GOS is present in human milk and contain a high amount of galactose as well as lactose at their reducing ends (Cho and Finocchiaro, 2010). GOS are produced *in vitro* from lactose (usually obtained from Cow's milk) using a transferase activity in β-galactosidase. GOS have been commercially available since 1950s (Shortt and O'Brien, 2004). Bifidobacteria and lactobacilli are able to produce a high level of β-galactosidase enzyme to synthesize GOS from lactose (Rabiu *et al.*, 2001).

GOS is a reducing sugar and does undergo Maillard reaction. It is highly soluble in water and stable after heat treatment (resistant to pasteurization and sterilization process in low pH) and therefore can be used as a food additive in milk-derived products (yogurt, juice drinks), bakery products and breakfast cereals. GOS is also used in infant milk formula because of its bifidogenic properties (Cho and Finocchiaro, 2010).

GOS is not hydrolyzed in the stomach and small intestine and can reach the colon intact. Rycroft and colleagues confirmed that this oligosaccharide can support fermentation. GOS significantly increases the number of bifidobacteria and lactobacilli species, however is not as selective as FOS (stimulating non-probiotic strains). During fermentation of GOS by probiotic bacteria, a high level of SCFA is obtained but with lower gas production than that observed with FOS (Rycroft *et al.*, 2001).

1.3.7 Novel oligosaccharides with prebiotic potential

The range of commercial and pre-commercial prebiotics has been growing very rapidly. Up to 2007 over 400 prebiotic food products have been reported and there are more than 20 companies which produce oligosaccharides and fibres used as prebiotics. According to the Frost and Sullivan report, the prebiotic market reached a value of 87 million euro in 2007 and they predicted a market worth 179.7 million euro in 2010 (FAO, 2007).

The expanding market for prebiotics became the main reason for the search and production of novel prebiotics. Most prebiotics are manufactured in Japan and are widely available on the Japanese market. The leaders on the European market are mainly inulin, FOS and GOS (Rastall and Maitin, 2002).

There are several possible methods of prebiotic production. Extraction from natural sources is widely used. Inulin extraction from chicory (very similar to extraction of sugar from beet) is one of the best examples. The three oligosaccharides (raffinose, stachylose and verbacsose) are isolated commercially from soybean whey by Calpis Food Industry Co. in Japan (Tannock, 2002).

Prebiotics can also be artificially synthesized. This principle may allow eventual production of bespoke prebiotics. Currently there is only one prebiotic produced by chemical reaction, lactulose. Lactulose is produced from lactose by isomerisation to form fructose residue in the presence of two catalysts, sodium hydroxide and sodium borate. Prebiotic lactulose is manufactured in Japan by The Morinage Milk Industry also in Europe by Solvay (Tannock, 2002).

Enzymatic production of prebiotics is also possible. This method is widely used in production of prebiotics and generally consists of two approaches: hydrolysis of polysaccharides or synthesis of oligosaccharides from disaccharides.

Fructooligosaccharides (FOS) can be produced from inulin by hydrolysis (see section 1.3.6.1). Xylooligosaccherides (XO) are also manufactured using hydrolysis method. These prebiotics are obtained from xylan. Xylan is a polysaccharide present in plant cells walls (corn cobs, straws, hulls malt, bagasses). There are three approaches to production of XO from xylan-rich materials (Mussatto and Mancilha, 2007):

a) Chemical treatment to causes the degradation of xylan by steam, acid or alkaline solution.
b) Enzymatic treatment of xylan-rich materials using exo-xylanase or/and β-xylosidase.
c) Separation and purification by adsorption to remove saccharide molecules. Ion-exchange and chromatographic separation is carried out to remove colour and desalt (Domínguez *et al.*, 2003).

XO can also be produced by microorganisms; however this method is not used commercially. *Pleurotus sp*. (a fungi) has been used for production of XO by enzymatic hydrolysis of xylan derived from oat-spelt (de Menezes *et al.*, 2009). This kind of

production of XO is based on xylan hydrolysis by endo-1,4-xylanase and β-xylosidase. Both enzymes are produced by *Pleurotus sp.*

1.3.7.1 Novel prebiotics from plant cell walls

Novel prebiotics can be derived from plant cell wall polysaccharides which are a rich source of NDO. Fibre-rich by-products and wastes (fruit pomace, cereal bran, potato fiber and so on) are also a rich source of polysaccharides. By enzymatic hydrolysis these polysaccharides can release many oligosaccharides with potential prebiotic effect (Mussatto and Mancilha, 2007). Specific glycanases have been used to produce novel prebiotics derived from plant cell wall polysaccharides (Table 1.10).

Table 1.10: Novel prebiotic derived from plant cell wall.

Novel prebiotic	Origin	Enzyme used
arabinogalacto-oligosaccharides	soybeans	endogalactanases
arabino-oligosaccharides	sugar beet	endoarabinases
rhamnogalacturono-oligosaccharides	apple	rhamnogalacturonases
arabinoxylo-oligosaccharides	wheat	xylanases
galacturono-oligosaccharides	poligalacturonic acid	endogalacturonases
cinnamoyloligosaccharides	wheat bran	endoxylanases

Source: (Rastall and Maitin, 2002).

All these novel prebiotics have been tested for their prebiotic activity. They are fermented by intestinal probiotic *Bifidobacterium* and *Lactobacillus* species. Bifidobacteria species utilize all these novel oligosaccharides except rhamnogalacturono-oligosaccharides and galacturono-oligosaccharides. Other intestinal strains such as *Clostridium sp., Bacteroides sp., Klebsiella pneumoniae* and *Escherichia coli* have been found to ferment some plant cell wall derived oligosaccharides (Van Laere *et al.*, 2000), which may not be a desirable characteristic.

1.3.7.2 Synthesis method production of prebiotics

Prebiotics such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactosucrose and izomaltooligosaccharides (IMO) are manufactured by enzymatic synthesis. This method requires sucrose or lactose as precursors (Tannock, 2002).

FOS production, already mentioned in section 1.3.6.1, is widely conducted on a large scale from sucrose. In this process the microbial enzyme fructosyltransferase derived from *Aspergulus niger* and *Aureobasidium pullulans* are used (Bucke, 1999). These two enzymes transfer fructosyl groups and in this way build more branched fructooligosaccharides from 60% fructose solution at 50-60°C (Tannock, 2002).

GOS are manufactured from lactose by using β-galactosidase. This is a hydrolase enzyme which also catalyzes transgalactosylation and forms galactooligosaccharides from 2 to 8 monosaccharides (Lin *et al.*, 2008). The enzyme β-galactosidase can be produced by bifidobacteria and lactobaccilli species. The largest manufacturer of GOS is Yakult in Japan and Borculo Domo Ingredients in Europe (Tannock, 2002).

Lactosucrose is manufactured from a mixture of lactose and sucrose in the presence of concentrated β-fructofuranosidase (Broekhoven *et al.*, 2001). Commercially, lactosucrose is produced in Japan by Hayashibare Shoji and Ensuiko Sugar (Tannock, 2002).

IMO are also produced by enzymatic synthesis. IMO are obtained from starch and their production requires two stage reduction: hydrolysis of starch by α-amylase to malto-disaccharides which are substrates of the second stage of production. Malto-disaccharides are then converted to IMO by β-amylase and α-glucosidase (Mussatto and Mancilha, 2007). IMO is produced in Japan by Showe Sangyu Co. (Tannock, 2002).
Starch can be precursor for manufacturing other novel oligosaccharides: cyclodextrins, gentiooligosaccharides, maltoologosaccharides. Figure 1.12 shows production of starch-derived oligosaccharides and types of enzymes used in their production.

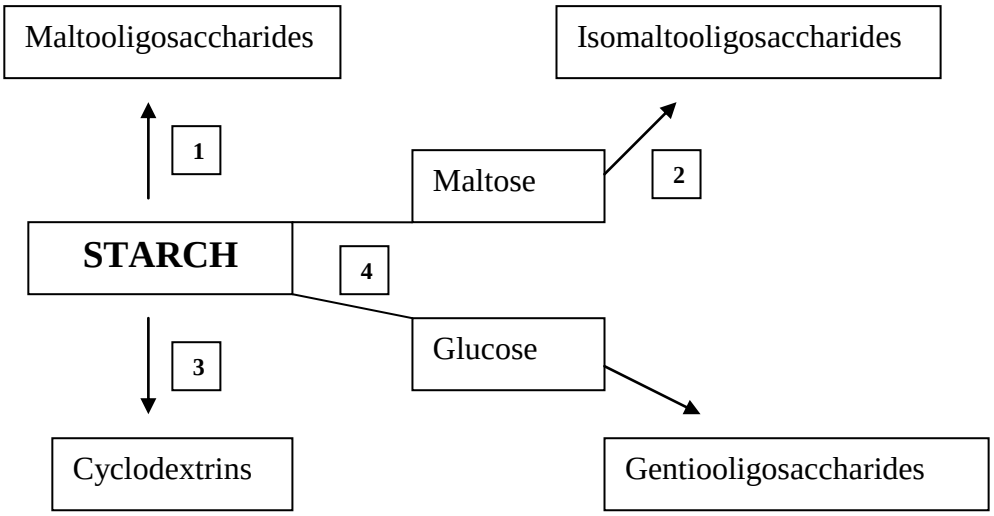


Figure 1.12: Oligosaccharides derived from starch; 1-Malto-oligosaccharide forming amylase, 2-Glucosidase, 3-Cycklodextrin glycosylotransferase (CGTase), 4-Glycosidase.
Source: (Nakakuki, 2002).

Other polysaccharides are also precursors of prebiotic production e.g. glucooligosaccharides can be produced from cellulose or cellobiose. Disaccharides such as maltose and sucrose can be precursors of synthesis of prebiotic glycosylsucrose (Mussatto and Mancilha, 2007).

1.4 Plant secondary metabolites

Natural products are compounds derived from living systems. Scientists, for many years, have tried to discover and elucidate structure, chemistry and properties of natural products. This interest is always directed towards production of *in vitro* active components derived from natural sources (Mann *et al.*, 1994). Secondary metabolites are frequently a particular subject of interest to researchers because of their bioactive properties. Medicinal plants are often used to obtain these bioactive components. According to the World Health Organisation (WHO), herbal medicine provides most of the healthcare needs for 80% of the population in developing countries (Arora, 2010). Herbal medicine is, therefore, a very important part of human health care and represents the oldest and most widespread form of medication (Gupta *et al.*, 2010). In addition herbal materials are the natural sources of many synthetic drugs.

Natural plant products can be divided into two groups:

1) Primary metabolites:

Those directly involved in the metabolism of the plant (growth, development and reproduction): carbohydrates, proteins, amino acids, nucleotides and nucleosides.

2) Secondary metabolites:

These components are specific and characteristic for limited ranges of species and include:

- polyketides and fatty acids
- terpenes and sterols
- phenylpropanoids
- alkaloids
- specialised amino acids and peptides
- specialised carbohydrates.

Secondary metabolites (SMs) in plants are derived from primary metabolites through different metabolic paths taking place mostly in cytosol. SMs are also produced in other organelles, for example, chloroplasts, mitochondria, vesicles, endoplasmic reticulum (Hanson, 2003).

The biological functions of these organic compounds are:

a) Self-protection agents against different pathogens: bacteria, fungi, other plants, insects (Blumwald *et al.*, 1998), (De Gara *et al.*, 2003) and herbivores (Wink, 2003).
b) Symbiosis with other organisms such as fungi (Antunes *et al.*, 2006) and bacteria (Hause and Schaarschmidt, 2009) needed for proper development of the plant
c) Pheromones (Barbosa *et al.*, 1991)
d) Production of enzymes in response to oxidative stress (Morita *et al.*, 2011)
e) Attractants for pollinators (Borg-Karlson and Groth, 1986)
f) Allelopathic agents (Li *et al.*, 2011).

1.4.1 Type of plant secondary metabolites

There are many types of plant secondary metabolites. Shimada (Shimada, 2001) reported that horse chestnuts contained considerable amounts of tannins and saponins. The following sections review the classes of secondary metabolites in general and then details those secondary metabolites most relevant to horse chestnut tissue. Horse chestnut contains triterpenoid saponins (notably escin), coumarins (notably esculin) and flavonoids. There are also tannins and flavonoids. The bark contains coumarins, glycoside, resin and pigment. Escin, the main active constituent, has anti-inflammatory properties. In the production of escin preparations for the treatment of chronic venous insufficiency, horse chestnut nuts are usually pre-treated by shelling and washing before the extraction.

1.4.1.1 Phenolics and Phenylpropanoids

Phenylpropanoids are the most studied class of plant secondary metabolites because of their biological activities and biosynthesis (Kazufumi, 2006). Phenylpropanoids are organic constituents belonging to large class of plant phenols. They are built from at least one aromatic ring (phenol group-Figure 1.13; Garcia-Salas *et al.*, 2010).

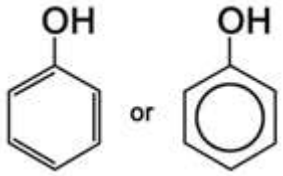


Figure 1.13: Phenol, the simplest phenolic compound.
Source: (Freeman and Beattie, 2008).

Phenolic molecules can also be associated with sugar residue (glucose, galactose, arabinose, rhamnose, xylose, mannose, apiose, allose, glucuronic and galacturonic acid) attached to one or more phenolic group (Mann *et al.*, 1994). Phenolic molecules represent more than 8000 various structures widely dispersed in the plant kingdom (Crozier *et al.*, 2007). The major classes of phenylpropanoids present in plants are presented in Table 1.11.

Table 1.11: The major of classes of phenolics in plants.

Number of carbon atoms	**Basic skeleton**	**Class**	**Example**	**Presence**
6	C6	Simple phenols	Catechol, arbutin	63% of higher plant
7	C6-C1	Phenolic acids	salicylic acid	Fruits, vegetables, herbs
8	C6-C2	Acetophenones	3-acetyl-6-methoxybenzaldehyde	Leaves of *Encelia Farinosa* (Gray and Bonner, 1948)
		Phelylacetic acids	p-hydroxyphenylacetic	Olive oils (Papadopoulos and Boskou, 1991)
9	C6-C3	Hydroxycinnamic acids	caffeic, ferulic	Cereals, coffee, vegetables, fruits (Maurya and Devasagayam, 2010)
		Phenylpropenes	myristicin, eugenol	*Myristica fragrance*, cloves oil, cinnamon leaves (Dewick, 2011)
		Coumarins	aesculetin, fraxin, scopoletin	*Aescullus hippocastanum* (Bombarelli and Morazzoni, 1996)
		Isocoumarins	bergenin	Cortex of *Mallotus japonicas (*Lim *et al.*, 2000)
		Chromones	eugenin	*Peucedanum japonicum* (Chen *et al.*, 1996)
10	C6-C4	Naphthoquinones	juglone, plumbagin	Roots of *Plumbago zeilanica* (Bothiraja *et al.*, 2011)
13	C5-C1-C6	Xanthones	mangiferin	*Mangifera indica* (Hou, 2011)
14	C6-C2-C6	Stilbenes	lunularic acid	*Lunularia cruciata*

				(Pryce, 1972)
		Anthraquinones	emodin	*Rheum officinale* (Tang *et al.*, 2007)
15	C6-C3-C6	Flavonoids	quercetin	Onion, red wine, apple, berris
		Isoflavonoids	genistein, daidzein	Soybeans, legumes
18	$(C6-C3)_2$	Lignans	podophyllotoxin	*Podophyllum sp*. (Gordaliza *et al.*, 2004)
30	$(C6-C3-C6)_2$	Biflavonoids	Amentoflavone	*Psilotum sp*. (Markham, 1984)
n	$(C6-C3)_n$	Lignins	pinoresinol	Fruits of *Forsythia koreana* (Jung *et al*., 2010)
	$(C6)_n$	Catechol melanins		.
	$(C6-C3-C6)_n$	Condensed tannins	anthocyanins	Red berries, grapes

Adapted from: (Mann *et al.*, 1994).

1.4.1.2 Phenolic acids

Phenolic acids (PHa), know as hydroxybenzoates, are simple "plant phenolics" which possess one carboxylic acid group. PHa are derived from hydroxybenzoic acid and hydroxycinnamic acids (Stalikas, 2007). The major components of phenolic acids and their subunits are presented in Table 1.12 and Table 1.13.

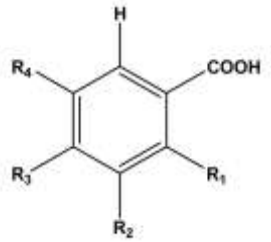


Table 1.12: Hydroxibenzoic acids and their substitution pattern

Name of phenolic acids	Subunits			
	R1	R2	R3	R4
benzoic acid	H	H	H	H
p-hydroxybenzoic acid	H	H	OH	H
vanillic acid	H	OCH_3	OH	H
gallic acid	H	OH	OH	OH
protocatechuic acid	H	OH	OH	H
syringic acid	H	OCH_3	OH	OCH_3
gentisic acid	OH	H	H	OH
veratric acid	H	OCH_3	OCH_3	H
salicylic acid	OH	H	H	H

Source: (Stalikas, 2007).

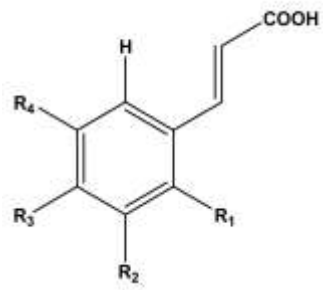


Table 1.13: Hydroxycinnamic acids and their substitution pattern.

Name of phenolic acids	Subunits			
	R1	**R2**	**R3**	**R4**
cinnamic acid	H	H	H	H
o-coumaric acid	OH	H	H	H
m-coumaric acid	H	OH	H	H
p-coumaric acid	H	H	OH	H
ferulic acid	H	OCH_3	OH	H
sinapic acid	H	OCH_3	OH	OCH_3
caffeic acid	H	OH	OH	H

Source: (Stalikas, 2007).

Only a minor fraction of phenolic acids exist in free acid form (found in fruits, vegetables and grains). Most phenolic acids are conjugated with other molecules making more complicated complexes (Stalikas, 2007).

1.4.1.3 The Flavonoids

Flavonoids are polyphenols containing two phenol rings connected by three carbon bridge (Crozier *et al.*, 2007). The basic flavonoid structure C_6-C_3-C_6 is presented in Figure 1.14 where parts A,B and C symbolize three rings to which other molecules can be attached depending on the structural variation of flavonoids. Hydroxylation, methoxylation, phenylation and glycosylation are the most likely pattern characteristics occurring in flavonoid components (Stalikas, 2007).

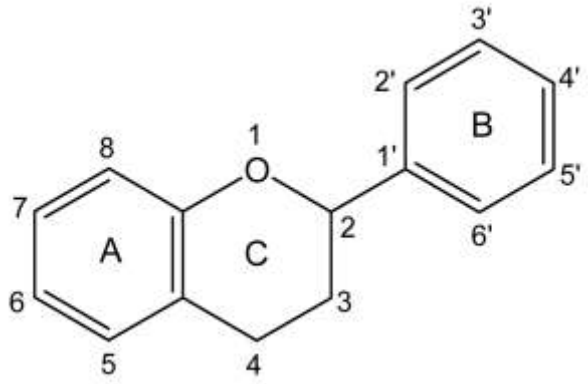
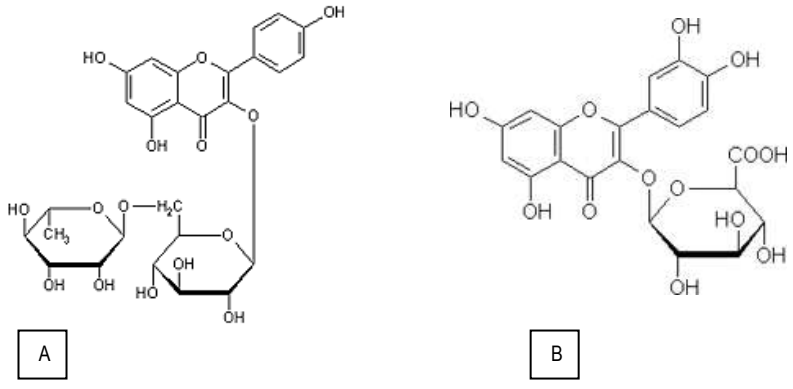


Figure 1.14: Basic structure of flavonoids.
Source: (Stalikas, 2007).

Over 4,000 flavonoids have been identified and the major class are: flavonols, flavones, flavanones, isoflavones, anthocyanidins, flavan-3-ols (Cook and Samman, 1996). Minor flavonoids are dihydroflavolones, chalcones, dihydrochalcones, coumarins, flavan-3,4-ols, coumarins, aurones (Crozier *et al.*, 2007).

Flavonols are widely distributed in plants. They are not present in fungi or algae. Flavonols can be found in fruits and vegetables, and their concentration depends on the climate, place of cultivation, storage and processing of the plant. They are also present in nuts and beverages (Hoffmann-Ribani *et al.*, 2009). Flavonols in large quantity are present in grape skins and contribute to the yellow colour of white and red wines however their presence in red wines is masked by red anthocyanin. Flavonols presents in wines are glycosylated, mostly as *O*-glycosides (Castillo-Muñoz *et al.*, 2010). Figure 1.15 presents flavonols in their *O*-glicoside form. Conjugation of aglycone with glycoside component in flavonols occurs more frequently at the C_3, as indicated in Figure 1.15 (Crozier *et al.*, 2007).



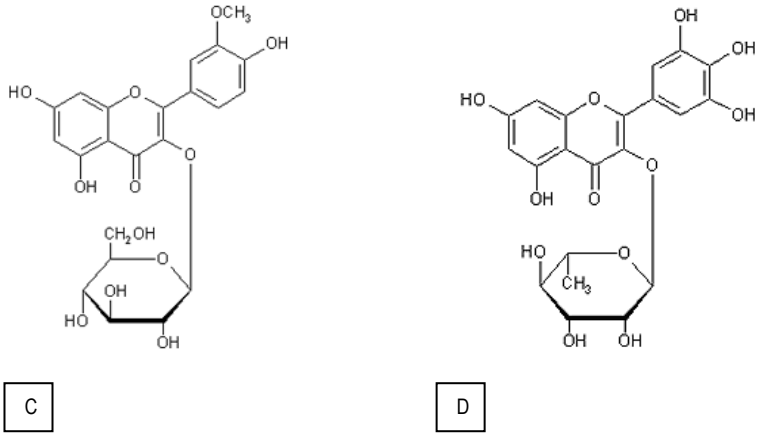


Figure 1.15: The flavonols: kaemferol (A), quercetin (B), isorhamnetin (C) and myricetin (D)
Source: (Extrasynthese, 2011).

The term "flavone" was first used by von Kostaniecki and Tambor who elucidated the structure of flavone. These phenolic components are structurally similar to flavonols. A wide range of substitution of the flavone molecules is possible (hydroxylation, methylation, isoprenylation, alkilation and glycosilation) (Martens and Mithafer, 2005). Luteolin and apigenin (Figure 1.16) are example of flavones without oxygen present at C_3 (A- and C-ring). Some flavones, such as as nobiletin and tangeretin, are polymethoxylated (Figure 1.17) and are widely distributed in citrus species (Crozier *et al.*, 2007).

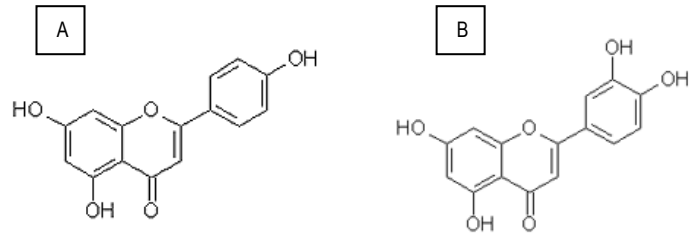


Figure 1.16: Structure of apigenin (A) and luteolin (B).
Source: (Extrasynthese, 2011).

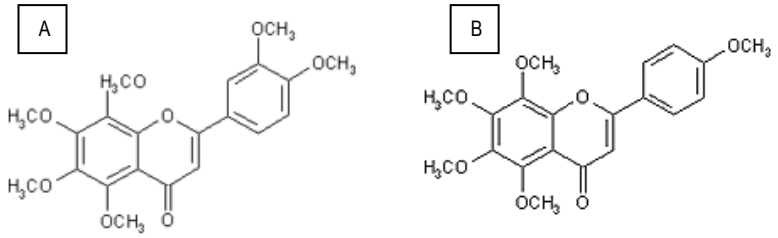


Figure 1.17: Structure of nobiletin (A) and tangeretin (B).
Source: (Extrasynthese, 2011).

Flavan-3-ols are a very large class of flavonoids. The characteristic property of these polyphenolic components is the presence of a non-polar element, a saturated C_3 on the C-ring. Other chemical modifications of flavan-3-ols are chiral centres at C_2 and C_3 which are responsible for creating isomers such as: (+)-catechin and (-)- epicatechin, (-)-catechin and (+)-epicatechin (Crozier *et al.*, 2007). Flavan-3-ols vary from simple monomers (catechins), monomeric isomers (epicatechin) (Figure 1.18) to more complicated structures, such as oligo,- and polymeric anthocyanidins (type A and B) which can comprise up to 50 units mainly consisting of catechins and epicatechins (Figure 1.19). Proanthocyanidins, (Figure 1.19) also named condensed tannins, are typical polymers of flavan-3ols. Other products of oligomerisation of flavan-3-ols are gallacto-catechins.

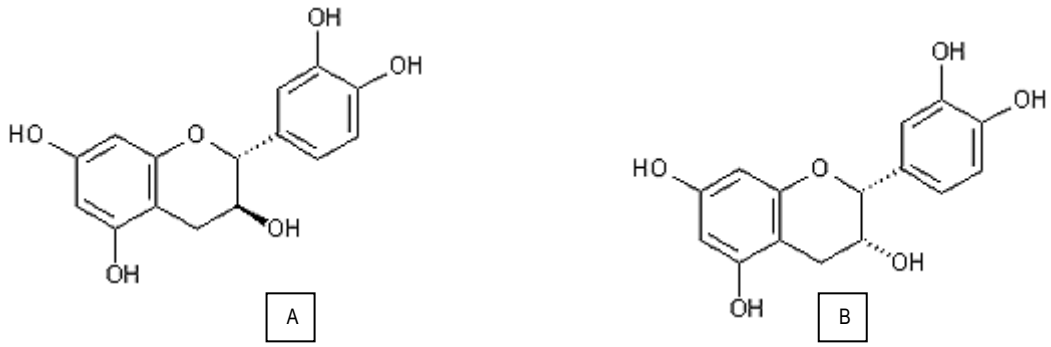


Figure 1.18: Simple isomers of flavan-3-ols: (+)-catechin (A) and (-)-epicatechin (B).
Source: (Extrasynthese, 2011).

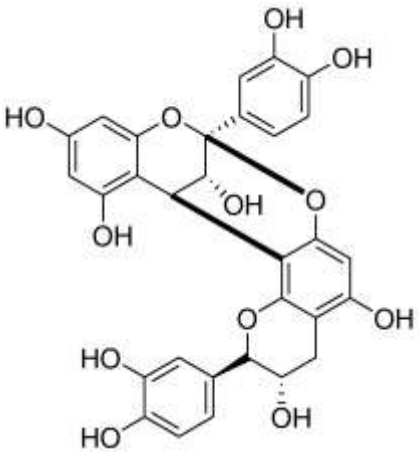


Figure 1.19: Structure proathocyanidin A_1.
Source: (Liu *et al.*, 2009).

Green tea (*Camellia sinensis*) is a rich source of catechins or flavan-3-ols. These polyphenolic components are a major subclass present in the leaves of green tea (Del Rio *et al.*, 2010). The concentration of catechins declines during fermentation of green tea leaves. Oxidation which takes place in fermented leaves (by polyphenol oxidase) gives raise a high-molecular thearubigins and theaflavins in black tea (Del Rio *et al.*, 2009).

The structure of flavanones differs to other flavonoids; the C-ring is attached to the B-ring at C_2 in α-conformation. There is no chiral centre at C_2 (Crozier *et al.*, 2007).
High concentration of flavanones is found in citrus fruits. Tasteless hesperidin is the principal flavanone found in lemon and *Citrus* species (Del Rio *et al.*, 2004). Other flavanones: neohesperidin and naringin are responsible for the bitter taste of some *Citrus* species. Bitter orange (*Citrus aurantium*) contains neohesperidin whereas the major flavonoid in grapefruit (*Citrus paradidi*) is naringin (Del Rio *et al.*, 1997).

1.4.1.4 Isolflavonoids

The characteristic structure of isoflavones is the presence of B-ring attached to the C_3 position (Crozier *et al.*, 2007). Genistein and daidzein (Figure 1.20) are examples of major isoflavones found in legumes. The best source of isoflavones genistein, daidzein, glycitin and their conjugated glycosidic forms is soybeans (Luthria *et al.*, 2007). These soy isoflavones may mimic the function the steroidal hormone oestradiol and have cognitive function on females (Lee *et al.*, 2005).

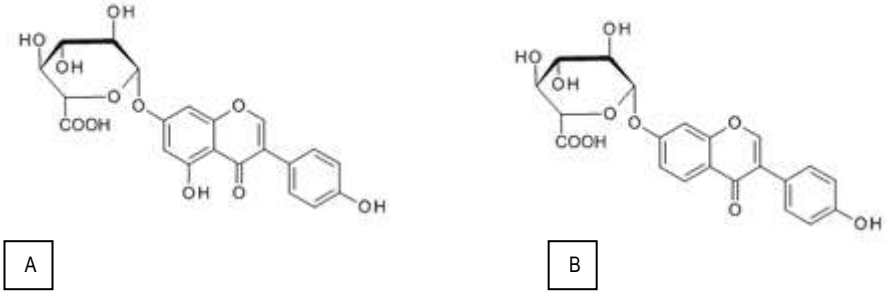
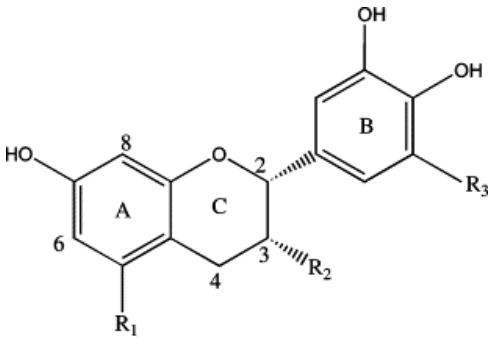


Figure 1.20: Structures of isoflavones: genistein (A) and daidzein (B).
Source: (Extrasynthese, 2011).

1.4.1.5 Tannins

Phenolic acids are precursors of tannins. Tannins with molecular mass 300-3000 Da can be divided into: gallotannins, ellagiotannins, complex tannins and condensed tannins (Li *et al.*, 2006). Gallotannins and ellagiotannins are hydrolysable and can be easy broken down by acids. Condensed tannins do not undergo hydrolysis (Hartzfeld *et al.*, 2002). Condensed tannins (proanthocyanidins, PAs) are oligomers and polymers of flavan-3-ols and flavan 3,4-diol units. They are built from groups of polyhydroxy-flavan-3-ol oligomers and other polymers attached to flavanol units by carbon-carbon bonds (Figure 1.21).



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 1.21: Basic structure of condense tannins.
R_1=R_2=O and R_3=H characteristic for (-) epicatechin.
R_1,R_2-as indicated in table characteristic for structure
included in table above. R_2=O gallolyl in the catechin gallates.
Source: (Schofield *et al.*, 2001).

Figure 1.22 shows the structure of condensed tannins. Condensed and hydrolysable tannins have large numbers of phenolic groups which are very reactive and may form strong bonds with proteins and carbohydrates (Silanikove *et al.*, 2001).

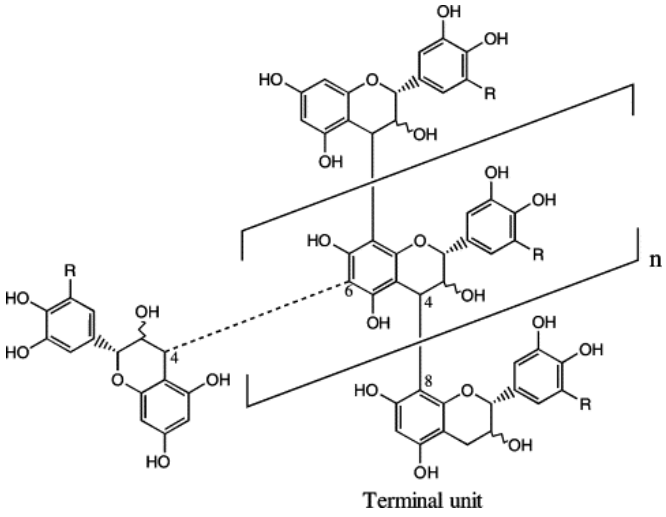


Figure 1.22: Structure of condensed tannins.
R=H or OH for procyanidin or prodelphinidin.
Bond 4→6 of interflavan.
Source: (Schofield *et al.*, 2001).

Groups R1, R2 and R3 (Figure 1.21) are particularly reactive thus tannins are able to bind proteins to form complexes. There are also able to precipitate proteins that have negative impact on appetite (Schofield *et al.*, 2001).

Tannins have been established to be responsible for astringency in natural products. They are able to bind to salivary proteins and give a sensation recognisable as astringent (McRae and Kennedy, 2011) and a taste of bitterness, and are present in a variety of foods (tea, wine, chocolate, fruits, nuts, soymilk; Lesschaeve and Noble, 2005).

Condensed tannins (proanthocyanidins, PAs) are widely distributed in red, blue and purple colour fruits such as berries and grapes. They very often build complexes with carbohydrates and comprise elements of cell wall structures. Some PAs exist alone as high molecular components. Because the majority of PAs are in-soluble and un-extractable, they reach the colon intact and are then fermented by colonic bacteria giving possible anti-oxidant effect. (Perez-Jimenez *et al.*, 2009, Goni *et al.*, 2005).

1.4.1.6 Coumarins

Coumarins (benzo α-pyrone) are natural members of plant derived simple phenolic components. Coumarins are components of essential oil in plants and therefore they are used as flavouring ingredients in food and beverages (Sproll *et al.*, 2008). For example sweet clover of melilot (*Melilotus sp*.) and sweet woodruff (*Galium odorata*) are rich sources of coumarin.

There are three classes of coumarins: hydroxicoumarins (e.g. coumarin, umbelliferone, esculetin and scopoletin), furanocoumarins (e.g. angelicin) and pyranocoumarins (e.g. psoralen; Hoffmann, 2003).

One of the simple coumarins is scopoletin and esculetin (Figure 1.23 A,B) present in horse chestnut (*Aesculus hippocastanum*). Esculetin (found in fruits and bark of horse chestnut) has an anticoagulant effect (Cassileth and Lucarelli, 2003) and therefore can be used in pharmaceutical preparation. Fraxin (Figure 1.23 C) is present mainly in the bark of *Aesculus hippocastanum*, but also is found in small quantities in the seeds. Both fraxin and esculetin, have fluorescent activity (Watts, 1871). Esculetin, fraxin and scopoletin have been shown to have anti-oxidative effect due to their action as free radical scavengers (N.K. Sinha *et al.*, 2010).

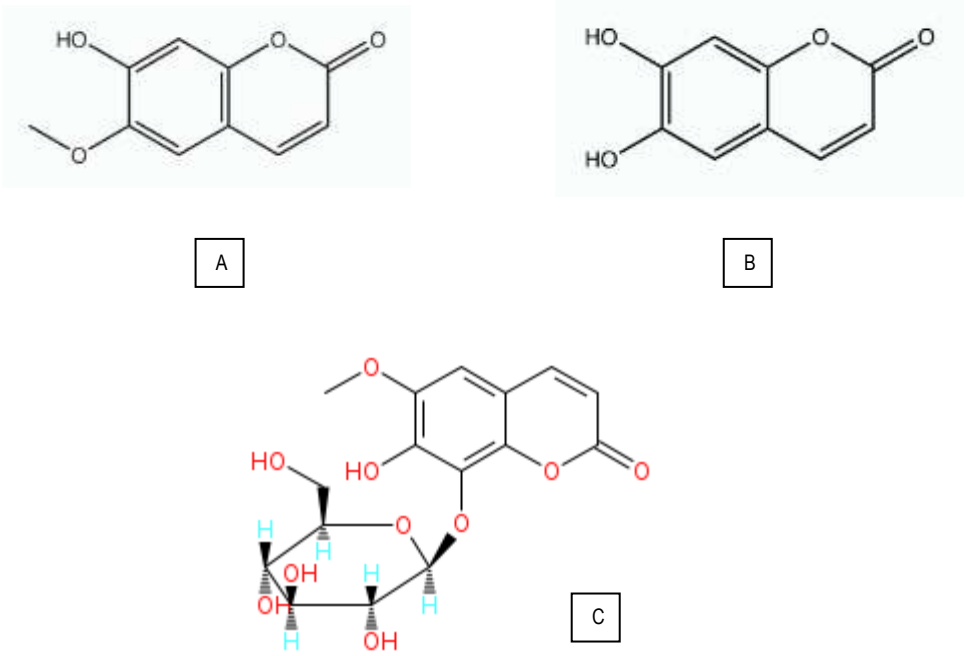


Figure 1.23: Structure of scopoletin (A), asculetin (B) and fraxin (C).
Source: (Cfm, 2012, Molecular, 2012).

1.4.1.7 Bioactivity of phenolic components:

Anti-mutagenic effect

Antimutagenic effect refers to the action of blocking and destruction of DNA-damaging mutagens. Anti-mutagens act outside the cell and prevent development of carcinogenesis at early stages (Horn and Vargas, 2003).The antimutagenic effect of some natural substances has been widely described by many researchers (Brockman *et al.*, 1992). Gallic acid is considered to have strong inhibitory effect on mutagenecity of 5 NFAA (3-5-nitro-2-furyl) acrylic acid and sodium azide. Ferulic acid, caffeic acid and syrigic acid also possess inhibitory effects on mutation induced by these two mutagens tested on *Salmonell*a *typhinurium* TA100 (Birosová *et al.*, 2005).

Natural phenolics extracted from beans (*Phaseolus vulgaris*) have been shown to have an antimutagenic effect on *S. typhinurium* (strain YG 1024). Methanolic extract of beans possess an inhibitory effect on the mutagenecity of 1-NP (De Mejía *et al.*, 1999).

Extract from pepper seeds (*Capsicum annum*) is also considered to have a high potency of antimutagenic activity against other mutagens, e.g. nitroarenes (1-NP, 1,6-DNP and 1,8-DNP). Rich in beta-carotene and xanthophylls, the extract showed more inhibition on mutagenecity of nitroarenes than pure trans-beta carotene (Brockman *et al.*, 1992).

The antimutagenic properties of extracts from *Maytenus ilicifolia* and *Peltastes peltatus* rich in flavonoids and tannins have also been investigated. Both extracts exhibited an anti-mutagenic properties against mutagens such as 4-oxide-1-nitroquinoline, sodium azide, 2-nitrofluorene, aflatoxin B_1, 2-aminofluorene and 2-aminoanthracene using the *Salmonella*/microsome assay (Horn and Vargas, 2003).

Antimutagenic activity is also found in horse chestnut extract (Sato *et al.*, 2005) The extract showed inhibition of the genotoxicities of furylfuramide, N-methyl-N-nitrosourea, methyl methanesulfonate, mitomycin C, 2-aminoanthracene and aflatoxin B1 at a concentration of 1 mg/ml or more.

Antimicrobial activity

Many studies are directed toward discovering new naturally-derived molecules which possess antimicrobial properties. Resistance of microbial pathogens to antimicrobial agents is still an important global problem. For example *Staphylococcus aureus* is a major cause of hospital acquired infection. This microorganism spreads very easily and usually is treated by β-lactam (or derived) antibiotics. The development of a methicillin-

resistant *Staphylococcus aureus* (MRSA) strain and community-associated MRSA (CA-MRSA) merits serious attention since these strains have been shown to be resistant to many antibiotics (Parasa *et al.*, 2011).

It has been demonstrated that some plant extracts have antibacterial activity against MRSA strains with therapeutic application in pharmaceutical formulation against *Staphylococcus* sp. (Mattana *et al.*, 2010; Karthy *et al.*, 2009).

Medicinal plants are also a rich source of antibacterial agents against other important infectious pathogenic strains: food-borne enteric *Salmonella typhimurum* (Ajayi and Akintola, 2010) and nosocomial *Klebsiella pneumoniae* (Sharma *et al.*, 2010).

Crude extracts from medicinal plants with potential antibacterial effect *in vitro* have been widely described by many researchers (El-Abyad *et al.*, 1990; Dall'Agnol *et al.*, 2003).

Some scientists have tried to isolate, qualify and quantify the single components of crude extracts possessing antibacterial properties. Antimicrobial activity of plant extracts may be attribute to the presence of phenolic compounds (phenolic acids, flavonoids, tannins etc.; Cushnie and Lamb, 2005). Table 1.14 presents some identified phenolic compounds derived from different parts of plant and their antibacterial activity against specified pathogenic strains.

Table 1.14: Plant phenolics and their antibacterial activity.

Indentified poliphenol compounds	**Origin**	**Tested pathogen organism**	**References**
quercetin	leaves of lotus	peridontitis bacteria: *Aggregaribacter actinomycetemcomitans, Actinomyces viscosus, Porphyromonas gingivalis, Fusobacterium nucleatum, Actinomyces. naeslundii*	(Li *et al.*, 2008)
rutin, epicatechin, dicaffeoylquinic acid, caffeic acid	ethanolic extract of peel apple of the Annurca (*Malus domestica var. Annurca*)	*Bacillus cereus*, *Escherichia coli* O157:H7	(Fratianni *et al.*, 2011)
anthocyanes, flavonoids, condensed tannins	ethanolic extract of leaves: *GuazumaulmifoliaLam*. (Sterculiaceae), *Acalypha guatemalensis* Pax & Hoffm (Euphorbiaceae), *Ocimum micranthum* Willd. (Lamiaceae) *Piper auritum* H.B.K. (Piperaceae) root: *Smilax spinosa* Mill. (Smilacaceae), leaves)	*Pseudomonas aeruginosa* ATCC 27853, *Salmonella, typhi* ATCC 14028 *Staphyloccocus aureus* ATCC6538	(Navarro *et al*., 2003)

phenolic acids consisting of C6-C1-COOH, and C6-C3-COOH	clonal oregano	*Helicobacter pylori*	(Chun *et al*., 2005)
hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside	leaves of olive (*Olea europaea*)	*Bacillus cereus,* *Escherichia coli,* *Salmonella enteritidis*	(Lee and Lee, 2009)
chlorogenic acids: 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid 4-O-caffeoylquinic acid	ethanolic extract of seeds of *Prunus mume*	*Staphylococcus aureus,* *Escherichia coli,* *Salmonella enterica,* *Virbio parahaemolyticus*	(Xia *et al.*, 2010)

An antimicrobial property of *Aesculus hippocastanum* has also been investigated. The study carried out by Roy *et al.* (Roy *et al.*, 2011a) showed that ethanolic and aqueous extract from horse chestnuts exhibited antifungal activity against *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigates*, *Mucor* sps. and *Penicillium morneffi*. The horse chestnut extracts were also effective against some oral bacteria (Roy *et al.*, 2011b).

Antioxidant effect

Antioxidants are those molecules that are able to inhibit the oxidation of other molecules. In response to the oxidation process, the body produces reactive oxygen species (ROS) and reactive nitrogen species (RNS), know as oxygen free radicals.

Overproduction of ROS resulted in oxidative stress which causes damage of important cell structures and components such as lipids, DNA and proteins (Valko *et al.*, 2007) and can lead to disturbances (both nuclear and mitochondrial) of redox-homeostasis. Imbalance of redox is often observed in cancer cells and is linked with DNA damage (Valko *et al.*, 2004; Valko *et al.*, 2006).

In response to oxidative stress the body develops several enzymatic defences (superoxide dismutase, catalases and glutathione peroxidases) and non-enzymatic cascades to protect the cytosol, nuclear, mitochondria, matrices and extracellular fluids (Sies, 1997).

Tocopherols, tocotrienols (Vitamin E) and carotenoids act as hydrophobic antioxidants in the lipid phase (membrane and LDL) where they protect against lipid peroxidation, whereas hydrophilic scavenger ascorbate is consider to be main antioxidant present in the cytosol, plasma or other body fluids. These molecules react with free radicals and thus prevent oxidative stress in the cell (Sies *et al.*, 1992; Chaudiere and Ferrari-Iliou, 1999).

The antioxidant activity of some polyphenols has been measured by ferric-reducing power determination by using a modified FRAP (ferric reducing/antioxidant power assay). Flavonoids (quercetin, rutin, and catechin), resveratrol, tannic acid, phenolic acids (gallic, caffeic, and ferulic) and carotenoids (β-carotene and zeaxanthine) have been tested. Tannic acid, quercetin, gallic acid and caffeic acid belong to those polyphenols that exhibited the highest antioxidant (AO) capacity in comparison to standards (trolox and BHA). In contrast resveratrol had the lowest AO. Carotenoids had no ferric reducing activity (Pulido *et al.*, 2000).

Another assay for screening of antioxidant activity uses α,α-diphenyl-β-picrylhydrazyl (DPPH). This method is based on reduction of DPPH in alcoholic solution by the antioxidant which acts as donor of hydrogen resulting in the formation of the non-radical DPPH-H (Sharma and Bhat, 2009). This method is the most frequently utilised procedure in determination of the antioxidant properties of plant extracts. For example the free radical scavenging activity of plant extract by DPPH assay was applied by Alviano *et al*. (Alviano *et al.*, 2008). The researchers investigated Brazilian medicinal plant extract from *Aristolochia cymbifera, Caesalpinia pyramidalis, Cocos nucifera* and *Ziziphus joazeiro* for potential antioxidant activity. All tested plant extracts indicated scavenging activities. Aqueous extracts of *Caesalpinia pyramidalis* and *Cocos nucifera* exhibited much better DPPH scavenging properties (antioxidant EC_{50}=15.2 and 10.0 µg/ml, respectively) in comparison to synthetic standard antioxidant butylated hydroxytoluene (BHT) (EC_{50}=86mg/ml).

Edible roots and stems of rhubarb (*Rheum ribes*) were also investigated for antioxidant activity by the DPPH method. Methanolic extracts of these exhibited significantly higher ($p \leq 0.05$) scavenging properties than BHT at concentrations ≥50µg/ml. Further investigation showed that both extracts were rich in flavonoids (Ozturk *et al.*, 2007).

Edible plants are a potential source of antioxidants due to their action as free radical scavengers and their ability to protect against lipid peroxidation (Arabshahi-D *et al.*, 2007; Gladine *et al.*, 2007).

Horse chestnuts are also source of antioxidant agents. In study carried out by Sato *et al*. (2005) the extract obtained from horse chestnut seeds exhibited an inhibitory effect on the autooxidation of linoleic acid (IC_{50}=0.2 mg/ml), moreover this effect was dose-dependent and the inhibition was almost complete at a concentration of 1 mg/ml. The extract also showed DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals and superoxide anions scavenging properties with EC_{50} of 0.65 and 0.21 mg/ml, respectively.

Role of phenolics in enzymatic inhibition of hyaluronidase and elastase

Hyaluronic acid (hyaluronan, HA) is a major glucoaminoglycan comprising monomers of D-glucuronate and N-acetyl-D-glucosamine connected by β-1,3 linkage (Nadarajah, 2008).

HA can be found in free form or as a component of proteoglycans and together with elastin, collagen and fibronectin forms a significant portion of the extracellular matrix. Due to its hydrophilic properties, HA is responsible for binding Ca^{2+} ions and holding water molecules (hydratation) thus keeping tissues moist and lubricated (Koolman and Rahm, 2005).

Elastin is a protein, mainly built (40%) from hydrophobic amino acids. Elastin, as a component of extracellular matrix, comprises elastic fibres which, in tissue of blood vessels, lung, skin and tendons, is responsible for elasticity (Mecham, 2011). Both hyaluronan and elastin undergo hydrolysiss by enzymes (hyaluronidase and elastase, respectively).

Hyaluronidase (HA-ase), an enzyme described in nomenclature as EC 3.2.1.35, belongs to the group of hydrolases, which cleave β 1,4 glycosidic linkage between N-acetyloglucosamine and D-glucuronate residues in hyaluronic acid (HA). HA-ase also hydrolyses 4-S and 6-S chondroitin sulphate and catalyses transglycosylation. HA-ase is mostly present in testes, liver lysosomes and serum (Garg and Hales, 2004).

Elastase (EC 3.4.21.36) is a protease that hydrolyses elastin. Elastase is produced in the pancreas in inactive form and is activated in the duodenum by trypsin. Another type of elastase is neutrophil elastase found in leucocytes and neutrophils (Nadarajah, 2008).

Imbalances between hyaluronidase and elastase production contribute to the degradation of HA and elastin in extracellular matrix and may lead to, for example, lung disorders, cardiovascular disorders, atherosclerosis and cancer (Royce and Steinmann, 2002). Skin ageing may also be caused by the action of elastase and hyaluronidase. Elasticity of the skin decreases when overproduction of elastase is observed and sagging process of skin

occurs more frequently, at the same time the destructive effect of hyaluronidase on hyaluronic acid is manifested by the appearance of wrinkles (Sahasrabudhe and Deodhar, 2010).

Inhibitory activity of some plant extracts on hyaluronidase and elastase enzymes has been investigated by Piwowarski *et al*. (Piwowarski *et al.,* 2011). Several medicinal plants were chosen and tested: Lythri herba (*Lythrum salicaria*- aerial part), Gei urbani radix cum rhizome (*Geum urbanum*- root, rhizome), Rubi idaei folium (*Rubus idaeus*- leaf), Quercus cortex (*Quercus roburr*- bark), Geranii pratenseae herba (*Geranium pratense*- aerial part), Rubi fruticosi folium (*Rubus fruticosus*- leaf), Geranii robertiani herba (*Geranium robertianum*- aerial part), Tormentillae rhizoma (*Potentilla erecta*- rhizome), Filipendulae ulmariae herba (*Filipendula ulmaria*- aerial part), Anserinae herba (*Potentilla anserine*- aerial part), Agrimoniae herba (*Agrimonia eupatoria*- aerial part) and Hippocastani cortex (*Aesculus hippocastanum*- bark).

It has been shown that aqueous extracts of those plants are rich sources of tannins and moreover possess inhibitory effects on elastase and hyaluronidase. The strongest inhibition on activity of hyaluronidase (64.9% at concentration of 10µg/ml) was observed for aqueous extract of Lythri herba. Bark of horse chestnut (*Aesculus hippocastanum*) contained 24.5% condensed tannins. It exhibited the strongest (among all plant extracts tested) inhibition on elastase (62% at concentration 10µg/ml) and this inhibitory effect was even greater than well-known elastase inhibitor- quercetin (46% inhibition at concentration of 10µg/ml). No anti-hyaluronidase activity for extract from horse chestnut bark was observed (Piwowarski *et al.*, 2011).

Phenolics as dietary components

Phenolic compounds of edible plants are very poorly absorbed in the small intestine and about of 90-95% dietary phenolics accumulate in the colon (Cueva *et al.*, 2010). It has been shown that a diet rich in polyphenols (especially in anthocyanins) have anti-thrombotic effect, manifested in reduction of platelet aggregation by 10-40% in humans. It is not known if polyphenols or their metabolites, or both are responsible for this effect, but dietary polyphenols are thought to play an important role in the prevention of diseases (Rechner and Kroner, 2005).

Tzounis *et al.* (Tzounis *et al.*, 2008) showed that some flavanols ((-)-epicatechin and (+)-catechin) stimulate the growth of gut microbiota. Bacteria present in the colon are able to metabolize the phenolic compounds which at the same time inhibit the

pathogenic strains. The research suggests that diets rich in phenolic compounds have a prebiotic effect on probiotic commensal bacteria in the human gut.

1.4.1.8 Terpenoids and sterols

Terpenes, known also as isoprenoids, are a large class of products of secondary metabolism. Over 30,000 terpenes are plant derived as listed on Dictionary of Natural Products (Crozier *et al.*, 2007).

Terpenes are derived from the 5-carbon alkene isoprene. Through the mevalonic acid pathway, isoprene is converted to more complicated structures giving products constructed from five carbon building units such that the final product may have a C_5, C_{10}, C_{15}, C_{20} , etc skeleton. Mevalonic acid is the precursor of all terpenoids. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are parents of classes of terpenes such as hemiterpenoids. Other parents of terpenes are geranyl pyrophosphate (GPP) – monoterpenoids; farnesyl pyrophosphate (FPP) – sesquiterpenoids; geranlgeranyl pyrophosphate (GGPP) – diterpenoids; geranylfarnesyl pyrophosphate (GFPP) – sesterterpenoids; squalene – triterpenoids; phytoene – carotenoids. This general rule of biosynthesis of terpenoids was considered by Rudzicka in 1953 (Mann *et al.*, 1994).

These molecules are of particular interests to scientists because of their important role as fragrances in perfumery (essential oil terpenes) and as flavours. Biological function of natural terpenes include antimicrobial agents, plant hormones, lipids membranes, and attractants for animal pollinators, anti-feedants, and electron transporters during photosynthesis (Crozier *et al.*, 2007).

1.4.1.9 Hemiterpenes and monoterpenes

Fifty hemiterpenes (C_5) have been isolated. Prenol is an example of a hemoterpene occuring in natural oils of flowers of the Cananga tree (*Cananga odorata*) and the oil of hops of *Humulus lupulus.* Oranges and grapefruits contain S-(-)-3-Methyl 3-buten-2-ol. The flavour of blackcurrant (*Ribes nigrum*) is due to the presence of other hemiterpene-4-Methoxy-2-methyl-2-butanthiol (Crozier *et al.*, 2007). Humulone is responsible for the bitter taste of hops (Mann *et al.*, 1994).

Monoterpenes (C_{10}) are distributed widely in plants and the number of well documented monoterpenes is 1,500. They are colourless, mostly volatile oils, giving flavours and aromas in plants. Most of them are essential oils playing important roles as attractants for pollinator animals, some of them are natural plant defences with insecticide, anti-feedant and antibacterial properties (Rameshwar Singh, 2010). Monoterpenes are also valuable for application in herbal medicine due to their antibacterial, antiseptic and antihelmintic properties (Crozier *et al.*, 2007; Breitmaie, 2006).

Monoterpenes have significance in perfumery and the flavour industry as they are volatile oils from different parts of plants (Baker, 1930).

1.4.1.10 Diterpenes

Diterpenes (C_{20}) represent terpenes with more folded and cyclised molecules than monoterpenoids. In addition they contain a higher number of double bonds (Crozier *et al.*, 2007).

Diterpenes play an important role as phytohormones (such as gibberellins) which are responsible for the growth and development of plants (stem elongation, flowering, seed development). Gibberellins are a large group of natural products, more than one hundred of these natural components are known to be distributed not only in higher plants, but also in fungi and bacteria (Hedden and Phillips, 2000). Taxol (Figure 1.24) is an example of an important diterpene due to its anticancer properties (Marth *et al.*, 1995).

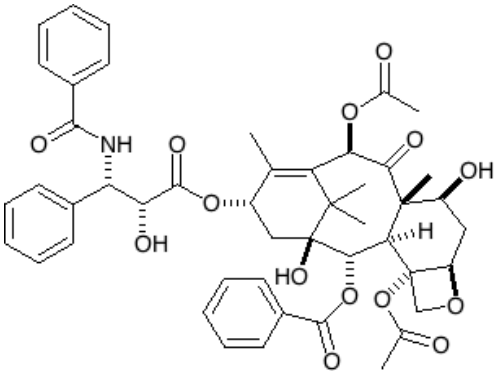


Figure 1.24: Structure of taxol.
Source: (Goodman, 2012).

1.4.1.11 Carotenoids

Carotenoids are C_{40} isoprenoids (tetraterpenes) mainly responsible for colouration (yellow and orange) of flowers and fruits, that make the plants more attractive for animals as well as playing an important role in photosynthesis as "light catcher" elements (Crozier *et al.*, 2007).

Humans cannot synthesise carotenoids therefore these natural components must be obtained from diet. A carotenoid rich diet is essential to maintain the health of the human body. Beta carotene, lutein, lycopene, β-cryptoxanthin and zeoxanthin are examples of carotenoids which possess bioactive properties (Britton *et al.*, 2009).

1.4.1.12 Triterpenes and sterols

Triterpenes are C_{30} polycyclic isoprenoids derived from condensation of two farnesyl pyrophosphates (FPP) to form squalene (Figure 1.25). Squalene may cyclized giving more complicated structures of triterpenes and sterols (Seigler, 1998).

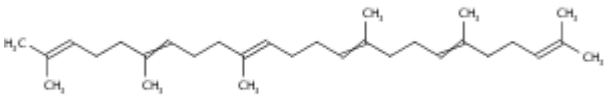


Figure 1.25: Structure of squalene.
Source: (BMRD, 2012).

More than 4,000 triterpenes have been isolated. Structurally triterpenoids can be divided into 40 different types (Seigler, 1998). Saponins (C_{30}) are a group of non-volatile triterpene glycosides widely distributed in plants. The name "saponins" is derived from Latin "*sapo*" meaning "soap". These natural secondary metabolites consist of non-polar aglycones paired with sugar moieties (with polar properties; Vincken *et al.*, 2007). This combination allows saponins to be distinguished from other glycosides and provides surface-active property that allows saponins to dissolve in water, making colloidal solutions. Saponins, due to their foam forming properties, have been used as soap for centuries (Mann *et al.*, 1994).

Based on the nature of aglycone, saponins can be divided into three groups: steroidal saponins (present mostly in monocotyledonous angiosperm), triterpenoids saponins (present mostly in dicotyledonous angiosperm) and steroidal alkaloids (Sparg *et al.*, 2004).

Another system of classification of saponins is based on the formation of carbon skeletons during metabolism of triterpenes and steroids. In this way 11 classes of saponins may be distinguished: dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids. The structure of the main saponins and their derivatives is presented in Figure 1.26. The dammaranes, lupines, hopanes, oleananes, ursanes and steroids can be divided into 16 subclasses due to the changes in their structural carbon skeleton in which fragmentation, homologation and degradation reactions occur.

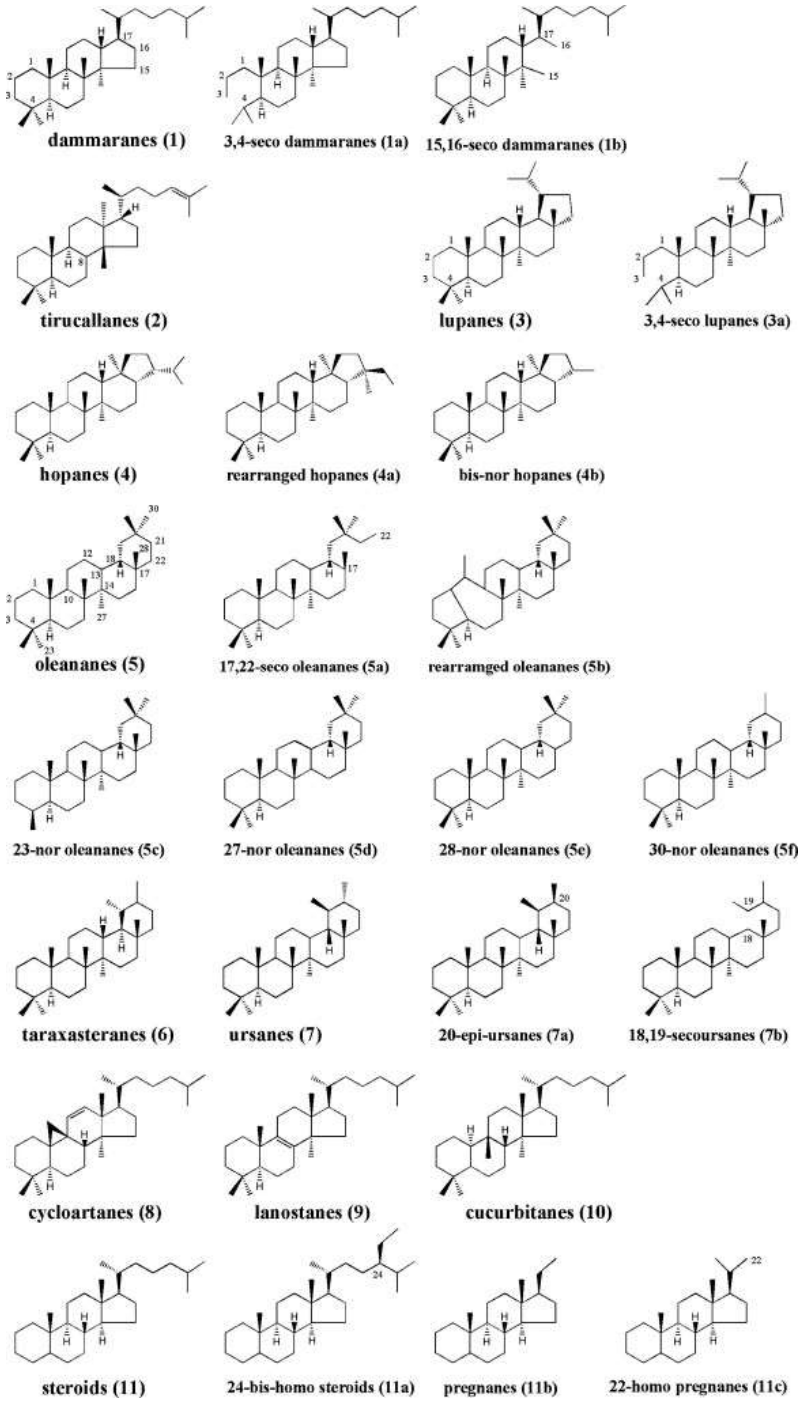


Figure 1.26: Structure of main 11 classes of saponins and their derivatives.
Source: (Vincken *et al.*, 2007)

The most common saponins found in plants are oleanane type (nr.5 in Figure 1.26). In oleananes, carbons at C_4, C_{17} and C_{20} have a diverse variety of substituents attached. The methyl (CH_3) and hydroxyl groups (OH) can also be attached at different positions. Some methyl groups can be processed and oxidized making other substituents such as: CH_2OH, CHO and COOH (Vincken *et al.*, 2007). An example of an oleanane type

saponin is escin molecule (see Figure 1.5), a naturally occurring triterpene saponin found in the seeds of horse chestnut tree (*Aesculus hippocastanum*). The structure of escin and its biological properties are described in section 1.1.6. The biological and pharmaceutical properties of some other saponins are presented in Table 1.15.

Table 1.15: Biological activity of plant saponins.

Biological activity	Examples	Source	References
Haemolitic activity	**escin saponin**	***Aesculum hippocastanum***	(Oda *et al.*, 2000)
		Zizyphus jujuba	
	oleanane type triterpene saponin	leaves of *Maesa lanceolata* (methanolic extract)	(Sindambiwe *et al.*, 1998)
	oleanane type triterpene saponin	bark of *Pometia ridleui* (ethanolic extract)	(Voutquenne *et al.*, 2003)
	saiksaponins (A,D,E)	roots of *Bupleurum falcatum*	(Ahn *et al.*, 1998)
Malluscicidal activity	monodesmosidic saponins of serjanic and spergulacenic acid	berries of *Phytolacca icosandra*	(Treyvaud *et al.*, 2000)
	maesasaponin VI_2	leaves of *Maesa lanceolata*	(Apers *et al.*, 2001)
Antiinflammatory activity	fruticesaponin A,C	*Bupleurum fruticescens*	(Just *et al.*, 1998)
	escin	***Aesculum hippocastanum***	**(Sirtori, 2001)**
	kalopanaxsaponin A, pictoside A	bark of *Kalopanax pictus*	(Li *et al.*, 2002)
	steroidal saponin	bulb of *Allium ampeloprasum var.porrum*	(Adao *et al.*, 2011)
	liniceroside C	aerial parts of *Lonicera japonica*	(Son *et al.*, 2003)
Antifungal/antiyeast activity	maesasaponin	*Maesa lanceolata*	(Sindambiwe *et al.*, 1998)
	jujubogenin saponin	stem of *Colubrina retusa* (ethanolic extract)	(Lee *et al.*, 1999, Li *et al.*, 1999)
	monodesmosidic saponins	*Hedera colchica*	(Mshvildadze *et al.*, 2000)
	saponin mixtrure	seeds of *Chenopodium guinoa*	(Woldemichael and Wink, 2001)
	furostanol saponins (capsicoide E,F,G)	seeds of *Capsicum annuum*	(Iorizzi *et al.*, 2002)
Antibacterial/ antimicrobial activity	oleanane type: nudicaucins A,B,C guaiacin D	*Hedyotis nudicaulis*	(Konishi *et al.*, 1998)
	5β-spirostan-3β-ol saponins	*Yucca schidigera*	(Killeen *et al.*, 1998)
	jujubogenin saponin	stem of *Colubrina retusa*	(ElSohly *et al.*, 1999)
Antiparasitic activity	glinosides A,B	aerial part of *Glinus oppositifolius*	(Traore *et al.*, 2000)
	α-hederin, β-hederin, hederacolchiside	*Hedera helix*	(Ridoux *et al.*, 2001)

Citotoxicity/Antitumor activity	furcreastatin	leaves of *Furcraea foetida*	(Itabashi *et al.*, 1999)
	bisdesmosidic spirostanol saponin: aculeosides B	underground parts of *Ruscus aculeatus*	(Mimaki *et al.*, 1998a)
	manogenins	rhisomes of *Hosta sieboldii*	(Mimaki *et al.*, 1998b)
	spirostanol, furostanol	rhisomes *of Dracaena angustifolia* Roxb.	(Tran *et al.*, 2001)
	oleanane derived saponins	root and bark of *Aralia dasyphylla* (ethanolic extract)	(Xiao *et al.*, 1999)
	ginseng saponine metabolite (IH-901)	*Panax ginseng*	(Lee *et al.*, 1999)
Antiviral activity	oleanane type triterpene saponin	Brazilien and Chinese plant	(Simões *et al.*, 1999)
		Fabaceae family	(Kinjo *et al.*, 2000)
	maesasaponin VI_2	leaves of *Maesa lanceolata*	(Apers *et al.*, 2001)
	arganine C	fruits of *Tighemella heckelii*	(Gosse *et al.*, 2002)
	mixture saponins	tea seeds of *Camellia sinensis var.sinensis*	(Hayashi *et al.*, 2000)
	escin (Ia,Ib), isoescin (Ia,Ib), escin IV(c-f)	seeds of *Aesculum chinensis*	(Yang *et al.*, 1999)
Other biological activity			
aphrodisiac	ginsenosides	root of *Panax ginseng*	(Nocerino *et al.*, 2000)
wound healing	ginseng saponine	*Panax ginseng*	(Kanzaki *et al.*, 1998)
anticomplementary	ginseng saponine	root of *Panax ginseng*	(Kim *et al.*, 1998)
immunopotentation	saponin fraction	root of *Polygala senega*	(Estrada *et al.*, 2000)
hepatoprotective	notoginsenosides O,P,Q,S,T	flower buds of *Panax notoginseng*	(Yoshikawa *et al.*, 2003)
antistress	ocotillol type saponins: majonside R2(MR-2)	Vietnamese ginseng	(Huong *et al.*, 1998)
neuroprotective	ginsenosides Rb1,Rg1	root of *Panax ginseng*	(Liao *et al.*, 2002)
antigenotoxic	soyasaponins B, DDMP soyasaponins	soybean molasses	(Berhow *et al.*, 2000)
hyperlipidemic	saponin fractions	rhisomes of *Acorus calamus*	(Parab and Mengi, 2002)

Steroids are derived from triterpenoids due to their basic skeleton (Mann *et al.*, 1994). Cholesterol is the most familiar example of steroid. Cholesterol plays a primary role in building human membranes, but it is also present in plants in very small quantities (Crozier *et al.*, 2007).

A large amount of sterols are accumulated in plants. Phytosterols (plants sterols) are widely distributed in vegetable oils, nuts, seeds, fruits and cereals. Phytosterols (Figure 1.27) have a similar structure to cholesterol and differ only by an addition of methyl

groups (campesterol) or ethyl group (sistosterol) at C_{24}, or additional double bond at C_{22} (brassicasterol and stigmasterol, respectively) (Jansen *et al.*, 2006).

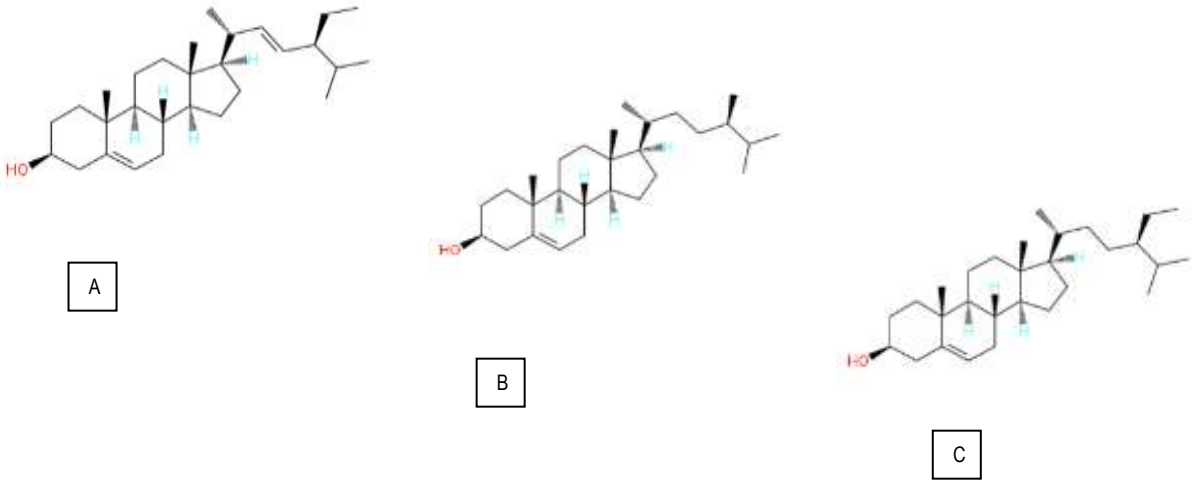


Figure 1.27: Structure of stigmasterol (A), campesterol (C) and sistosterol (C).
Source: (Molecular, 2012).

Sistosterol, campesterol and stigmasterol (Figure 1.27) belong to plant phytosterols present in phospholipid membrane of cells playing important roles in integrity and rigidity of the membrane. Plant sterols lower cholesterol level, especially LDL fraction, thus having significance in decreasing the risk of coronary disease (Katan *et al.*, 2003). It has also been found that diets rich in phytosterols can protect against cancer (Ifere *et al.*, 2009; Mendilaharsu *et al.*, 1998).

1.4.1.13 Sesquiterpenes

Sesquiterpenes (C15) are terpenes derived from farnesyl pyrophosphate (FPP) and comprise the most numerous of all terpenes with 5,000 known compounds (Seigler, 1998). These natural secondary metabolites are produced by fungi, marine organisms and *Streptomyces sp.* but a large number of flowering plants also produce sesquiterpenes where their major function is as components of essential oils (Crozier *et al.*, 2007). Insect attractants, antifeedants, phytoalexins, allomones and allelopathy are also characteristic biological roles of sesquiterpenes (Araceli *et al.*, 2007; Gonzalez *et al.*, 1997; Seigler, 1998), while many sesquitrepenes also have therapeutic properties.

1.5 Horse chestnut residue

The Horse Chestnut by-product referred to in this thesis was produced by Temmler Ireland (Ltd.) at their facility in Killorglin, Co. Kerry. Horse chestnuts were extracted seven times with absolute alcohol. The extract was isolated and reduced by heating to a specified concentration and colour. A seed extract of *Aesculus hippocastanum* contains escin, a triterpene saponic, as its active component. The purpose of the alcoholic extraction is to remove escin which is purified for pharmaceutical use. The remaining horse chestnut material (Figure 1.28) is collected by filtration and allowed to dry. The residue was collected from Temmler. The sample residue, subjected in this study, derived from one single batch collected in March 2008 and then packed in plastic bags and stored in a freezer at -20°C until use.

Figure 1.28: Horse chestnut residue.

1.6 Objectives of Research

Horse Chestnut extract is a novel waste produced by a local company in Co. Kerry following extraction of pharmaceutical grade product. Its composition was unknown and a preliminary objective of this work was to carry out a proximal analysis on the residue. The second major objective was to investigate the feasibility of adding some commercial value to a waste material which is currently supplied to local farmers for animal feed.

Following a literature review, it was decided that there were a number of possible avenues of exploration for use of this material:

1. As a fertiliser or soil conditioner.

2. Animal feed or supplement. The residue was, however, already being sold to farmers as a feed but at a low cost. It was felt that this application would provide only limited additional value.
3. Due to the high carbohydrate content and anecdotal evidence from farmers of improvements in animals fed on the horse chestnut extract, a possible role as a prebiotic.
4. Investigation of antimicrobial activities or effects.
5. As a potential source of enzyme inhibitors of therapeutic interest.

The studies carried out were with a view to finding an alternative use or carrying out further extractions on the Horse Chestnut residue in order to gain added value.

1.7 Introduction to the analytical techniques used in this study

1.7.1 Analysis of horse chestnut waste

1.7.1.1 Moisture content

Moisture content of a sample is called "water content" and can be established by gravimetric analysis. This method involves measuring the weight of a sample residue before and after a suitable duration of heat treatment.

1.7.1.2 Ash content

Ash content is the inorganic residue remaining after burning of organic material.

1.7.1.3 Determination of metal elements by atomic emission/absorption and colorimetric techniques

The concentration of metal elements in a sample can be determined by atomic absorption/emission spectroscopy as well as various colorimetric techniques. Atomic absorption spectroscopy (AAS) is based on absorption at a specific wavelength of light by an element. It is a very sensitive method and consists of the following steps:

1) First the sample is atomized (i.e. converted into ground state free atoms).

2) The cloud of atoms is produced by aspirating the sample in solution into a flame.
3) A beam of light of the appropriate wavelength is passed through the vaporized sample. The light is generated by a hallow cathode lamp and its wavelength is specific to the particular atom.
4) Light is absorbed by atoms in the sample and the absorbance is directly proportional to the number of atoms.
5) The light is directed onto a detector that records electrical signals proportional to the light intensity. Absorbance of the light by atoms is then calculated by the spectrophotometer automatically.
6) Using standard solutions of minerals of known concentrations allows construction of standard curves, which are used in calculation of the mineral concentration in the unknown sample.

Some minerals such as potassium and sodium can be determined by emission spectrophotometry (called also flame photometry). The method consists of two main stages (James, 1999):

1) Heating of mineral solution in a flame. Electrons of metals are excited to higher energy level and visual colour is observed (characteristic for each metal).
2) Electrons drop back to the lower energy levels, releasing excess energy as light (wavelength) and its intensity corresponds to the concentration of mineral present.

Atomic emission was used for the determination of sodium and potassium. This method does not use a hollow cathode lamp. Atomic emission has good specificity although precision is considered to be low.

Colorimetric techniques for determination of minerals are based on changing colour of sample after treating with appropriate reagent. HACH offers many different methods for determination of mineral content, one of which is determination of phosphate concentration by the Ascorbic Acid Method. This method uses a mixture of ascorbic acid and sodium molybdate. Sodium molybdate forms a complex with phosphate ion which is subsequently reduced by the ascorbic acid and forms a blue species which can be detected with a spectrophotometer at 890nm. The intensity of the blue color is

proportional to the concentration of phosphate in the sample. A phosphate standard solution is used for the checking the accuracy of the test (HACH, 1990).

1.7.1.4 Protein content

The reference protein assay (Kjeldahl method) is the determination of nitrogen content and subsequent conversion to protein content. Generally, pure protein solution contains 16% of nitrogen. This factor is very useful for calculation of protein content which is established by multiplying nitrogen content by the factor 6.25=100/16 (Hui, 2006). Conversion factor may vary for specific proteins, however. The method was established by Kjeldahl in 1883 and estimates the total nitrogen content present as –NH- in the sample. The result is then converted into % of protein using the appropriate formula.
The procedure has three different steps:

1) Digestion - the sample is digested in sulphuric acid (at 400°C) until carbon and hydrogen is oxidized. All nitrogen in proteins is converted into ammonium sulfate:

$$\text{N - from proteins} + H_2SO_4 \rightarrow (NH_4)_2SO_2 + H_2O + CO_2 + SO_2$$

2) Distillation - the ammonium sulfate solution is alkalized by adding sodium hydroxide. The ammonia is then removed from ammonium sulfate under steam distillation and goes to a flask with boric acid (Figure 1.29) where the complex $NH_{4+}{:}H_2BO_3$ is formed:

$$(NH_4)_2SO_2 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

$$NH_3 + H_3BO_3 \rightarrow NH_4^+{:}H_2BO_3 + H_3BO_3$$

3) Titration – ammonium is ousted from the complex $NH_{4+}{:}H_2BO_3$ by adding hydrochloric acid. The amount of hydrochloric acid used is proportional to the amount of nitrogen present in the sample (1 mole of HCl = 14 grams of nitrogen). Because the complex makes boric acid alkaline, using a pH indicator it is possible to see the moment of end-point by titration with hydrochloric acid (Cole-Parmer, 2010):

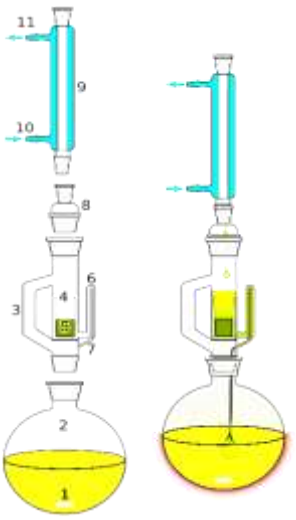
$$NH_{4+}{:}H_2BO_3 + HCl \rightarrow NH_4Cl + H_3BO_3$$

1. Turn on/off
2. Steam generator
3. Kieldahl tube
4. Conical flask with boric acid
5. Pumps
6. Cooling water sensor
7. Flask with boric acid

Figure 1.29: 2100Kjeltec Distillation Unit.
Source: (Busan, 2006).

1.7.1.5 Lipid content

One of the methods used for determination of lipids is petroleum ether extraction. Petroleum ether is an organic solvent in which lipids are soluble and is more selective towards true lipids. The Soxhlet method was used based on extraction with petroleum ether. The solvent in a flask is heated then evaporates and in turn is cooled in a condenser. Petroleum ether passes through the crushed sample solubilising lipids and is collected in a round bottom flask (Figure 1.30). After extraction petroleum ether is removed by evaporation. Total lipid content was determined gravimetrically. The experiment was carried out in triplicate.



1. Extraction solvent
2. Extraction pot
3. Distillation path
4. Cellulose thimber
5. Extracted sample
6. Syphon arm inlet
7. Syphon arm outlet
8. Adapter
9. Condenser
10. Cooling water in
11. Cooling water out

Figure 1.30: Soxhlet apparatus.
Source: (AbsoluteAstronomy.com, 2010b).

1.7.1.6 Analysis of fatty acids by gas chromatography

There are many of methods of fatty acids analysis but gas chromatography (CG) offers many advantages over other procedures is great for the following reasons:

- small amount of sample is used
- fast and simple preparation of sample
- good qualitative and quantitative procedure for fatty acids

Described by James and Martin (1952) gas chromatography showed separation of normal saturated fatty acids but only up to 12 carbon atoms in chain (James and Martin, 1954). In 1953 Cropper and Heywood further developed the method by separating methyl esters of fatty acids up to behenic acids (Cropper and Heywood, 1953). Now it is possible to detect and estimate methyl esters of fatty acids with up to 34 carbon atoms in chain.

The procedure involes:

a) Methanolysis in the presence of alkaline. Lipids are hydrolyzed to their constituent fatty acids.
b) Methylation in the presence of boron trifluoride which consequently leads to esterification of fatty acids. The methyl esters of most fatty acids have higher volatility and are more easily separated by gas chromatography.
c) Separation of methyl esters in gas chromatography column. The machine should be set to an appropriate program to obtain the best separation result. Fatty acids are identified by comparison of their retention times with standards. They are also quantified by measurement of peak area (James, 1999).

1.7.1.7 The cultivation of probiotic bacterial strains

A large number of different media and agars for bacterial cultivation are used in microbiology. The choice of the correct growth medium is important in optimising growth of different bacterial strains. *Lactobacillus* species belong to Gram-positive, non-spore forming, catalase negative strains. They are able to ferment carbohydrates and produce lactic acid, therefore these strains have specific growth requirements. The production of high concentrations of lactic acid by *Lactobacillus* can reduce pH to a level that inhibits growth of other undesirable microorganisms. *Lactobacillus* species

are also facultatively anaerobic and grow best on MRS agar/broth. Additionally, a carbon dioxide rich atmosphere and incubation temperature of 35-37°C are desirable. *Bifidobacterium* species are also Gram-positive, catalase negative, anaerobic bacterial strains. A carbon dioxide rich atmosphere improves their growth significantly. Thus they may be cultivated under the same condition as *Lactobacillus* species.

1.7.1.8 Determination of the prebiotic potential of horse chestnut waste

One of the main aims of this experiment was to measure the prebiotic effect of horse chestnut waste extract in an *in vitro* model. To this end, utilisation of prebiotic carbohydrates widely available on the market was compared with fermentation of horse chestnut waste extract. The prebiotic effects were monitored by measuring optical densities of the culture, which reflects the growth in bacterial numbers, and by determination of pH level, which decreases with increasing short chain fatty acid production. The quantity of material extracted from horse chestnut residue was also determined in different extraction conditions.

1.7.2 Analysis of lyophilised horse chestnut aqueous extract

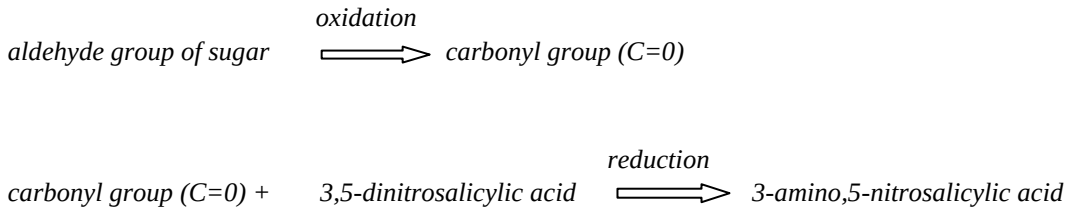
1.7.2.1 Determination of protein content by Bradford method

Marion M. Bradford method is based on absorbance change of Coomassie Brillant Blue (CBB) G-250 from 465nm to 595nm after binding with protein. CBB G-250 in acid solution is red in colour, but after reaction with protein, a visible blue colour change is observed and its intensification is proportional to the protein concentration (Bradford, 1976). Bradford is a rapid and sensitive method between 5-200 micrograms protein.

1.7.2.2 Determination of reducing sugars by dinitrosalicylic colorimetric method

Reducing sugars (e.g. glucose) contain aldehyde functional group or ketose functional group (e.g. fructose). In dinitrosalicylic (DNS) colorimetric method these functional

group of sugars are first oxidized to form free carbonyl groups (C=O) which subsequently reduce 3,5 dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid under alkaline conditions:



During alkaline reduction of DNS red-brown color is observed as a result of forming 3-amino,5-nitrosalicylic acid. Absorbance at 546nm is proportional to the amount of reducing sugars in a sample (Nam Sun Wang, 2009).

1.7.2.3 Determination of phenolic content by Folin-Ciocalteau method

The total phenolic content in lyophilised of horse chestnut aqueous extract was determined by Folin-Ciocalteau (FC) method. Folin-Ciocalteau method has been used for estimation of total concentration of antioxidants. The method is based on forming a coloured product by reaction of Folin reagent (mixture of tungsten and molybdenum oxides) with phenolic groups of tested molecules. The products are blue in colour and are indicated by the absorbance at 750nm, and their intensity is proportional to the concentration of phenolic content (Waterhouse, 2002).

1.7.2.4 Analysis of lyophilised horse chestnut aqueous extract by Anion/Cation Exchange Chromatography

Chromatography refers to the method of separation of a mixture of compounds in a flowing system on a column with a specialist absorbent (Weston and Brown, 1997). In a flowing system there is a mobile phase which can be liquid or gas. There are many different absorbent materials developed for use in a column and the choice is dependent on the types of components in a mixture. Liquid chromatography (LC) is used to separate a mixture in solution. In this case the mobile phase is a liquid. In High Pressure Liquid Chromatography (HPLC), the mobile phase is mechanically pumped into the

column. This system is supplied with an injector, a pump, a column and a detector (Stavros, 2000).

Different liquid chromatographic methods are classified according to their mechanism of sample retention as follows: Adsorption chromatography (Normal Phase Chromatography), Partition chromatography (Reverse Phase Chromatography), Size Exclusion Chromatography, Affinity Chromatography and Ion Exchange Chromatography (Weston and Brown, 1997).

Ion Exchange Chromatography was used to determine sugar content in the reported work, and therefore this process is described below:

An ion-exchange chromatography column has a special stationary phase to which ionic components are attached. These ionic "elements" can be different and their specific activity site depends on their ionic character (e.g. presence of carbonyl or sulphonate groups) which gives the column cation exchange properties (Cation Exchange Chromatography). The presence of, for example, ammonium groups gives anionic exchange character (Anion Exchange Chromatography; James, 1999). The most suitable sample components to be separated on this type of column are ionized. Ionic forms of constituents present in a sample pass through the column and exchange with stationary phase ions. This process allows separation of charged molecules from other components in a sample. Molecules which have stronger charge bind more strongly to the stationary phase and consequently are eluted more slowly from the column.

Detection of components of a sample is obtained by different HPLC detectors and the choice of detector depends on the characteristics of the sample of interest.

Refractive Index (RI) detector, used in this research, is based on slowing the speed of light which passes through the separated molecules. The beam of light passes through the mobile phase at a particular angle, but this beam is refracted (bent) when it passes through a molecule of different density to the mobile phase (Figure 1.31). The changes in the RI of the mobile phase containing a particular compound are proportional to the concentration of the compound present (Watson, 1999)

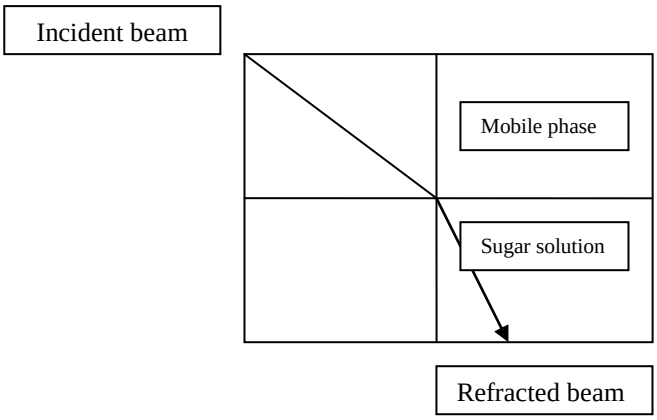


Figure 1.31: Refraction of beam of light passing from mobile phase to a sugar solution.

This method of detection is very useful for the study of components such as sugars. Moreover it allows also rapid measurement of sugar content by direct calibration.

1.7.2.5 Analysis of lyophilised horse chestnut aqueous extract by Size Exclusion Chromatography using FPLC system

Fast Protein Liquid Chromatography (FPLC) was developed for protein separation but can also be used to separate other biologically important molecules (nucleic acids, sugars). FPLC is similar to HPLC system with some differences:

- Lower operating pressure range of 0-4 MPa in comparison with HPLC 1-40 MPa (this permits the use of a wider range of columns).
- Solvent flow rate of 1-499 ml/hr while HPLC pump typically operates at 0.6-600ml/hr
- Mostly used for separation of biological components (Doonan, 1996).

FPLC permits a wide range of column matrices. In this research FPLC was used for size exclusion chromatography (SEC; also known as gel filtration or gel permeation chromatography).

This chromatographic method separates molecules based on their size and shape. The stationary phase (matrix) has a specific pore size. Small molecules, which pass through the column and penetrate inside the pores of the matrix, are retained and consequently are eluted from the column later whilst large molecules are not retained because they are unable to penetrate the pores and are eluted quickly (Dubin, 1988). A variety of

stationary phases are available (cross-linked dextran, polyacrylamide for low pressure SEC and styrene-divinylbenzene copolymers, silica or porous glass for high pressure SEC). Each has a strictly defined fractionation range (M_r range) which is dependant on pore size (Å) and is related to the chain length of a molecule just large enough to be totally excluded from all the pores of the gel (Sandie, 1992).

In size exclusion chromatography, molecular standards (molecules with well know molecular weight) are used. Important criteria in molecular mass determination are geometric column volume (V_C) determined by suppliers and included in the specification of the column, and void volume (V_O), the volume of mobile phase that is external to the stationary phase particles. This value is usually obtained by injecting and detecting a compound of known molecular weight which falls above the fractionation range of the designated column, and the volume of liquid that passes through the column from the time of injection to detection is the void volume. Elution volume (Ve), named also as retention volume (V_R), is the volume needed to elute a molecule from the point of injection to the centre of elution peak. These 3 values (V_C, V_O and Ve) are used for calculation of partition coefficient (K_{av}) and construction of a calibration curve (K_{av} vs. log molecular weight of standards; Healthcare, 2011). The component of interest is treated in a similar manner to determine K_{av} value. The calculation of the molecular mass is carried out using the calibration curve derived from molecular standards.

1.7.2.6 Determination of sugar content in fractions of lyophilised horse chestnut aqueous extract by Dubois assay

The main aim this part of the research was to establish total sugar content in fractions of the lyophilised horse chestnut aqueous extract (lHCE). The Dubois assay is a simple and sensitive method and allows effective determination of the presence of sugar monomers, complex soluble carbohydrates and even crystalline cellulose in a sample (Taylor, 1995). Simple sugars, oligosaccharides and also their derivatives (methyl esters) when treated with phenol-concentrated sulphuric acid mixture give an orange colour which is stable and detected on spectrophotometer at 490nm The intensity depends on sugar (or their derivatives) content in a sample (DuBois *et al.*, 1956).

1.7.2.7 Determination of protein content in fractions of lyophilised horse chestnut aqueous extract by Bradford assay

Bradford assay, previously described in section 1.7.2.1 of this thesis, a simple and fast method for determination of total protein content, was used to analyze the fraction from gel filtration chromatography by FPLC. The Bradford assay is based on dye-binding of Coomassie Brilliant Blue G-250 to protein present in a sample. The optical character of the protein-dye complex is different to the protein or dyes alone, and allows quantitative determination of the protein present in a sample.

1.7.2.8 Estimation of molecular mass of sugar fraction of lyophilised horse chestnut aqueous extract by size exclusion chromatography

The lyophilised horse chestnut aqueous extract (lHCE) was subjected to molecular mass analysis by size exclusion chromatography using the FLPC system. The experiment consisted of two steps. First dextran standards with known molecular weight were fractionated on size exclusion column and then all fractions were subjected to analysis for total sugar content by modified Dubois assay. The fractions consisted mainly of carbohydrate, therefore an assumption is made that the molecular weight described refers to the carbohydrates present.

1.7.3 Determination of the prebiotic index (PI) for horse chestnut aqueous extract (HCE)

The ability of the residue to support growth of a broad range of probiotic strains such as *Lactobacillus* and *Bifidobacterium* species was investigated. A comparison of performance to existing commercially available prebiotics (Fructooligosaccharide, Galactooligosaccharide, Inulin) was also carried out. In order to compare the efficacy of prebiotics for specific probiotic strains, a quantitative approach was required. The method used was based on incubation of lactobacilli and bifidobacteria in MRS medium enriched with different prebiotics, HCE or glucose under defined conditions (time,

temperature, gas composition) and measurement of the change in cell biomass (as optical density at 600nm). The growth of probiotic strains is then compared to growth of non-probiotic, enteric strains (e.g. *E. coli*) grown in M9 medium under the same conditions.

The prebiotic activity score, also referred to as prebiotic index (PI), represents the ratio of probiotic growth on prebiotics relative to their growth on non-prebiotic sugar (glucose) and relative to the the growth of a non-probiotic organism (*E. coli*).

The determination of prebiotic activity score was carried out following the procedure established by Huebner *et al.* (Huebner *et al.*, 2007) with some changes. The formula thus derived provides a method of calculating a PI scores for each prebiotic:

PI =

[(probiotic OD at 24h on prebiotic – probiotic OD at 0h on prebiotic) – (probiotic OD at 24h on control MRS – probiotic OD at 0h on control MRS)] /

[(probiotic OD at 24h on glucose – probiotic OD at 0h on glucose) – (probiotic OD at 24h on control MRS – probiotic OD at 0h on control MRS)]

divided by

[(enteric OD at 24h on prebiotic – enteric OD at 0h on prebiotic) – (enteric OD at 24h on control M9 – enteric OD at 0h on control M9)] /

[(enteric OD at 24h on glucose – enteric OD at 0h on glucose) – (enteric OD at 24h on control M9 – enteric OD at 0h on control M9)]

The growth of prebiotic and enteric strains was also monitored in MRS/M9 medium without carbohydrates added. A positive PI score is obtained where probiotic strain growth is better on a prebiotic than on glucose and/or at the same time the probiotic did not support the growth of an enteric strain (*E. coli*). Very low or negative PI scores were obtained for those prebiotics that did not support the growth of prebiotic (in comparison to the growth on a control, glucose) and/or supported the growth of enteric strains better than the probiotic tested.

1.7.4 Determination of the effect of different processing conditions on prebiotic activity of horse chestnut extract

The desired functionality of commercial prebiotics requires them to be both chemically and biologically stable under a variety of processing conditions. This ensures that, at

minimum, the prebiotic retains its growth enhancing effect on probiotic bacteria. In some cases processing treatments (such as heat) can improve the stimulation of the growth of bifidobacteria in the gut (Böhm *et al.*, 2006).

The lyophilised horse chestnut extract (lHCE) was investigated for chemical and functional stability under different processing treatments. The research procedure was based on previous work established by Huebner *et al.,* in 2008 (Huebner *et al.*, 2008). The study was divided into three:

a) Examination of the effect of pH: lHCE was dissolved in the buffer pH 3.0, 4.0, 5.0, 6.0 and held at ambient temperature for 24 hours.
b) The effect of pH and heat: lHCE was dissolved in the buffers of pH 3.0, 4.0, 5.0, 6.0 and heated at 85ºC for 30 min.
c) lHCE was dissolved in the buffer with glycine and allowed to undergo the Maillard reaction.

After treatment the prebiotic activity of the lHCE was determined. For comparison, commercial prebiotics (FOS, GOS) were also treated in similar manner. The stimulatory effect of treated lHCE/commercial prebiotics was observed when compared to untreated controls. Probiotic *Lactobacillus* and *Bifidobacterium* species were the test organisms as these were known to respond positively to each of the prebiotic candidates. The growth of an enteric strain (*E. coli*) is also monitored. The prebiotic activity was determined as in previous experiment (section 1.7.3). Observation of the change in cell density (as OD at 600nm) after 24 and 48 hours of incubation in each prebiotic or lHCE was carried out. PI for all commercial prebiotics and lHCE were calculated using modified version of the original formula (Huebner *et al.*, 2008).

1.7.5 Qualitative and quantitative analysis of organic acids in fermentation broths

Ion exchange chromatography was used for separation of organic acids in fermented medium of *Lactobacillus* and *Bifidobacterium* species. A cation exchange column was used to perform the separation. This column is usually packed with cross-linked polymers containing negatively charged functional groups on the surface (such as sulphonic acid) which attracts cations. Positively charged compounds are held by the

negatively charged surface of the column. Elution is achieved by competitive displacement of ions derived from the mobile phase which has the same charge as bound compounds. The compounds can then be eluted from the column. The order depends on the strength of the ionic bond between the column and compound (Marvin, 1994). Equilibration of the column is carried out with weak acid as mobile phase. All sulfonated functional groups bind with cationic hydrogen and then the column is ready for separation.

In this research the column was equilibrated with 0.1% phosphoric acid. A solution of organic acid standard in the mobile phase (0.1% phosphoric acid) was then injected. The same mobile phase is used to elute fractions. The organic acids are delayed in their elution from the column because at the pH of the phosphoric acid solution their acidic groups are protonated and unionised. Passing through the column, mobile phase hydrogen ions begin to compete with the bound organic acid on the sulfonate sites. The mechanism of ion exclusion of organic acid on cation exchange stationary phase is presented as follow:

$$R\text{-}COO^- + H^+ \leftrightarrow R\text{-}COOH$$

(Repelled) (Retained)

The organic acids are displaced down the column and finally eluted into the detector.

The organic acids with the strongest charge bind the tightest and therefore are eluted last. A UV-Visible detector at wavelength of 210nm was used to detect organic acids.

1.7.6 Determination of the effect of lyophilised horse chestnut aqueous extract on hyaluronidase activity

Hyaluronidase (HA-ase) is an enzyme belonging to a class of endoglycosidase that degrades hyaluronic acid (HA), chondroitin and their sulphates (El-Safory *et al.*, 2010). Hyaluronan (hyaluronic acid, hyaluronate, HA) is a non-sulphated glycosaminoglycan widely found in the extracellular matrix of epithelial and connective tissues. It is a high molecular mass carbohydrate polymer and plays an important role in the viscoelasticity of cells with stabilizing and hydrating properties. It also regulates interaction between

cells by providing elasticity in the external matrix of tissues. Hyaluronan also has an important role in wound repair (Kim *et al.*, 1995). Degradation of HA can led to increased inflammation, angiogenesis, fibrosis and scar formation (Bleacher *et al.*, 1993).

Enzymatic degradation of hyaluronan can be measured with Ehrlich reagent. In this model sodium hyaluronate is degraded by HA-ase into *N*-acetylglucosamine. For the quantitative estimation of *N*-acetylglucosamine, a colourimetric method is used. The reaction of *p*-dimethylaminobenzaldehyde-DMAB (called also as Ehrlich's Reagent) with *N*-acetylglucosamine produces a reddish-purple colour product (Morgan and Elson, 1933) and the amount can be quantified spectrophotometrically (Bonner Jr and Cantey, 1966). Hyaluronidase activity can be inhibited by heparin. Heparin belongs to the highly sulphated glycosaminoglycans (GAGs) which are a family of carbohydrates (Wolf *et al.*, 1984). The inhibition of HA-ase by heparin is non-competitive (Mio and Stern, 2002). Heparin is therefore used as an anticoagulant as it not only prevents formation of clots but is helpful in wound healing. When the enzyme is inhibited by heparin, low level of product is produced and the absorbance is decreased.

The inhibitory effect of the lyophilised horse chestnut aqueous extract (lHCE) on hyaluronidase activity was investigated. Heparin sodium was used as positive control.

1.7.7 Determination of inhibitory effect of lyophilised horse chestnut aqueous extract on elastase activity

Elastin is a protein component of the connective tissue of lungs, skin, arteries and ligaments. It is highly flexible, hydrophobic and together with collagen determines the structural flexibility of tissue (Shinguh *et al.*, 1998). Elastase is a protease (peptidase) which is able to degrade elastin. This enzyme naturally occurs in human neutrophils and macrophages, however, the body also produce inhibitors against elastase and thus provides homeostasis. If the balance between production of elastase and its inhibitor is shifted in favour of elastase the body tissue is subsequently destroyed and not renewed. This factor contributes to human pathology and in consequence leads to many abnormalities. Excessive elastase production is connected with some diseases: adult respiratory distress syndrome (ARDS), cystic fibrosis, pulmonary emphysema, smoking

related chronic bronchitis, rheumatoid arthritis (Rode *et al.*, 2006) and chronic obstructive pulmonary disease (COPD; He *et al.*, 2010).

The lyophilised form of horse chestnut aqueous extract (lHCE) was evaluated for elastase inhibitory activity. The EnzChek Elastase Assay Kit (E-12056) was used of screening for inhibitory activity in a high-throughput format. The kit provides non-fluorescent labelled substrate (DQ elastin – soluble bovine neck ligament elastin) which can be digested by elastase yielding highly fluorescent fragments measurable by a microplate reader fitted with fluorescein filters. The kits also contain the selective inhibitor (*N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) that irreversibly inhibits elastase and is used as control inhibitor when screening for novel elastase inhibitor.

1.7.8 Organic extracts of horse chestnut waste

1.7.8.1 Extraction of raw compounds from horse chestnut residue using organic solvents

Solvents are classified according to their polarity: non-polar and polar. These properties of solvents depend on dielectric constant. Solvents with dielectric constant less than 15 are considered as non-polar (e.g. chloroform, dichloromethane); in contrast acetone, ethanol and methanol are polar solvents (Lowery and Richardson, 1987). Extraction of plant residues is based on the ability of the solvents to dissolve compounds present according to polarity. An important aim of the research was to obtain a raw extract from horse chestnut residue for subsequent analysis and characterisation. A known amount of horse chestnut residue was extracted at room temperature with solvents of increasing polarity: chloroform, dichloromethane, acetone, ethanol and methanol.

1.7.8.2 Preliminary screening of the raw horse chestnut organic extracts by thin layer chromatography

Thin layer chromatography (TLC) is a simple, cost effective and non sophisticated method widely used in chromatography since 1958 (Stahl, 1983). This technique permits preliminary screening of different mixtures of compounds in terms of their chemical properties. Lipophilic mixtures as well as mixtures of alkaloids, sugars,

glycosides and amino acids can be separated quickly and simply on silica gel layers, moreover high sample throughput can be separated in a short time. TLC also has other important advantages: storage of the analytical information on TLC plates can be long term and it permits further analysis of separated compounds by mass spectrophotometry (MS, NMR, IR). TLC can be both qualitative and quantitative for determination of constituents of interest in a crude sample. TLC plates are made of glass polyester or aluminium sheets coated in a thin layer of silica gel (MN, 2011). The sample to be separated is usually applied onto the TLC plates to form a small spot which is allowed to dry. Following the sample application the plate is placed in a chromatography tank containing suitable solvent, the level of which is below the position of the samples. The solvent is drawn up the plate migrating between silica particles and separating the components of the sample. In contrast to HPLC, this technique does not need any pressure to achieve separation of components. The developing solvent is usually a mixture different liquid organic solvents the polarity of which can be varied. Solvents can be polar and non-polar. Their position on the polarity scale are affected by the dielectric constant, dipole moment, hydrogen bonding and polarizability of the solvents (Lowery and Richardson, 1987). Miscibility of solvent mixtures is an important factor and the solvent miscibility table (Phenomenex, 2011) must be used in the preparation of the development mixture. The final location of the sample components on the TLC plate is dependant on their polarity and the developing mixture.

After chromatography, the TLC plate is treated and heated to stain and visualise the spots. The choice of the visualization reagent depends on the type of compounds or structure which can be detected with the specific reagent (AnalChem, 2000). The plate can also be viewed under normal light and UV lamp (at short and long wavelength) to determine any active spots. For qualitative evaluation of separated spots the retention factor (R_f) is used and is defined as follow:

$$R_f = \frac{\text{distance (cm) traveled by the compound}}{\text{distance (cm) traveled by the solvent front}}$$

R_f refers to the distance travelled by the compound divided by the distance travelled by the solvent (UC, 2011). Larger R_f values correspond to greater distance travelled by the compound. This occurs where a component travels more readily with the solvent rather than interacting with the silica gel. The rate of travel by the sample is related to its polarity. Polar spots react strongly with the silica gel and their movement is retarded

while non-polar or less polar components are carried more readily by the solvent. R_f values can, therefore, be used to predict the polarity of the sample components.
In this research the organic extracts of horse chestnut residue (MetHCE, EtHCE, AcHCE, DiHCE and ChHCE) are subjected to TLC using different developing mixtures. The chromatograms were then retained for further analysis.

1.7.8.3 Disc diffusion assay

Agar diffusion is a method of determination of antibiotic activity. It is based on diffusion of antimicrobial agents from discs into solid nutrient medium (Bonev *et al.*, 2008). The original procedure established by Kirby and Bauer (Bauer *et al.*, 1966) was modified by Clinical Laboratory Standards Institute and it is currently applied in microbiology as a standard method (CLSI, 2006). The bacterial pathogen is grown on Mueller-Hinton agar (MHA) in the presence of various antimicrobial compounds impregnated on paper discs. Inhibition zones are observed unaided by eye and the measurement of zone diameter in mm can determine the degree of inhibition. The susceptibility or resistance of the pathogen to the test drug can be interpreted as: susceptible (S), intermediate (I) or resistant (R). Interpretation of inhibition zone (zone diameter interpretative standards) is different for each bacteria tested.
In this research the agar diffusion method was used for the primary screening of the residue extracts against target pathogens. The horse chestnut extracts (methanolic MetHCE, ethanolic EtHCE, acetone AcHCE, dichloromethane DiHCE and chloroform ChHCE) were applied onto paper discs and placed on inoculated surface of MHA. The procedure was carried out following the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol by Hudzicki (Hudzicki, 2009).

1.7.8.4 Bioautography assay

The bioautography assay consists of two steps. First the components of interest are separated by thin layer chromatography (TLC) using silica gel and then the spots are subjected to investigation for potential inhibitory effect using a thin layer of agar distributed over the TLC plates which contains the test pathogen (bacteria or fungi). Bioautography is a very fast and inexpensive method used in microbiology. It gives

some information on the chemical properties of active components (such as polarity) and at the same time indicates the degree of inhibition of microorganisms. This method permits the determination of the bioactivity of the spots (separated constituents of extract) and facilitates further analysis.

In this research the antimicrobial activity of constituents of the horse chestnut organic extracts (MetHCE, EtHCE, AcHCE, DiHCE and ChHCE) was determined. Both Gram-positive and Gram-negative bacteria were employed in the assays.

1.7.8.5 Checkerboard assay

Checkerboard assay is one of the methods often used in microbiology for antimicrobial susceptibility testing to determine minimal inhibitory concentration of antibiotic/drug tested. Minimal inhibitory concentration (MIC) of antibiotic can be obtained by broth microdilution techniques performed in 96-well microplates according to Clinical Laboratory Standards Institute (CLSI; Drago *et al.*, 2007). The checkerboard assay is also useful for assessing antibacterial potential of plant extracts. The advantages of this method over the agar diffusion method are: convenience, sensitivity, ease of diffusion of the extract into the media (Karthy *et al.*, 2009) Another very important property of the checkerboard method is quantitative determination of Minimal Inhibitory Concentration (MIC). The drug with potential inhibitory activity against bacteria or fungi is added to the wells. Serial dilutions (usually two fold dilutions) are used. Determination of the presence of viable and non-viable cells is carried out by using different colourimetric indicators such as p-iodonitro tetrazolium violet (INT) (Mohammed *et al.*, 2009; Karthy *et al.*, 2009), AlamarBlue (Habeeb *et al.*, 2007a; Karuppusamy and Rajasekaran, 2009) and 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) (Hamid *et al.*, 2004).

A checkerboard assay using AlamarBlue was carried out in this research. This method is based on measurement of cell viability by the fluorometric/colourimetric indicator resazurin. The active ingredient of AlamarBlue is a non-toxic, aqueous dye, blue in colour. Upon entering viable cells, resazurin is reduced to resorufin (pink in colour) during cellular respiration. Resorufin can be measured spectrophometrically (at 570nm using 600nm as reference wavelength) or fluorometrically (exc.540/emiss.590nm) and the amount of reduced resazurin is proportional to the number of viable cells present

(Karuppusamy and Rajasekaran, 2009). This method is very sensitive (can detects as few as 50 cells per well). Fluorescence measurement of metabolically active cells is much more sensitive than colourimetric and is therefore the preferred detection method (Technologies, 2011).

In this research the checkerboard assay was applied to determine MIC of the horse chestnut organic extracts (MetHCE and EtHCE). AlamarBlue reagent was used for indication of living cells with fluorescence detection for determination of cell viability.

2 Material and methods

2.1 Chemical composition of horse chestnut waste

A fundamental aim of the research was the proximal analysis of horse chestnut residue. The main components: water, ash, fat, protein and carbohydrate content were determined. All procedures for measuring the various parameters were quite rapid and well documented analytical techniques.

2.1.1 Moisture content

Apparatus

- Drying oven at 101-103 °C (Lab-line Instruments, Inc., USA)
- Crucible
- Desiccator
- Balance (Mettler Toledo AG 245, Switzerland)

Procedure

a) Dry, clean crucibles were prepared by placing them in an oven at 103ºC for 30 minutes followed by cooling to room temperature in a desiccator.
b) Immediately before use, each crucible was weighed. Five grams of sample (horse chestnut waste) was placed into the crucible. The crucible with sample was weighed again and dried in an oven at 101-103°C overnight. Next day crucible was removed and cooled in a desiccator.
c) The weight of dish plus content was recorded. The samples were returned to the oven until a constant weight was recorded on two consecutive weightings.
d) The moisture content was calculated by assuming that loss of weight of the sample during drying corresponded to the moisture content. The experiment was carried out in triplicate for each sample.
e) The percentage of water content was established by using following formula:

$$\% \text{ water content} = \left(\frac{w_2 - w_3}{w_2 - w_1} \right) \text{x } 100$$

w_1 – weight of empty crucible

w_2 – weight of crucible plus sample before drying

w_3 – weight of crucible plus sample after drying

% total solids = 100 - % water content

2.1.2 Ash content

Apparatus

- Muffle furnace at 550°C (Carbolite, UK)
- Desiccator
- Crucible with sample (dried horse chestnut waste)

Procedure

a) A pre-weighed crucible with dried horse chestnut waste (method 2.1.1) was weighed and transferred to the muffle furnace at 550°C.

b) The sample was left in until reduced to a white ash (about 5 hours).

c) The crucible with ash was placed in a desiccator and then reweighed. The experiment was carried out in triplicate for each sample.

d) The amount of ash content was calculated as follows:

$$\% \text{ ash content} = \left(\frac{w_3 - w_1}{w_2 - w_1} \right) \times 100$$

w_1 – weight of empty crucible

w_2 – weight of crucible plus sample before ashing

w_3 – weight crucible plus ash

2.1.3 Determination of metal elements by atomic emission/absorption and colorimetric techniques.

Apparatus

a) Atomic Absorption Spectroscopy (AAS)

- Atomic absorption and atomic emission spectrophotometer (Perkin Elmer, AAanalyst 100 Spectrometer, USA)
- Volumetric flasks (250ml, 100ml)
- Fume Hood

b) Colorimetric technique

- Direct reading spectrophotometer (HACH DR/2000)
- Cuvettes (HACH)

Reagents

a) Atomic Absorption Spectroscopy (AAS)

- AAS standard solutions:
  - Magnesium Standard Solution (Reagecon, AAMgH)
  - Potassium Standard Solution (Merck, 1.19505.0500)
  - Sodium Standard Solution (Merck, 19507.0500)
  - Calcium Standard Solution (Merck, 19778.0500)
  - Copper Standard Solution (Merck, 19786.0500)
  - Iron Standard Solution (Merck, 1.19781.0500)
  - Nickel Standard Solution (Merck, 1.19792)
  - Manganese Standard Solution (Merck, 19789.0500)
  - Zinc Standard Solution (Merck, 1.19806.0500)
- Concentrated nitric acid (BDH, 293356C)

b) Colorimetric technique

- PhosVer3 Phosphate Reagent Powder Pillows (HACH Permachem Reagents, 2209-99)

Procedure

a) A know quantity of horse chestnut waste ash (22.2mg), from experiment 2.1.2, was placed in a volumetric flask (100ml).
b) Two milliliters of concentrated nitric acid was added in a fume hood. After the ash was completely dissolved, deionized water was added to final volume of 100ml.

c) The ash solution was used the determination of mineral content of the original sample.

d) Standard solutions were prepared for the appropriate amounts of the minerals. Concentrations ranged from 1-5 ppm. Stock solution of each standard was prepared by adding 1.25 ml of original standard solution (1000mg/L) to 250 ml volumetric flask and deionized water was added to give a final volume of 250ml. In this way 5 ppm stock solution was obtained.

e) The following standard solutions were made by adding appropriate amount of stock solution (5 ppm) and deionized water, as shown Table 2.1.

Table 2.1: Preparation of standard solutions of minerals.

Stock solution 5ppm (ml)	**Deionized water (ml)**	**Finale volume (ml)**	**Concentration (ppm)**
100	0	100	5
80	20	100	4
60	40	100	3
40	60	100	2
20	80	100	1

f) Standard solutions of each of the nine elements tested were prepared at the following concentrations: 1ppm, 2ppm, 3ppm, 4ppm, 5ppm.

g) A detector recorded absorbance values (3 replicates for each concentration of standard solutions) and then automatically calculated the mean and standard deviation from 3 readings.

h) Standard curves were prepared for each mineral using concentration of standard solutions to give absorbance in the linear range. Standard curves of mineral standards are included in Appendix A.

i) Using the formula $y = mx+c$ derived from each standard curve, a known sample absorbance (y-value) was used for calculation of mineral content (x-value) in the ash solution.

j) If the ash solution did not fall within the standard curve range, the ash solution was diluted as necessary.

k) Potassium and sodium concentration were established by atomic emission.

Colorimetric technique:

Determination of phosphorus content was measured by colorimetric method using HACH equipments.

b) One milliliter of ash solution (22.2mg/100ml) was placed into HACH cuvette then deionized water was added to 25ml mark.
c) One pillow of PhosVer3 Phosphate Reagent was added to the sample cell and mixed immediately.
d) After a two-minute reaction the result was measured on a direct reading spectrophotometer at 890nm.
e) The displayed result in % PO_4^{3-} was converted to the phosphorus content in the ash solution and then multiplied by the appropriate factor dilution.

2.1.4 Protein content

Apparatus

- Kjeldahl tubes
- Digestion unit (Tecator digestor 2006, Sweden)
- Distillation unit (2100Kjeltec Distillation Unit, Foss Tecator, Sweden)
- Conical flask (250ml)

Reagents

- Kjeldahl reagent tablets (1000 KjelTabs, Thompson & Capper Ltd. Code AA 09)
- Concentrated Sulphuric Acid (HCC- Riedel-de Haën)
- Sodium hydroxide 40% (AnalaR, BDH, Prod. 102525P)
- Boric acid 0.1% (Sigma Aldrich, 10043-35-3)
- Hydrochloric acid 0.1M (HCC- Riedel-de Haën)
- pH indicator (Hopking & Williams, 886033)

Procedure

a) About 3 grams of dried horse chestnut waste was accurately weighed and placed into Kjeldahl tube then two Kjeldahl reagent tablets and 25ml of concentrated

sulphuric acid were added. A control tube was also prepared, containing reagents and no sample.

b) Each tube was placed in a heating block and heated at about 400°C (for 2 hours) until a clear solution was observed.
c) After this step the tube was removed from heating block and cooled to room temperature then placed in the Kjeldahl distillation unit which automatically dispensed 40% (w/v) sodium hydroxide directly to digested sample.
d) The Kjeldahl unit was used to carry out the steam distillation and capture of ammonia in 100ml of 1% (w/v) boric acid. Distillation time was set at 4 minutes.
e) The conical flask was removed from Kjeldahl unit and a few drops of pH indicator were added.
f) The contents of the flask (ammonium borate solution) were titrated with 0.1M hydrochloric acid until a yellow end-point was reached. The experiment was carried out in triplicate for each sample.
g) Protein content present in the sample was established using following formula:

$$\text{X ml of HCl x } \left(\frac{\text{0.1 mol HCl}}{\text{1000ml HCl}}\right) \text{ x } \left(\frac{\text{14g nitrogen}}{\text{1 mol HCl}}\right) \text{ x } \left(\frac{\text{100g protein}}{\text{16g nitrogen}}\right)$$

X – volume of 0.1M hydrochloric acid used in the titration

2.1.5 Lipid content

Apparatus

- Soxhlet apparatus (round bottom flask and extractor unit)
- Cellulose thimble (Lennox, single thickness, 18x55cm)
- Heating mantle (Electothermal, England)
- Condenser
- Evaporator with water bath (Bibby Sterilin, England)

Reagents

- Petroleum ether (40-60°C; Romil-SpS Super, Purity Solvent, H601)

Procedure

a) A soxhlet distillation flask (250ml) was weighed prior to use.
b) Dried horse chestnut waste sample was first crushed and placed into cellulose thimble. The amount of sample was recorded in grams.
c) The thimble was placed in the extractor and the apparatus set up with a condenser.
d) The solvent (100ml) was boiled gently by electric heater for about 2 hours. There were approximately 70 cycles of extraction per hour.
e) After this time petroleum ether was removed from the flask using a vacuum evaporator with water bath. The flask with crude lipid residue on the bottom was reweighed.
f) The total (crude) lipids content was calculated according to the following formula:

$$\% \text{ lipid content} = \left(\frac{w_2 - w_1}{w_3} \right) \times 100$$

w_1 – weight of empty Soxhlet flask

w_2 – weight of flask plus lipids

w_3 – weight of dried sample in thimble before extraction

2.1.6 Analysis of fatty acids by gas chromatography

Apparatus

- GC chromatograph (Shimadzu GC-17A, Japan)
- GC Software : Class-VP 7.2.1 SP1
- GC Column JW Scientific D81301
- Bottle 20ml
- Test tube
- Heating plate (Stuart heat-stir CB 162, England)
- Steam bath (Grand GD-100, England)
- Pipette

Reagents

- Sodium hydroxide in methanol 0.5M (AnalaR, BDH, 102525P)
- Boron trifluoride in methanol 12.5% (Merck, 801663)
- Saturated sodium chlorite solution (AnalaR Normapur, 27810.262)
- n-Hexane 95% (Romil-SA)
- Standards fatty acids :
    - Supelco Component FAME Mix C4-C24 (18919-1AMP)
    - Supelco Component FAME Mix C18-C20 (18916-1AMP)
    - Supelco Component FAME Mix GLC-20 (1892-1AMP)

Procedure

Methyl esterification of lipids

a) To the round bottom flask with crude lipids from Soxhlet extraction, 8ml of 0.5M sodium hydroxide in methanol was added. The sample was heated on steam bath until the lipids completely dissolved (about 5 min.).
b) Four milliliters of this mixture was placed into 10ml in a glassbottle, then 5ml of 12.5% (v/v) boron trifluoride in methanol was added. The bottle with content was boiled for 2 minutes on an universal hotplate to facilitate esterification.
c) The sample was then cooled and 5ml of saturated sodium chloride solution was added.
d) The methyl esters of fatty acids were extracted with 2ml of n-hexan and transferred by pipette to a clean test tube.
e) The extract was ready for analysis by gas chromatography.

Gas chromatography (GC)

Commercially produced standard mixture

a) A standard mixture (Supelco Component FAME) of the methyl esters of fatty acids was used for gas chromatography; the standards were diluted in n-hexane to the different concentrations.
b) One microlitre of standard mixture was injected into GC.

c) The peak areas of three different concentrations of each standard (2.5mg, 5mg and 10mg) were plotted for each fatty acid.
d) Standard curve of peak area vs. concentration (mg/ml) was potted.
e) Standard curves of fatty acids were included in Appendix B.

Sample mixture

a) Sample mixture following methyl esterification process was injected into GC column in 1µl volume.
b) Qualitative and quantitative analysis of fatty acid composition of the sample was performed by comparison of the retention times with those of the standards.

Parameters of the chromatography process

a) Column parameters:

Length	30m
Inner diameter	0.25mm
Film thickness	0.25µm
Carrier gas	helium, 32cm/sec.
Column flow	1.3ml/min

b) Detector

Flame Ionisation Detector (FID)

c) Oven temperature parameters and their graphical presentation (Figure 2.1) :

Rate (°C/min)	**Temperature (°C)**	**Wait (minutes)**
Initial	70	3.0
8.0	150	0.0
2.0	175	0.0
1.0	185	0.0
2.0	260	3.0
40.0	70	0.0

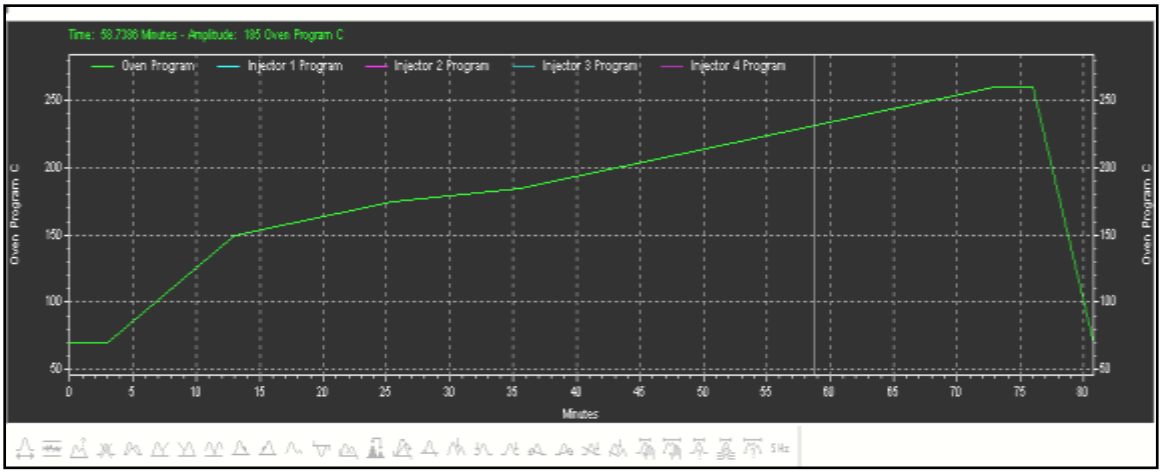


Figure 2.1: Temperature profile of gas chromatography.

2.2 Prebiotic potential of horse chestnut waste

Due to the high proportion of complex carbohydrate present and anecdotal evidence of the beneficial effects of the residue when used as cattle feed, it was decided to determine if the residue contained potential prebiotic compounds. The influence of prebiotics on probiotic bacterial strains is well established. Prebiotics are these food ingredients that have a beneficial effect on probiotic strains e.g. *Lactobacillus* and *Bifidobacterium* species. Prebiotics stimulate the growth of some bacteria present in the gut and thus have beneficial effect on human health.

The prebiotic effect of horse chestnut aqueous extract (HCE) in an *in vitro* model was investigated. The growth of probiotic in medium supplemented with the extract from horse chestnut waste was compared to bacterial growth in medium enriched with commercial prebiotic FOS.

2.2.1 The cultivation of probiotic bacterial strains

Apparatus

- Sterile Petri dishes
- Class II cabinet (Holten Lamin-Air)
- Anaerobic jar
- Incubator
- 1L bottle (glass)

Reagents

- MRS agar (Fluka, 69946) commercially available contained:
    - Agar (12 g/L)
    - Diammonium hydrogen citrate (2 g/L)
    - Dipotassium hydrogen phosphate (2 g/L)
    - D(+)-glucose (20 g/L)
    - Magnesium sulfate (0.1 g/L)
    - Manganous sulfate (0.05 g/L)
    - Meat extract (5 g/L)

- Sodium acetate (5 g/L)
- Universal peptone (10 g/L)
- Yeast extract (5 g/L)
- Final pH6.5±0.2 (25 °C)

- Sodium thioglycolate (Sigma, T0632)
- Anaerocult® A , reagent for the generation of an anaerobic medium in anaerobic jars, (Merck, 113829)
- Bacterial strains:

Lactobacillus sps. strains

- *Lactobacillus delbrueckii subsp. lactis*
- *Lactobacillus plantarum* ATCC 8014
- *Lactobaciullus acidophilus* ATCC 4356
- *Lactobacillus rhamnosus* ATCC 7469

Bifidobacterium sps. strains:

- *Bifidobacterium infantis* ATCC 15697
- *Bifidobacterium angulatum* ATCC 27535

Lactobacillus strains were purchased from Medical-Supply Co. Ltd., Ireland. The product codes were: MBL0235P, MBL0234P, MBL0243P and MBL0233P respectively. All strains were delivered in freeze dried form. *Bifidobacterium* strains were purchased from DSMZ GmbH, Germany with following product codes: ACC No. 20088 and 20098.

Procedure

Preparation of Petri dishes with MRS agar:

a) MRS agar was prepared with the addition of 0.05% (w/v) sodium thioglycolate. The thioglycolate is added to maintain anaerobic conditions.
b) After being autoclaved (121ºC/1atm/15minutes) the agar was cooled to approximately 50ºC and 20ml poured into Petri dishes. This was carried out in a Class II cabinet to maintain sterility.
c) The agar plates were allowed to set and then incubated overnight at 37°C.
d) The following day all Petri plates with MRS agar were examined for contamination. Any contaminated plates were autoclaved and discarded.
e) Sterile plates were covered with parafilm and stored at 4ºC.

Streaking of Petri dishes with bacterial strains:

a) The resuscitation of freeze-dried *Lactobacillus* and *Bifidobacterium* strains was carried out by streaking with the attached sterile cotton swab into MRS agar.
b) Immediately the petri dishes were labelled and placed in an anaerobic jar with a carbon dioxide sachet (Anaerocult® A).
c) The jar was placed in an incubator at 37°C for 48±4 hours.
d) The petri dishes with the bacterial cultures were then covered in parafilm and stored at 4ºC for use as stock cultures.

2.2.2 Determination of the prebiotic potential of horse chestnut waste

Apparatus

- Glass universal bottles (30ml)
- Centrifuge (Falcon 6/300, MSE ,UK)
- Beakers (500ml)
- Sterile 0.20µm pore-size filters (Sarstedt, 83.1826.001)
- Freeze drier (Labconco, Freeze Dry system/Freezone 2.5, USA)
- Crucibles

Reagents

- Minimal medium (MM)– based on Garch's medium (Bielecka *et al.*, 2002)
    - L-glutamic acid (1g/L ; Sigma, G1251-100G)
    - Potassium phosphate (0.25g/L ; M&B, MU6078)
    - Magnesium sulphate . 7 H_2O (0.1g/L ; Philip-harris, S521 C54)
    - Sodium chloride (0.1g/L ; AnalaR, 27810.262)
    - Meat peptone (1g/L ; Oxoid, LP0029)
    - L-cysteine hydrochloride (0.04g/L ; Sigma, C-1276)
    - Final pH 7.0

All ingredients were dissolved in deionized water and the pH was adjusted up to 7.0 using 2M NaOH.

- MRS broth (Fluka, 69966) commercially available contained:
    - Polypeptone (10g/L)
    - Beef extract (10g/L)

- Yeast extract (5g/L)
- Glucose (20g/L)
- Tween-80 (1.08g/L)
- Dipotassium phosphate (2g/L)
- Sodium acetate (5g/L)
- Ammonium citrate (2g/L)
- Magnesium sulphate (0,2g/L)
- Manganese sulphate (0.05g/L)
- Final pH 6.4 at 25°C

- Horse chestnut waste
- Fructooligosaccharides from chicory (Sigma, F8052-50G)

Procedure

Preparation of raw material (horse chestnut residue)

Horse chestnut waste used in this experiment was prepared as follows:

a) Raw material stored at -20°C was placed into a freeze drier over night.
b) Dried horse chestnut waste was ground in a mill for about 2 minutes to achieve powder form.
c) The powdered material was then stored at -20°C until required.

Preparation of commercial prebiotic in minimal medium (MM)

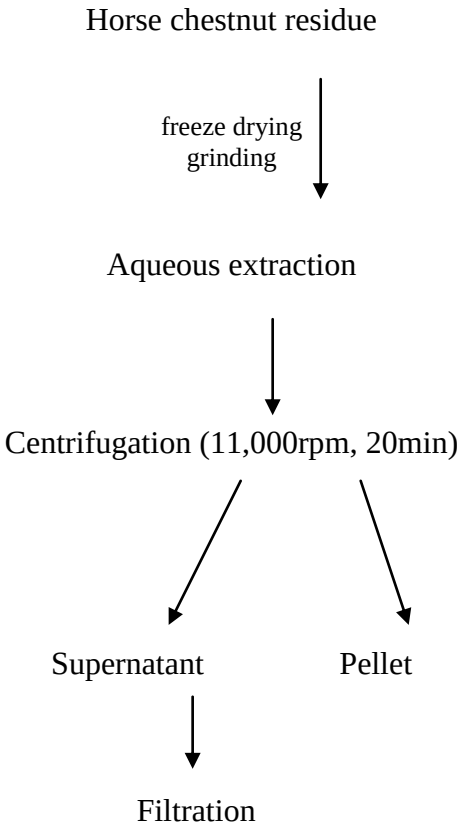
Fructooligosaccharides from chicory (FOS) was dissolved in minimal medium (MM) to 1% (w/v) and 10 ml aliquots were dispensed into universal bottles prior to autoclaving (121ºC/1atm/15minutes).

Preparation of horse chestnut aqueous extract (HCE) in minimal medium (MM)

a) Two grams of dried and powdered horse chestnut residue was added to three beakers each containing 200ml of minimal medium.
b) Three different extraction methods were used:
    - Stirring at 20ºC for 1 hour
    - Stirring at 90ºC for 1 hour
    - Autoclaving at 121ºC for 15 minutes.
c) Each extract was then centrifuged at 11,000 rpm for 20 minutes.

d) The supernatants were filtered, using non-pyrogenic sterile 0.20µm pore-size filters (Sarstedt, 83.1826.001) directly into sterile universal bottles. An aliquot of 10 ml was dispensed into each bottle.

e) The flow chart below shows preparation of horse chestnut extract (HCE):



Preparation of inoculum of *Lactobacillus* and *Bifidobacterium* strains

a) One colony of each probiotic bacterial strain was transferred by inoculating loop from MRS agar plates into separate flasks containing 100ml MRS broth with 0.05% (w/v) sodium thioglycolate.

b) Flasks were placed in an orbital incubator at 37ºC/50rpm overnight.

Inoculation of test medium

a) Inoculum (100µl) was added into universal bottles with 10ml of MM + 1% (w/v) of FOS and 10ml of MM with 1% (w/v) horse chestnut extract (HCE).

b) As a control 100µl was also inoculated into 10ml of unsupplemented minimal medium (MM).

c) All bottles were placed in an orbital incubator at 37ºC/100rpm.

d) Optical density (at 600nm) and pH were measured every 2 hours during the incubation.

Estimation of water soluble fraction of horse chestnut waste

To establish the percentage of soluble fraction extracted from horse chestnut residue at three different temperatures (20ºC, 90ºC or 121°C) as described in previous part of this thesis.

a) The supernatants were removed following centrifugation and pellets were placed into previously dried and weighed crucibles.
b) The crucibles with pellets were placed in an oven at 101-103ºC overnight.
c) Next day crucibles were cooled in a dessicator and reweighed.
d) Weight of water soluble fraction was calculated as follows:

$$\text{Weight of soluble fraction (g)} = 2 - [2 - (w_2 - w_1)]$$

w_1 – weight of empty crucible (g)

w_2 – weight of crucible plus pellet after drying (g)

2.3 Analysis of composition of horse chestnut extract water soluble fraction

Previous experiment with probiotic strains showed that active compound(s) which stimulate growth of bacterial cells were constituents of a water soluble fraction obtained from horse chestnut waste. Therefore the next main aim was to establish the composition of the fraction (moisture content, ash, minerals, protein, poliphenols, carbohydrate). To further characterize the fraction Gel Filtration and Anion/Cation Exchange Chromatography were also performed.

To obtain a purer aqueous fraction from horse chestnut residue, crude, wet material was lyophilised. Dry horse chestnut waste was crushed in a mill for about 3 minutes. After this time powdered sample was placed into a beaker and deionized water was added to obtain 1% (w/v) mixture. The mixture was stirred for about 1 hour on a universal plate at room temperature and then centrifuged at 11000rpm (N x g) for 20 minutes. The supernatant was filtered into a glass Petri dish using 0.2μm pore-size filter (Sarstedt).

The filtrate was lyophilised again to remove water, which resulted in a white powder. The powder (lyophilised horse chestnut aqueous extract (lHCE) from horse chestnut waste) was placed into plastic tubes and stored at 4°C until use.

2.3.1 Moisture content

Determination of moisture content was described in part 2.1.1 of this thesis. The experiment was carried out in triplicate for each sample.

2.3.2 Ash content

Similar to water content, ash content was determined using the procedure from part 2.1.2.

2.3.3 Determination of metal elements by atomic emission/absorption and colorimetric techniques.

The concentration of metal elements present in the lyophilised horse chestnut aqueous extract (lHCE) was established according the procedure described in part 2.1.3. The mineral standard curves were used for determination of minerals in the sample. The ash solution used contained 17.4mg of ash.

2.3.4 Determination of protein content by Bradford method

Apparatus

- Eppendorf tubes
- Spectrophotometer (Shimadzu, UVmini-1240, China)

Reagents

- Sodium hydroxide (1M)
- Bradford reagent:

- Coomassie Brillant Blue G-250 (100mg; Sigma, B-113I)
- Ethanol 95% (50ml ; J.T. Baker, 3408)
- Phosphoric acid (100ml 85% (w/v) ; Merck, 573.2500)
- Deionized water (1 liter)
- Reagent was filtered, Whatman no.1 paper just before use

- Standard protein – bovine serum albumin –BSA (VWR, BDH, 421501I)
- Lyophilised horse chestnut aqueous extract (lHCE)

Procedure

a) Stock solution of standard protein BSA (1mg/ml) was prepared (25mg of BSA was dissolved in 1M sodium hydroxide in a 25ml volumetric flask).
b) Dilutions of BSA in range 1-0.1 mg/ml were prepared using 1M sodium hydroxide as diluent (Table 2.2).

Table 2.2: Preparation of BSA solution.

Concentration of stock solution BSA (mg/ml)	**Volume of stock solution (ml)**	**Volume of 1M sodium hydroxide (ml)**	**Concentration of BSA standard (mg/ml)**	**Final volume of BSA standard (ml)**
1				
	1	0	1	1
	0.75	0.25	0.75	1
	0.5	0.5	0.5	1
	0.25	0.75	0.25	1
	0.1	0.9	0.1	1

Source: (UG, 2009).

c) BSA was used to prepare a standard curve for protein determination.
d) Five milligrams of water soluble fraction from horse chestnut residue was dissolved in 1ml of 1M sodium hydroxide.
e) To separate Eppendorf tubes were added 0.02ml each of BSA standard or sample and 1ml of Bradford reagent. At the same time, a blank was prepared by mixing 0.02ml 1M of sodium hydroxide and 1ml of Bradford reagent.
f) All tubes were gently vortexed and incubated for 10 minutes at room temperature.
g) After this time the absorbance was measured at 595nm.

h) The standard curve was constructed (Appendix E) and shows linear correlation (R^2=0.9954) between concentration of BSA protein and the absorbance at 595nm.

i) Protein content in lHCE was established using formula obtained from standard curve of BSA.

j) The experiment was carried out in triplicate for each standard and sample.

2.3.5 Determination of reducing sugars by dinitrosalicylic colorimetric method

Apparatus

- Test tubes
- Spectrophotometer (Shimadzu, UVmini-1240, China)
- Water bath (LAUDA, AQUAline, AL18, UK)
- Burette
- Pipette
- Volumetric flask (1000ml, 100ml)

Reagents

- 1% Dinitrosalicylic Acid Reagent Solution (DNS-reagent)
    - Dinitrosalicylic acid (10g ; Sigma, 609-99-4)
    - Sodium sulfite (0.5g ; BDH, 302264C)
    - Sodium hydroxide (10g)
    - Potassium sodium tartrate (300g color stabilizator)
    - Deionized water
- Glucose anhydrous (Fisher Scientific G/0450/53)
- Fructooligosaccharides from chicory (Sigma, F8052-50G)

Procedure

a) Glucose standard solutions were prepared in concentrations of 5, 10, 15 and 20μmol glucose per milliliter

b) Ten milligrams of the sample (lHCE) and 15 mg FOS product were dissolved in 1ml of deionized water in separate tubes.
c) Standard glucose solutions 1ml (5-20µmol/ml) was added to the separate test tubes.
d) DNS reagent (1ml) was added to each standard and sample tube.
e) Blank sample was also prepared and contained only 1ml of deionized water and 1ml of DNS reagent.
f) All test tubes with were heated gently in boiling water (in water bath) for 5 minutes to stabilize the colour as a result of the reaction between glucose and DNS.
g) After cooling 8ml of deionized water was added to each test tube using a burette.
h) The absorbance of each solution was measured on a spectrophotometer at 546nm.
i) Standard curve was created, by plotting absorbance at 456nm against concentration of glucose per 10ml (Appendix F) Using formula y = mx+c the amount of reducing sugars in the samples was established.
j) The experiment was carried out in triplicate.

2.3.6 Determination of phenolic content by Folin-Ciocalteau method

Apparatus

- Microplate Reader (Varioskan Flash-Thermo Scientific, USA)
- Microplate (Multiple Well Plate 96-Well Flat Bottom, Sarstedt, 82.1581)
- Multichannel Pipette
- Pipette

Reagents

- Folin-Ciocalteau Reagent (Merck, 9001)
- Sodium carbonate (20% ; Merck, 1.06398.1000)
- Phloroglucinol (1% (w/v) polyphenol standard)

Procedure

The original procedure was modified in-house (Dr. Agnieszka Kowalska, 2009- private communication) for use in a microplate reader. In this case a smaller amount of all reagents was used which also reduced laboratory waste.

Preparation of reagents:

a) Folin-Ciocalteau reagent was diluted (4x; 500µl Folin-Ciocalteau with 1500µl deionized water).
b) Sodium carbonate was diluted (2x; 500µl 20% sodium carbonate with 500µl deionized water).
c) Phoroglucinol was diluted (200x; 5µl 1% (w/v) phloroglucinol with 995µl deionized water), to give 0.005% (w/v) of phoroglucinol solution.

Preparation of sample:

a) Fifteen milligrams of lHCE was dissolved in 300µl of deionized water, to give a 5% (w/v) solution.
b) Phloroglucinol standard: In wells A2-A12 50µl water was added using a multi-channel pipette. Diluted phloroglucinol (100µl) was pipetted into the A1 well and then serial dilutions were made by taking 50µl of solution from A1 and adding to the A2 well, etc. This mixture was gently mixed using the same pipette.
c) Exactly the same action was repeated in wells A2-A11 but from A11 well 50µl of solution was removed, leaving only water in A12. The action was carried out in triplicate (rows A,B,C).
d) In rows E and G serial dilution of the sample solution (lHCE) were prepared in the same way.
e) To all wells A, B, C, E, G 20µl of ¼ strength FC reagent was added and 30µl of diluted sodium carbonate was added by using multi-channel pipette.
f) Mixtures were mixed well and incubated for 1 hour at room temperature.
g) After this time absorbance was measured at 750nm on Varioskan Flash Microplate Reader.
h) The Varioskan Flash Microplate Reader automatically measured and recorded the absorbance of phloroglucinol standards.

i) Standard curve for phloroglucinol was constructed (Appendix G) and shows concentration (mg/ml) vs. absorbance at 750nm. Linear regression and correlation were determined.

j) Concentration of phenolic content in lHCE was established using formula from phloroglucinol standard curve.

2.3.7 Analysis of lyophilised horse chestnut aqueous extract by Anion/Cation Exchange Chromatography

Apparatus

- HPLC system with refractive index detector
    - pump (Waters 510 Pump)
    - detector (Waters 410 Differential Refractionometer)
    - printer (Shimazu Chromatopac C-R6A, Japan)
- Column- Supelco 25cm x 4,6mm x 5µm, SUPELCOSIL-LC-NH2, cat.no. 58338 for anion exchange chromatography
- Column- Supelco, 30cm x 7,8mm x 9µm, SUPELCOGEL C-610H, cat.no. 59320-U for cation exchange chromatography
- Degas system (Cole, Parmer,8893)
- Hamilton syringe (50µl)
- Volumetric flasks (100ml)
- Bottles 1l (glass)
- PVDF membrane 0.45µm pore-size filter; Acrodisc, LC 25mm Syringe Filter (PALL, 4408)

Reagents

- Mobile phases

  Anion Exchange Chromatography (acetonitrile:water 75:25)
    - Acetonitrile(HiPerSolv Chromanorm, HPLC gradient grade, VWR, BDH, 20060.320)
    - Filtered water endotoxin free

  Cation Exchange Chromatography (0.1% (v/v) phosphoric acid in water)
    - Phosphoric acid (Sigma-Aldrich, P5811-500G)

- Lyophilised horse chestnut aqueous extract (lHCE)
- Standard sugars

  Monosaccharides:
    - Glucose anhydrous (Fisher Scientific G/0450/53)
    - D(+)- Fructose (Merck, 1.05321.1000)
    - D(+)- Galactose (Aldrich, 11,259-3)
    - D(+)- Xylose (Lancaster, 11203)

  Disaccharides
    - Maltose (VWR, BPH, 25188.291)
    - Lactose monohydrate (Merck, 1.076565.5000)
    - D(+)- Sucrose (AppliChem, A1125.1000)

  Oligosaccharides
    - Raffinose (BDH, 38056)
    - Dextrin 15 maltodextrin (Fluka, 31412-100g)
    - FOS (Fructooligosaccharides from chicory, Sigma, F8052-50G)
    - GOS (Vivinal GOS, DOMO®, 502675)
    - Inulin (Inulin from chicory, Sigma-Aldrich, I2255)
- Phosphate-citrate buffer
    - Di-sodium hydrogen orthophosphate (Sigma-Aldrich, 04276)
    - Citric acid (BDH, AnalaR, 100081)

Procedure

Preparation of phosphate-citrate buffer for anion exchange chromatography

A 0.2M solution of di-sodium hydrogen orthophosphate (MW=141.96g/mol) was prepared be dissolving 2.84g in 100ml deionized water in volumetric flask. To prepare 0.1M citric acid solution, 2.1g of citric acid (MW=192.12g/mol) was dissolved in 100ml. To obtain phosphate buffer, the two solutions were mixed: 58ml of di-sodium hydrogen orthophosphate (0.2M) and 42ml of citric acid (0.1M). The pH was adjusted to 5.5-6.0 (Pearse and Stoward, 1980).

Preparation of mobile phases

Anion Exchange Chromatography (AEC)

Acetonitrile (HPLC grade) was added to filtered deionized water to a final concentration of 75% (v/v).

Cation Exchange Chromatography (CEC)

Phosphoric acid (0.1% (v/v)) was prepared in filtered deionized water by taking 1.176ml of 85% phosphoric acid and making up to 1 litre with water.

The solutions were degassed for 20 minutes using a sonicator bath.

Preparation of standard sugars

Anion Exchange Chromatography

Standard sugars were dissolved in phosphate buffer. Their concentrations (w/v) were as follows: glucose 1%, galactose 1%, fructose 1%, GOS 1%, FOS 1%. All sugar solutions were filtered using 0.45µm pore-size PVDF membrane filter.

Cation Exchange Chromatography

Standard sugars (mono,- di,- and oligosaccharides) were dissolved in mobile phase (0.1%; v/v phosphoric acid) to concentrations of 5% (w/v). The solutions were filtered through 0.45µm PVDF membrane filter.

Preparation of horse chestnut extract sample

Lyophilised chestnut aqueous extract (lHCE) was dissolved in phosphate buffer or 0.1% (v/v) phosphoric acid to obtain 5% (w/v) solution. This sample solution was filtered using 0.45µm pore-size PVDF membrane filter prior to use.

Chromatographic process

The HPLC system was purged with an appropriate solvent (mobile phase) for 10 minutes then a column, was fitted and 1 hour of flushing process was carried out at a flow rate of 1ml/min (AEC) or 0.4ml/min (CEC).

After this time, 20µl of sample (5% (w/v) lHCE) was injected into the HPLC column using a Hamilton syringe. The peaks were recorded on Chromatopac integrator. Solutions of all sugar standards were treated in similar manner. The presence of sugar in the sample was identified by comparison of retention time of the sample peaks with those of the standard sugar solution.

2.3.8 Analysis of lyophilised horse chestnut aqueous extract by Size Exclusion Chromatography using FPLC system

2.3.8.1 Fractionation of lyophilised horse chestnut aqueous extract

The solution of lyophilised horse chestnut aqueous extract (lHCE) was subjected to fractionation by size exclusion chromatography. The fractions obtained from lHCE were subjected to further analysis.

Apparatus

- FPLC system –AKTA purifier (Sweden)
  - pump (P-900)
  - detector (OV-900), pH/C-900, BOX-900
  - fractionator (F-950)
- Column- Superdex™ 200 10/300 GL- GE Healthcare (17-5175-01)
- Eppendorf tubes
- PVDF membrane 0.45µm pore-size filter; Acrodisc, LC 25mm syringe filter (PALL, 4408)

Reagents

- Running buffer
  - Sodium acetate (0.2M; Sigma Aldrich, S2889)
  - Sodium chloride (0.15M; SciChem, 7647-14-5)
  - Deionized water to 1000ml volume
  - pH 4.5
  - Filtered through No.5 Whatman filter

Procedure

a) Running buffer was prepared in the following way: 16.4g of sodium acetate (MW=82.08g/mol) and 8.75g of sodium chloride (MW=58.44g/mol) was dissolved in 800ml of deionised water in volumetric flask (1000ml) then pH was adjusted to 4.5 using hydrochloric acid. Deionised water was then added to the

flask up to 1000ml mark. The buffer was filtered through a Whatman No.1 filter and degassed prior to use.

b) The sample (lHCE) was dissolved in running buffer to a concentration of 5% (w/v) and then filtered using 0.45μm pore-size PVDF filter.
c) The FPLC system was purged with buffer. The column was fitted to the system and equilibrated with running buffer using two column volumes (CV).
d) Eppendorf tubes were placed on the fractionator which automatically collected the fractions from the column. 250μl sample volumes were injected into the system.
e) Size exclusion chromatography parameters were as follows: flow rate-0.40ml/min, fraction volume-0.5ml per one Eppendorf tube.

2.3.8.2 Determination of sugar content in fractions of lyophilised horse chestnut aqueous extract by Dubois assay

Apparatus

- Microplate Reader (Varioskan Flash-Thermo Scientific, USA)
- Microplate 96-Well Plate Flat Bottom (Sarstedt, 82.1581)
- Pipette
- Eppendorf tubes
- Volumetric flask 25ml
- Water bath (LAUDA, AQUAline, AL18)
- Vortex

Reagents

- 5% (w/v) phenol solution (Fluka, 77608)
- Concentrated sulphuric acid (Sigma-Aldrich, 07208)
- Standard sugar - Glucose anhydrous (Fisher Scientific G/0450/53)

Procedure

Glucose standard curve:

a) Glucose standard solution was prepared with the following glucose concentration (w/v): 0.001%, 0.002%, 0.003%, 0.005%, 0.007% and 0.01%.
b) Glucose stock solution (0.1% (w/v)) was prepared by dissolving 25mg of glucose anhydrous in deionised water up to 25ml mark in a volumetric flask.
c) The stock solution was diluted to obtain desirable concentrations (Table 2.3)

Table 2.3: Preparation of glucose standards solutions.

Concentration of glucose stock solution	**Volume of stock solution**	**Volume of deionized water**	**Concentration of glucose standard solution**	**Final volume of glucose standard solution**
(%)	**(ml)**	**(ml)**	**(%)**	**(ml)**
0.1				
	0.25	24.75	0.001	25
	0.50	24.50	0.002	25
	0.75	24.25	0.003	25
	1.25	23.75	0.005	25
	1.75	23.25	0.007	25
	2.5	22.50	0.01	25

d) Each glucose standard (50µl) was placed into Eppendorf tube in triplicate and 50µl of 5% (w/v) phenol solution was added to each tube.
e) For a blank sample deionized water was used instead of standard.
f) The mixtures were mixed by vortex and incubated at 4°C for 10 minutes.
g) Concentrated sulphuric acid (250µl) was then added to each tube and mixed by vortex. The tubes were placed in a water bath and boiled for 15 minutes.
h) Once the orange colour developed, 150µl of each standard was placed in a 96-well plate. Samples were prepared in triplicate.
i) Absorbance was recorded at 490nm using a Varioskan Flash Microplate Reader. Results given were exported to Excel and a calibration curve was constructed.
j) Glucose standard calibration curve shows absorbance of glucose at 490nm against glucose concentration.
k) Standard curve shows linear correlation between concentration of glucose (%) and absorbance (λ=490nm); R^2=0.9986 (Appendix H).

<u>Fractions from lHCE:</u>

Fractions obtained from the gel filtration process (91 fractions per run) were examined for sugar content. The experiment was carried out at the same time as glucose standards. Every second fraction, in triplicate, was examined for total sugar content by Dubois

assay; 50µl of each fraction was used. The procedure was carried out as described above. Absorbance of the fractions was obtained and total sugar content was calculated using the equation of the line from calibration curve of glucose (y = 27.614x + 0.0463).

2.3.8.3 Determination of protein content in fractions of lyophilised horse chestnut aqueous extract by Bradford assay

Apparatus

- Eppendorf tubes
- Microplate 96-Well Plate Flat Bottom (Sarstedt, 82.1581)
- Pipette
- Microplate Reader (Varioskan Flash-Thermo Scientific, USA)
- Vortex

Reagents

- Bradford Reagent (Amresco, E-530)
- Standard protein – bovine serum albumin, BSA (VWR,BDH, 421501I)

Procedure

Preparation of calibration curve of BSA:

a) Standard protein (BSA) was prepared in concentration range 1-0.1mg/ml as previously described (section 2.3.4). Concentration of BSA standard was converted from mg/ml into percentage.
b) Absorbances of BSA standards were recorded at 595nm on Varioskan Flash Microplate Reader. Results given were exported to Excel and a calibration curve was constructed (Appendix I).
c) Standard curve shows linear correlation between concentration of BSA protein (%) and absorbance (λ=595nm); R^2=0.9882. The formula of the line, y=2.5333x+0.0069 was used in the estimation of protein content in lHCE fractions.

Fractions from lHCE:

a) Every second fraction obtained from gel filtration chromatography (section 2.3.8.1) was examined for total protein content; 20µl of fraction was placed into separate Eppendorf tubes in triplicate.
b) 1ml of Bradford Reagent was added to each tube, all samples were mixed (vortex).
c) After 10 minutes of incubation at room temperature, 150µl of sample from each tube was placed into microplate wells (each fraction and standard in triplicate).
d) Absorbance was measured on Varioskan Flash Microplate Reader at 595nm.

2.3.8.4 Estimation of molecular mass of sugar fraction of lyophilised horse chestnut aqueous extract by size exclusion chromatography

Apparatus

- Size exclusion column –Superdex 200 10/300 GL – (GE Healthcare, 17-5175-01; (Column specification: fractionation range $1x10^4$ to $6x10^5$ for globular protein, $1x10^3$ to $1x10^5$ for dextran)
- FPLC system- AKTA purifier (Sweden)
  - Pump (P-900)
  - Detector (OV-900), pH/C-900, BOX-900
  - Fractionator (F-950)
- PVDF membrane 0.45µm pore-size filter; Acrodisc, LC 25mm Syringe Filter (PALL, 4408)
- Mega blocks 96-Well plate: 1.2ml (Sarstedt, 82.1971.002)
- Microplate 96-Well Plate Flat Bottom (Sarstedt, 82.1581)
- Water bath (LAUDA, AQUAline, AL18)
- Microplate Reader (Varioskan Flash-Thermo Scientific, USA)
- Multichannel pipette
- Adhesive sealing tape, aluminium, non-transparent (Sarstedt, 95.1995)
- Pipette tips

Reagents

- Dextran standards from *Leuconostoc mesenteroides* (Fluka)

  - MW 5 000 (Fluka, 31417)
  - MW 12 000 (Fluka, 31418)
  - MW 80 000 (Fluka, 31421)
- Dextran blue from *Leuconostoc ssp*. MW 2 000 000 (Fluka, 31393)
- Lyophilised horse chestnut aqueous extract (lHCE)
- Running buffer
  - Sodium acetate (0.2M; Sigma-Aldrich, S2889)
  - Sodium chloride (0.15M; SciChem, 7647-14-5)
  - pH 4.5 adjusted with HCl
  - Double deionised water to 1000ml mark, filtered through No.1 Whatman filter and degassed
- Ethanol 20% (v/v) - storage solution
- Phenol solution 5% (w/v; Fluka, 77608)
- Concentrated sulphuric acid (Sigma-Aldrich, 07208)

Procedure

Size exclusion chromatography

a) Running buffer was prepared using 0.2M sodium acetate and 0.15M sodium chloride, adjusted to pH 4.5. The preparation was described in part 2.3.8.1 this thesis.
b) The size exclusion column was first equilibrated with two column volumes (48ml) of running buffer at a flow rate of 0.4ml/min.
c) Solutions of all dextran standards and lyophilsed horse chestnut extract (lHCE) were prepared by dissolving in running buffer to the following concentrations: 10mg/ml (dextran 5,000), 10mg/ml (dextran 12,000), 5mg/ml (dextran 80,000), 5mg/ml dextran blue and 50mg/ml lHCE.
d) Standard solutions and the sample were filtered (0.45µm) and injected (into FPLC system) separately using a 100µl loop. Flow was set at 0.4ml/min.
e) After use the column was stored in 20% (v/v) ethanol solution.
f) Fractions were collected into a mega block in 0.5ml aliquots per well. Two column volumes were used for elution of dextran standards and 94 fractions were collected.

g) After size exclusion chromatography each fraction were tested for total sugar content by Dubois assay. The procedure was similar to that described in part 2.3.8.2 of this thesis with some changes. The Dubois assay was performed in mega blocks instead in Eppendorf tubes so that every fraction could be tested conveniently. The advantages of this modification to the method are savings of time, costs and equipment.

Modified Dubois assay using Mega Block 96-Well

a) Using a multichannel pipette, 50µl of each fraction (in duplicate) was transferred into a mega block 96-well and 50µl of 5% (w/v) phenol solution was added.
b) The well contents were mixed thoroughly using a multichannel pipette and incubated for 10 min at 4ºC.
c) After this time 250µl of concentrated sulphuric acid was added and mixed by multichannel pipette. The mega block was then covered with adhesive aluminium tape and placed on the surface of boiling water in the water bath.
d) After 15 min the mega block was removed and seal was discarded. Using multichannel pipette 150µl of mixture from each well was replaced into a 96-well microplate.
e) Absorbance was measured at 490nm by plate reader.
f) Fractions from lHCE were treated in a similar manner and were subjected to molecular weight analysis.
g) Results were exported to Excel program and a calibration curve of dextran standards was constructed (Appendix J). The curve shows K_{av} vs. the logarithm of molecular weight of dextran standards.
h) The molecular weight of lHCE sugar fraction was determined from the calibration curve values of K_{av} and calculated from the specific formula:

$$K_{av} = \frac{V_R - V_O}{V_C - V_O}$$

V_O-void volume
V_C-bed volume
V_R-retention (elution) volume of separated sample

2.4 Determination of the prebiotic index (PI) for horse chestnut aqueous extract (HCE)

Results from the characterisation of prebiotic-like activity of horse chestnut extract (HCE) showed that the aqueous extract had a beneficial effect on *Lactobacillus* and *Bifidobacterium* species. The degree of utilization of HCE by probiotic organisms was strain dependant. The ability of strains to ferment HCE was also compared to other commercial prebiotic- fructooligosaccharides (FOS) as presented in section 2.2.
The next objective of the research was to determine a quantitative score to describe the prebiotic activity of the horse chestnut extract (HCE).

Apparatus

- Spectrophotometer (Shimadzu, UVmini-1240, China)
- pH meter, (VWR symphony 5B70P)
- Class II cabinet (Holten Lamin-Air)
- Sterile 0.45µm pore-size filters, (Sarstedt, 83.1826)
- Sterile Eppendorf tubes (1ml)
- Universal glass bottles (30ml)
- Pipettes and sterile pipette tips
- Inoculation loops, (Sarstedt, 86.1562.010)
- Centrifuge (Falcon, 6/300, MSE, UK)
- Anaerobic chamber (Electrotek Anaerobic Workstation AW 200SG, Mini Micro, UK)

Reagents

- Probiotics strains :
  - *Lactobacillus plantarum,* ATCC 8014
  - *Lactobacillus rhamnosus,* ATCC 7469
  - *Lactobacillus acidophilus,* ATCC 4356
  - *Lactobacillus casei,* ATCC 393
  - *Lactobacillus fermentum,* ATCC 9338
  - *Bifidobacterium angulatum,* DSM 20098 (ATCC 27535)
  - *Bifidobacterium infantis,* DSM 20088(ATCC 15698)
  - *Bifidobacterium longum,* DSM 20219 (ATCC 17930)

- *Bifidobacterium breve,* DSM 20091 (ATCC 15698)

- Enteric strain :
  - *Escherichia coli* ECOR1 ATCC 35320

Lactobacillus strains were purchased from Medical-Supply Co. Ltd., Ireland. *Bifidobacterium* strains were derived from DSMZ GmbH, Germany. All strains were supplied in freeze-dried form.

Escherichia coli strain was purchased in freeze-dried form from ATCC-LGC Standards, UK.

- Commercial prebiotics :
  - GOS : VIVINAL®GOS, (Friesland foods, 502675)
  - FOS : Fructooligosaccharides from chicory, (Sigma-Aldrich, F8052-50G)
  - Inulin from chicory, (Sigma-Aldrich, I2255)

- Horse chestnut residue

Agar:

- MRS agar commercially available (Fluka, 69964) contained:
  - Agar (12 g/L)
  - Diammonium hydrogen citrate (2 g/L)
  - Dipotassium hydrogen phosphate (2 g/L)
  - D(+)-glucose (20 g/L)
  - Magnesium sulphate (0.1 g/L)
  - Manganous sulphate(0.05 g/L)
  - Meat extract (5 g/L)
  - Sodium acetate (5 g/L)
  - Universal peptone (10 g/L
  - Yeast extract (5 g/L)
  - Final pH 6.5±0.2 (25 °C)

- Nutrient Agar commercially available (Fluka, N9405) contained:
  - Peptic digest of animal tissue (5g/L)
  - Beef extract (3g/L)
  - Agar (15g/L)
  - Final pH 6.8±0.2 (25 °C)

Broths:

- MRS medium prepared manually, recipe from DSMZ GmbH website (DSMZ, 2007). The medium was sterilised by autoclave (15min/1atm/121ºC).
    - Bacteriological peptone (10g/L ; Oxoid, LP0037)
    - Meat extract (10g/L ; Fluka, 70164-500g)
    - Yeast extract (10g/L ; Oxoid, LP0021)
    - Tween 80 (1g/L ; Sigma-Aldrich, P1754)
    - Disodium hydrogen phosphate (2g/L ; Sigma-Aldrich, 04276-1kg)
    - Sodium acetate (5g/L ; Sigma-Aldrich, S8750-1kg)
    - Magnesium sulphate heptahydrate (0.2g/L ; Sigma-Aldrich, M1880-500mg)
    - Manganese sulphate monohydrate (0.05g/L ; BDH, 291474-500mg)
    - Distilled water (1000ml)
    - Sodium thioglycolate (0.5g/L ; Sigma, T0632)
    - Final pH (6.2-6.5)
- M9 broth prepared manually, recipe from AMRESCO website (AMRESCO, 1998). The medium was sterilised by autoclave (15min/1atm/121ºC)..
    - Sodium phosphate dibasic (6g/L ; Sigma-Aldrich, 04276)
    - Potassium phosphate monobasic (3g/L ; AppliChem, A1364,100)
    - Sodium chloride (0.5g/L ; Scientific and chemical, 7647-14-5)
    - Ammonium chloride (1.9g/L ; Sigma, A9434)
    - Distilled water (1000ml)
    - Final pH 7.5±0.2 at 25ºC

  Additional supplements added after autoclaving of M9 broth
    - Magnesium sulphate (1M, 0.2ml/L ; Sigma-Aldrich, M7506)
    - Calcium chloride (1M, 0.1ml/L ;Aldrich, 499608)
    - Supplements were autoclaved by filtration
- Bifidobacterium medium, prepared manually, recipe from DSMZ GmbH website, (DSMZ, 2008). The medium was sterilised by autoclave (15min/1atm/121ºC).

- Yeast extract (5g/L ; Oxoid, LP0021)
- Meat extract (5g/L ; Fluka, 70164)
- Bacto soytone (5g/L ; BD, 243620)
- Dipotassium hydrogen phosphate (2g/L ; Merck, 1.05101.1000)
- Magnesium sulphate heptahydrate (0.2g/L ; Sigma-Aldrich, M1880-500mg)
- Manganese sulphate monohydrate (0.05g/L ; BDH, 291474W-500g)
- Tween 80 (1ml/L ; Sigma-Aldrich, P1754)
- Sodium chloride (5g/L ; Scientific and chemical, 7647-14-5)
- L-Cysteine hydrochloride monohydrate (0.5g/L; AppliChem, A1702,0050)
- Salt solution (see below) (40ml/L)
- Resazurin (25mg/100ml; 4ml, Aldrich, 199303-5g)
- Distilled water (950ml)

Note: The cysteine was added after the medium had been boiled and cooled under CO_2, pH was then adjusted to 6.8 using 8 N NaOH.

Salt solution:

- Calcium chloride dihydrate (0.25g/L, Sigma-Aldrich, C3881)
- Magnesium sulphate heptahydrate (0.5g/L, Sigma-Aldrich, M1880-500mg)
- Dipotassium hydrogen phosphate (1g/L, Merck, 1.05101.1000)
- Potassium dihydrogen phosphate (1g/L, AppliChem, A1364,100)
- Sodium hydrogen carbonate (10g/L, Sigma-Aldrich, S6014-1kg)
- Sodium chloride (2g/L, Scientific and chemical, 7647-14-5)
- Distilled water (1000ml)

The solution was sterilised by autoclave (15min/1atm/121ºC).

Starter broths:

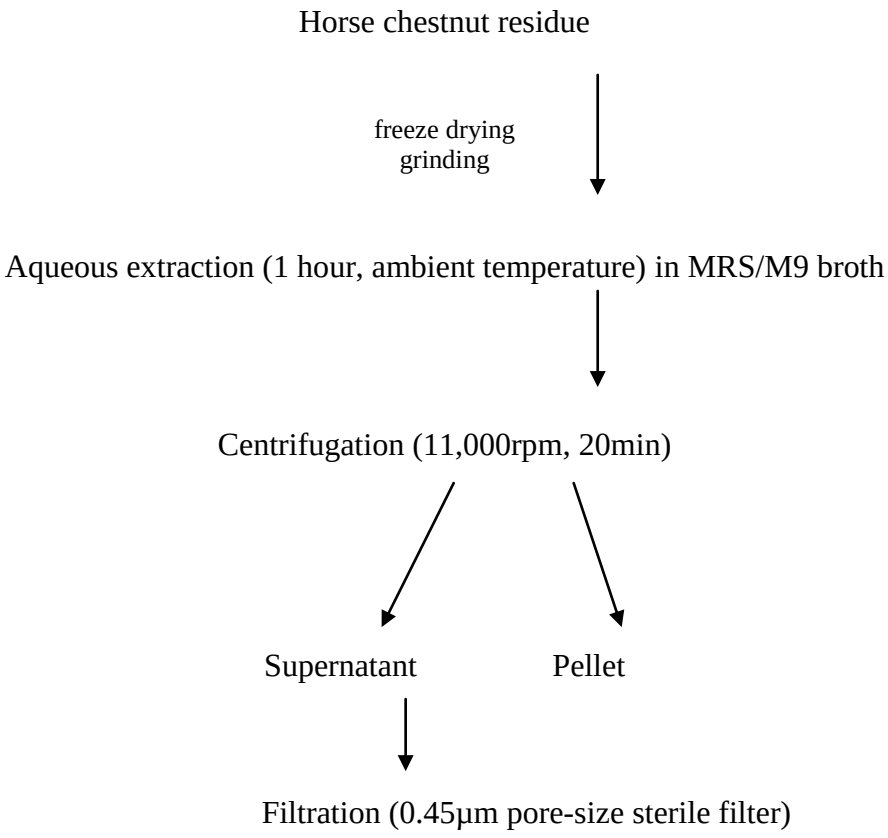
- MRS broth commercially available (Fluka, 69966) composition is shown in section 2.2.2.
- TSB broth commercially available (Fluka, 22092-500G) contained:
  - Casein peptone (pancreatic), (17 g/L)

- Dipotassium hydrogen phosphate (2.5 g/L)
- Glucose (2.5 g/L)
- Sodium chloride (5 g/L)

Procedure

Preparation of horse chestnut aqueous extract (HCE)

a) Extract was prepared from dry and powdered horse chestnut residue by dissolving it in MRS and M9 broth to obtain 1% (w/v) concentration.
b) The mixture was stirred for one hour on a magnetic stirrer at room temperature.
c) After this time, extract was centrifuged (11,000 rpm, 20 minutes) and supernatant was filtered (using sterile 0.45µm pore-size filters, Sarstedt) into sterile universal bottles in 10 ml volumes.
d) Flow chart below shows preparation of the HCE:



Preparation of starter cultures of probiotic and enteric strains

a) All strains were revived from freeze-dried form by streaking with the attached sterile cotton swab into Petri dishes contained MRS agar (for probiotic strains) or Nutrient Agar (for enteric strain).
b) The Petri dishes with probiotic bacteria were placed in an anaerobic chamber and incubated at 37ºC for 24 hours. The enteric strain was incubated in aerobic conditions at 37ºC for 24 hours.
c) All strains were inoculated by taking one isolated colony from Petri plates and suspending it in appropriate broths.
d) *Lactobacillus* strains were inoculated in commercial MRS broth (Fluka). For *Bifidobacterium* strains Bifidobacterium medium (DSMZ) was used.
e) Enteric strain was inoculated in commercial TSB broth.
f) Probiotic *Lactobacillus* strains and *E. coli* were incubated aerobically at 37°C overnight. *Bifidobacterium* were incubated at 37ºC in anaerobic chamber (10% H_2+10% CO_2+80% N_2). Sodium thioglycolate (0.05% (w/v)) was added in order to maintain anaerobic conditions.

Cultivation of probiotic and enteric strains in medium supplemented with prebiotics, horse chestnut extract and glucose

a) Bacterial starter cultures (100µl) were added into bottles containing 10 ml of MRS (DSMZ) for *Lactobacillus* and *Bifidobacterium* strains or 10 ml of M9 broth for *E. coli*.
b) All broths were previously supplemented with 1% (w/v) of prebiotic (FOS, GOS, or Inulin), 1% (w/v; which corresponds to 0.16% soluble matter) of HCE or 1% (w/v) glucose. As controls, starter cultures were added into appropriate broths with no additional sugars.
c) Lactobacilli and bifidobacteria were incubated at 37°C in shaking incubator at 50 rpm. In addition, bottles with *Bifidobacterium* strains were incubated in an anaerobic chamber at 37°C (10% H_2+10% CO_2+80% N_2).
d) The experiment was carried out in triplicate.
e) For each strain, optical densities (600nm) were measured at 0, 24 and 48 hours post inoculation using a spectrophotometer.

f) In all fermentation broths, pH was measured at the end of incubation (48hr) and 2ml of each broth were taken for organic acid analysis.

g) Broths were filtered using sterile 0.45µm pore-size filters (Sarstedt) into sterile Eppendorfs and stored at 4°C until use.

h) The prebiotic activity score was calculated using the modified version of the original formula (Huebner *et al.*, 2008):

$$\text{Prebiotic activity score} = \left[\frac{(\text{probiotic OD at 24h on prebiotic} - \text{probiotic OD at 0h on prebiotic}) - (\text{probiotic OD at 24h on control MRS} - \text{probiotic OD at 0h on control MRS})}{(\text{probiotic OD at 24h on glucose} - \text{probiotic OD at 0h on glucose}) - (\text{probiotic OD at 24h on control MRS} - \text{probiotic OD at 0h on control MRS})} \right]$$

divided by

$$\left[\frac{(\text{enteric OD at 24h on prebiotic} - \text{enteric OD at 0h on prebiotic}) - (\text{enteric OD at 24h on control M9} - \text{enteric OD at 0h on control M9})}{(\text{enteric OD at 24h on glucose} - \text{enteric OD at 0h on glucose}) - (\text{enteric OD at 24h on control M9} - \text{enteric OD at 0h on control M9})} \right]$$

i) The prebiotic activity score was also calculated using the original formula established by Huebner *et al.* (2008):

$$\text{Prebiotic activity score} = \left[\frac{(\text{probiotic log O.D. on the prebiotic at 24h} - \text{probiotic log O.D. on the prebiotic at 0h})}{(\text{probiotic log O.D. on glucose at 24h} - \text{probiotic log O.D. on glucose at 0h})} \right]$$

minus

$$\left[\frac{(\text{enteric log O.D. on the prebiotic at 24h} - \text{enteric log O.D. on the prebiotic at 0h})}{(\text{enteric log O.D. on glucose at 24h} - \text{enteric log O.D. on glucose at 0h})} \right]$$

j) The same experiment was repeated but different concentrations of the horse chestnut extract (HCE) was tested ranging from 1% to 5% (w/v). PI scores were calculated using the modified formula

2.5 Development of a novel method for determination of prebiotic efficacy

A novel method for screening of prebiotic potential has been developed. 96-well microtitre plates offer an opportunity to rapidly assay large numbers of samples. This would potentially allow for high throughput screening of samples.

Apparatus

- Sterile 96-Well Plate with Flat Bottom and Lid (Sarstedt, 82.1581.001)
- Multichannel Pipette and sterile tips
- Class II cabinet (Holten-Lamin-Air)
- Microplate reader (Varioskan Flash-Thermo Scientific, USA)
- Tray
- Sterile reservoirs (Reagent Reservoir for multi-channel pipettes, Sigma-Aldrich R1525-100EA)

Reagents

- Probiotic strain *Lactobacillus plantarum* ATCC 8014
- Sterile MRS broths (DSMZ, 2007): unsupplemented and supplemented with 1% (w/v) glucose, GOS, FOS or horse chestnut extract (HCE). Broths were prepared as described in previous section.

Procedure

a) Starter culture of *Lactobacillus plantarum* was prepared as described in previous experiment (section 2.4).
b) All actions were carried out in sterile conditions using Class II cabinet.
c) Sterile MRS broths (unsupplemented and supplemented) were poured into separate sterile reservoirs.
d) To sterile microplate 96-wells 190µl of sterile unsupplemented MRS (without glucose) broth and sterile MRS broth supplemented with 1% (w/v) sugar (glucose, GOS or FOS) or 1% (w/v; which corresponds to 0.16% soluble matter) HCE was added to separate rows using multichannel pipette.
e) For background measurements, 200µl water was also added to separate wells
f) Ten microlitres of overnight starter culture of *L. plantarum* was added to each row (except for rows used to zero the reading).
g) All incubation mixtures were mixed thoroughly.
h) The plate was covered with a sterile lid and optical density at time 0h was immediately measured in microplate reader at 600nm.

i) Microplate was placed in tray filled with wet tissue to prevent formation of condensation on the lid and moisture loss.

j) The tray with microplate was incubated at 37ºC (no shaking) for 24 hours.

k) After this time the incubation mixtures were mixed again using multichannel pipette to generate smooth suspension of bacterial cells and permit correct OD measurement.

l) Optical density (OD) was taken after 24 hours and the values were transformed to Excel to calculate the increase in cell density for all sugars and HCE tested.

m) The OD values for background (water) were subtracted from all OD values at 0 and 24 hours.

2.6 Determination of the effect of different processing conditions on prebiotic activity of horse chestnut extract

Apparatus

- Microplate reader, (Varioskan Flash-Thermo Scientific, USA)
- Spectrophotometer (Shimadzu, UVmini-1240, China)
- Sterile Multiple Well Plate 96-Well Flat Bottom with Lid (Sarstedt, 82.1581.001)
- Multichannel pipette and sterile tips
- Sterile 0.45μm pore-size filters (Sarstedt, 83.1826)
- Water bath (LAUDA, AQUAline, AL18)

Reagents

- Sodium hydroxide 2M
- Glycine (Riedel-de Haen, 15527)
- Glucose (Fisher Sccientific G/0450/53)
- Commercial prebiotics
    - GOS (Vivinal GOS, DOMO®, Frieslandfoods, 502675)
    - FOS (Fructo-oligosaccharides from chicory, Sigma-Aldrich, F8052-50G)
- Lyophilised aqueous extract from horse chestnut residue (lHCE)
- Probiotic strain (*Lactobacillus plantarum* ATCC 8014)

- Enteric strain (*Escherichia coli* ECOR1, ATCC 35320)
- Broths
    - MRS medium (DSMZ, 2007) double strength-2X
    - M9 broth (AMRESCO, 1998) double strength -2X
- McIlvaine citrate/phosphate buffer made by mixing of :
    - Solution A: Citric acid (0.1M, BDH, AnalaR, 100081)
    - Solution B: Disodium hydrogen phosphate (0.2M, Sigma-Aldrich S7907)

Procedure

Citrate-phosphate buffer was prepared using citric acid and disodium phosphate according to the procedure established by McIlvaine (McIlvaine, 1921). To prepare 0.1M citric acid solution (solution A) 1.92 g of citric acid (MW=192.12g/mol) was added to flask and filled with water up to 100ml mark. To prepare 0.2M disodium hydrogen phosphate solution (solution B) 2.84g of Na_2HPO_4 (MW=141.96g/mol) was placed into flask and made up 100ml with water. Depending on pH needed the two components were mixed together for preparation of 20ml of buffer as described below:

- pH 3.0 (4.1ml-B and 15.9ml-A)
- pH 4.0 (7.7ml-B and 12.3ml-A)
- pH 5.0 (10.3ml-B and 9.7ml-A)
- pH 6.0 (12.6ml-B and 7.4ml-A)
- pH 7.0 (16.5ml-B and 3.5ml-A)

The citrate-phosphate buffers (pH 3.0, 4.0, 5.0, 6.0, 7.0) were diluted to 20mM as described in the procedure by Huebner *et al*. (2008).

<u>Determination of the effect of low pH on prebiotic activity of lHCE</u>

a) Prebiotics (FOS, GOS) and powdered lHCE were added to 20mM citrate-phosphate buffer at pH 3.0, 4.0, 5.0 and 6.0 to obtain a final concentration of 2% (w/v).
b) Samples were filter-sterilized (0.45µm, Sarstedt) and stored for 24 hours at room temperature in darkness.
c) After this time pH was adjusted to 7.0 with 2M sodium hydroxide. Samples were filter-sterilized and stored in freezer at -80°C until use.

d) Non-treated samples (GOS, FOS and lHCE) were also prepared in a buffer solution at pH 7.0 at a concentration of 2% (w/v).
e) The experiment was carried out in triplicate.

Determination of the effect of low pH and high temperature in combination on prebiotic activity of lHCE

a) To 20mM citrate-phosphate buffer at pH 3.0, 4.0, 5.0 and 6.0 commercial FOS, GOS or lHCE were added to a final concentration of 2% (w/v).
b) Samples were filter-sterilized (0.45µm, Sarstedt) and held at 85ºC for 30min. in a water bath.
c) Treated samples were removed from the water bath, allowed to cool to room temperature and pH was adjusted to neutral with 2M sodium hydroxide. The samples were then filter-sterilized and stored at -80ºC until use.
d) Untreated samples (GOS, FOS and lHCE) were also prepared in a buffer solution at pH 7.0 at a concentration of 2% (w/v).
e) The experiment was performed in triplicate.

Determination of the effect of Maillard reaction on prebiotic activity of lHCE

a) To 20mM citrate-phosphate buffer at pH 7.0 glycine was added to give a concentration of 1% (w/v).
b) Prebiotic FOS and powdered lHCE were added to 20mM citrate-phosphate buffer at pH 7.0 with 1% (w/v) glycine, to obtain 2% (w/v) FOS/lHCE in the buffer. As a control solution, 2% (w/v) glucose in the buffer was also prepared.
c) The samples were held at 85ºC in a water bath and then subsequently removed after 1, 2 and 3 hours, allowed to cool after which the absorbance at 420nm of 1ml aliquot of each sample was measured. The remainder of each sample was filter-sterilized (0.45µm, Sarstedt) and stored at -80ºC until further use.
d) Untreated samples (FOS, lHCE and glucose; 2% (w/v)) were also prepared in a buffer solution at pH 7.0 + 1% (w/v) glycine.
e) The experiment was carried out in triplicate.

f) Treated and untreated samples (FOS, lHCE and glucose) were also tested for the presence of browning products using the following formula:

$$\text{percent relative browning products} = \frac{\text{Average ABS at 420nm of prebiotic at time } t_a}{\text{Average ABS at 420nm of glucose at time } t_b} \times 100\ \%$$

t_a time at which sample was removed (1,2,3, hours)
t_b time the final time at the end of experiment (3h)

Determination of prebiotic activity of treated and untreated samples (FOS, GOS and lHCE)

a) *L. plantarum* ATCC 8014 and *E. coli* ATCC 35320 strains were inoculated in appropriate starter broths (MRS/ TSB) to obtain starter cultures and then were incubated overnight at 37°C.
b) The prebiotic activity assay was carried out in a sterile 96-well microplate. One hundred microlitres of 2% (w/v) treated or untreated FOS, GOS or lHCE was added into wells with 100µl of double strength MRS/M9 sterile broth. The contents of each well were mixed using a multichannel pipette.
c) As a positive control, a mixture of 100 µl of 2% (w/v) glucose in citrate-phosphate buffer at pH 7.0 and 100µl double strength MRS/M9 broths was prepared.
d) A blank sample (100 µL of sterile double strength MRS/M9 and 100µL sterile citrate-phosphate buffer, pH 7.0) was also prepared.
e) To all wells, 10µL of starter bacterial culture of *L. plantarum*/*E. coli* was added.
f) Microplates were covered with sterile lid and placed into a small container filled with wet tissue to maintain high humidity and at the same time prevent loss of water during incubation at 37ºC, for 24 hours.
g) Measurements of bacterial growth (as OD at 600nm) were taken at 0 and 24 hours of incubation using the microplate reader.

2.7 Qualitative and quantitative analysis of organic acids in fermentation broths

Apparatus

- Cation exchange column (SUPELCOGEL C-610H HPLC Column, 30cm x 7.8 mm ID, Sigma-Aldrich, 58338)
- HPLC system, (LC-20AT prominence liquid chromatograph, Shimazu, Japan)
- PVDF membrane 0.2µm pore-size filters; Acrodisc, LC 13mm Syringe Filter (PALL, 4455T)

Reagents

- Organic acid standards
  - Acetic acid, (FLUKA, 71251-5ml-F)
  - Isovaleric acid, (Aldrich, 129542-100ml)
  - Valeric acid, (Aldrich, 24,037-0)
  - Formic acid, (Fluka, 06440-100ml)
  - Lactic acid, (Fluka, 69785-250ml)
  - Propionic acid (Fluka, 94425-5ml-F)
  - Butyric acid (Fluka, 19215-5ml)
  - Isobutyric acid (Fluka, 58360-100ml)
- Mobile phase (0.1% (v/v) phosphoric acid in water)

  Phosphoric acid (Sigma-Aldrich, P5811-500G)

Procedure

Preparation of organic acid standard curves

a) Organic acid standard solutions were prepared at the following concentrations: 0.1M, 0.05M, and 0.025M, by dissolving organic acids standards in 0.1% (v/v) of phosphoric acid.
b) All organic acid solutions were filtered using PVDF filters (0.2µm).

Chromatographic process

a) The HPLC system fitted with column was first equilibrated using mobile phase (0.1% (v/v) phosphoric acid).

b) Broths (20µl; section 2.4) were injected into the column.
c) Solutions of all organic acid standards were treated in similar manner.
d) The set parameters were: flow 0.6 ml/min, UV detection at 210 nm.
e) The presence of organic acid in the samples was identified by comparison of retention time of the sample peaks with those of the standard organic acid solutions.
f) Standard curves of each organic acid were prepared and the quantity of organic acid present in fermentation broths was determined using the formula of the line obtained from standard curves. Samples were diluted where necessary.
g) The peak areas both for LA and AA at three different concentrations (0.1, 0.05 and 0.025M) were plotted using organic acid standards.
h) Appendix K shows retention time and peak area obtained for lactic acid (LA) standard. Standard curve of peak area vs. concentration (M) for LA is also presented. The values obtained for acetic acid (AA) are shown in Appendix K.

2.8 Determination of the effect of lyophilised horse chestnut aqueous extract on hyaluronidase activity

Apparatus

- Fume hood
- Water bath (LAUDA AQUAline AL18)
- Glass test tubes (75x10mm)
- Microplates 96-Wells
- Microplate Reader (Varioskan Flash-Thermo Scientific, USA)
- Volumetric flask (100ml)

Reagents

- Acetate buffer pH 3.5 contained:
    - Glacial acetic acid (0.1M)
    - Sodium acetate (0.1M)
- Calcium chloride dehydrated (12.5mM; Fluka, 21075-1kg)
- Hyaluronidase enzyme (801U/mg; Sigma-Aldrich, H-3506-1g)

- Hyaluronic acid potassium salt from human umbilical cord (Sigma-Aldrich, HI504-500mg)
- Heparin sodium (50µg/ml; Wexport Ltd., Littleisland, ED4071)
- DMAB reagent (10x stock solution) colour stabiliser contained:
    - DMAB (1g; 4-dimethyloamino benzaldehyde, Fluka, 39080-100g)
    - Glacial acetic acid (10ml) with 12.5% 10N HCl
- Sodium hydroxide (0.4N; AnalaR NORMAPUR, 28244.295 1kg)
- Potassium tetraborate tetrahydrate, 99% (0.4M; Sigma-Aldrich, 289795-1kg)
- Lyophilised horse chestnut aqueus extract (lHCE)
- FOS (fructooligosaccharides from chicory, Sigma, F-8052-50G)

Procedure

a) To prepare acetate buffer pH 3.5, 0.1M acetic acid and 0.1M sodium acetate solutions were prepared: according to Practical Chemistry (Lambert and Muir, 1968).

Preparation of acetic acid acid solution

0.1M acetic acid solution: 0.6ml glacial acetic acid prepared in 100ml flask

Preparation sodium acetate solution

0.1 M sodium acetate solution: 820mg sodium acetate (MW=82.03g/mol) in 100ml flask.

The acetate buffer pH 3.5 was prepared by mixing 92ml acetic acid (0.1M) with 8ml sodium acetate; pH was monitored on pH meter.

b) Preparation of calcium chloride solution as HA-ase activator

12.5M calcium chloride solution: 140mg of dehydrated calcium chloride (MW=110g/mol) in 100ml flask.

c) Preparation of sodium hydroxide solution

0.4N sodium hydroxide solution: 1.6g of sodium hydroxide (MW=40g/mol) in 100ml flask.

d) Preparation of potassium tetraborate solution

0.4M of potassium tetraborate solution: 12.14g of $K_2B_4O_7x4H_2O$ (MW=303.53g/mol) in 100ml flask.

e) Preparation of hyaluronidase (HA-ase) enzyme solution

Original activity of enzyme in vial was 801U/mg.A working solution of HA-ase was prepared with 7900U/ml in acetate buffer pH 3.5. The mixture had to be freshly prepared before each assay. To obtain 7900U/ml enzyme working solution of HA-ase 9.86mg of HA-ase was dissolved in 1ml of acetate buffer pH 3.5.

f) Preparation of heparin solution as model inhibitor of HA-ase

The inhibitor (heparin sodium) was prepared in acetate buffer pH 3.5 to a concentration of 50µg/ml.

g) Preparation of hyaluronic acid (HA) solution as substrate

HA substrate should be prepared fresh before use. The concentration was 1.2mg/ml in acetate buffer pH 3.5.

h) Preparation of DMAB reagent as colour stabilizer

One hundred millilitres of 10xDMAB was prepared as follow: To a glass bottle (dipped in cold water): water 0.167ml, HCl (37%) 12.333ml, glacial acetic acid (87.5ml) and DMAB (10g) were added in this sequence. The reaction between the added components is exothermic therefore this step was done in a fume hood.

DMAB (10x) was stored at 4°C. The working solution of DMAB used for each assay was 1x.

Enzymatic assay

a) The assay was carried out in glass tubes (75x10mm).
b) The lyophilised horse chestnut aqueous extract (lHCE) was dissolved in acetate buffer pH 3.5 to the following concentrations (50, 40, 30, 20, 10 and 5mg/ml).
c) To determine potential inhibitory effect of commercial prebiotic on hyaluronidase activity, fructooligosaccharide (commercial FOS) was also tested.

The concentrations in acetate buffer pH 3.5 were as follows: 50, 40, 30 and 20 mg/ml.

d) Standard inhibitor (as positive inhibition control) heparin was also prepared in acetate buffer pH 3.5 at concentrations of 0.5, 0.4, 0.3, 0.2, 0.1 and 0.05mg/ml.
e) One hundred microliters of sample (lHCE or FOS) and 50µl of HA-ase (7900U/ml) were added to a tube and mixed with pipette.
f) As enzymatic control only 100µl acetate buffer pH 3.5 and 50µl of HA-ase (7900U/ml) was applied.
g) As a control for inhibition (with commercial inhibitor) the following pattern was used: 100µl heparin solution at different concentration, and 50µl HA-ase (7900U/ml).
h) As blank (background) only 100µl of acetate buffer/sample (FOS or lHCE)/heparin was placed into separate tubes.
i) All samples and controls were prepared in duplicate.
j) To all tubes 100µl of 12.5mM $CaCl_2$ was added and each was incubated at 37ºC for 20minutes in a water bath.
k) After this time 250µl of 1.2mg/ml of HA was added to all tubes and mixtures were vortexed.
l) The tubes were incubated again at 37ºC for 40min. in water bath.
m) To stop enzymatic reaction, 100µl of 0.4N NaOH was added to each tube.
n) For all blank controls 50µl of HA-ase (7900U/ml) was added.
o) One hundred microliters of 0.4M $K_2B_4O_7x4H_2O$ was placed in the reaction mixtures and then incubated at 100ºC for 3 min.
p) After cooling to room temperature, 3ml of 1xDMAB was added to each tube in the fume hood before incubation at 37ºC for 20min.
q) Two hundred microliters of each reaction mixture was placed into microplate 96-wells and absorbances at 585nm were taken using microplate reader.
r) Inhibitory effect of lHCE or FOS on hyaluronidase activity was calculated using the following formula:

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$$\% \text{ of inhibition} = \frac{(\text{control ABS at 585nm} - \text{sample ABS at 585nm})}{\text{control ABS at 585nm}} \times 100\%$$

2.9 Determination of inhibitory effect of lyophilised horse chestnut aqueous extract on elastase activity

Apparatus

- Microplate Reader (Varioskan Flash-Thermo Scientific, USA) with fluorescence reading
- Assay Plate, 96 Well , No lid, Flat Bottom Black Polystyrene (Costor, 391)
- Multichannel pipette
- Tips

Reagents

- EnzChek® Elastase Assay Kit (E-12056) by Molecular Probes
    - Component A – DQ elastin from bovine neck ligament, BODIPY FL conjugate (in original vial 1mg substrate lyophilizated from 1ml phosphate-buffered saline, pH 7.2 (PBS)
    - Component B – 10x Reaction Buffer 28ml of 1M Tris-HCl, pH 8.0 containing 2mM sodium azide
    - Component C – Elastase from pig pancreas, 50 units (1U=amount of enzyme needed to solubilize 1mg of elastin in 20 min. at pH 8.8 and 37ºC)
    - Component D – *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, MW=503, inhibitor of elastase, 500µg
- Dimethylsulfoxide (Sigma, D-5879-500ml)
- Stock solution of lyophilised horse chestnut aqueous extract (200mg/ml)
- Double deionized water

Procedure

The experimental protocol used in this thesis is based on existing protocol included in the assay kit

Preparation of 1x Reaction Buffer

The original 10x Reaction Buffer (Component B) was diluted to obtain 1x working Reaction Buffer and stored at 4ºC.

Preparation of DQ elastin stock solution

To the original vial with lyophilised DQ elastin (Component A) 1ml of double deionized water was added and the content was mixed using a vortex mixer. The mixture (with concentration of 1mg/ml) was divided into aliquots (100 µl) and placed in Eppendorf tubes. DQ stock solutions were stored in -20ºC.

Preparation of DQ elastin working solution

The elastin working solution was prepared by dilution of elastin stock solution in 1x Reaction Buffer. The concentration of substrate needed was 100µg/ml.

Preparation stock solution of enzyme (elastase)

To prepare 100U/ml elastase stock solution 0.5ml of double deionized water was added to the content of the original vial (Component C). The working enzyme solution was stored at -20ºC.

Preparation of elastase inhibitor stock solution (Component D)

To the original vial with component D 50µl of anhydrous dimethylsulfoxide was added to obtain 10mg/ml concentration of inhibitor. The inhibitor stock solution was stored at -20ºC.

Enzymatic assay

The assay was carried out in a black microplate suitable for fluorescence measurement. To determine inhibitory effect of lHCE and commercial inhibitor (*N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) on elastase activity, different serial dilutions of lHCE and inhibitor were used in the assay, whereas the concentration of enzyme (elastase) and substrate (DQ- elastin) remained the same.

Assay of elastase in the presence of commercial inhibitor (N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone).

The inhibitor stock solution was prepared in 1x reaction buffer. Because the assay was carried out in a microplate and 50µl volume of the inhibitor was used for each 200µl reaction (per well), the inhibitor stock solution was 4x strength. The original vial with inhibitor (Component D) contained 10mg/ml of inhibitor. According to the guide (included to the kit) the final inhibitor concentration which inhibits elastase by 100% was established as 0.1M. Using the molecular weight of inhibitor (MW=503g/mol), 0.1M concentration of inhibitor was calculated as 0.0503mg/ml. To prepare 4x inhibitor working solution (0.2mg/ml) 10µl of inhibitor stock solution was diluted in 490µl 1x reaction buffer (volume of 500µl of 4x inhibitor working solution was sufficient for the assay).

a) The assay was done in duplicate.
b) Fifty microlitres of 1x reaction buffer was added to wells 1,2 (rows B-H) and 100µl of 4x inhibitor working solution was added to 1 and 2 (row A).
c) From wells 1,2(row A) 50µl of inhibitor working solution or lHCE solution was taken and two fold dilution was made in wells 1 and 2 (row B). The same action was done in wells 3 and 4 (wells A-H).
d) To wells 1 and 2, 100µl of enzyme (0.2U/ml) and to wells 3 and 4 (as no-enzyme control), 100µl 1x reaction buffer were added using a multichannel pipette and mixed very well.
e) The reaction mixtures were incubated at room temperature for 20 minutes and protected from light.
f) After this time 50 µl of 100µg/ml DQ elastin working solution was added to each assay well.
g) Because the reaction is continuous the fluorescence was measured at multiple time points. Measurements were taken every 10 minutes for a period of 120 minutes to give 13 readings.
h) The Varioskan Plate Reader was set at the following parameters: Excitation-495nm, Emission-515nm, room temperature.
i) From each time point of enzymatic reaction the background fluorescence (derived from enzyme free control) was subtracted.

2.10 Preparation of organic extracts of horse chestnut residue and assessment of antimicrobial activity

2.10.1 Extraction of raw compounds from horse chestnut residue

Apparatus

- Glass bottles (500ml)
- Round-bottom flasks (250ml)
- Celite 545 filter (Celite® Analytical Filter Aid II, Supelco, 11485-U)
- Glass funnel
- Vacuum pump (diaphragm vacuum pump, type MZ 2C, Vacuubrand GMBH + CO Germany,)
- Fume hood
- Rotary vacuum evaporator (Bibby Sterilin Ltd., England)
- Small brown glass bottles

Reagents

- Methanol (Reagecon, RSA007)
- Ethanol (99%, denatured, J.T. Baker, 3408)
- Acetone (Lenox, SA-010-1614)
- Dichloromethane (Sigma-Aldrich, 24233-2.5L-R)
- Chloroform (BDH, AnalaR NORMAPUR, analytical reagent 22711.324)

Procedure

Material preparation

Fresh horse chestnut waste was lyophilised. The residue was then powdered using a grinder and kept at -20 C until use.

Preparation of crude extracts from horse chestnut residue using organic solvents

In this procedure pure organic solvents (methanol, ethanol, acetone, dichloromethane and chloroform) were used for extraction.

a) The dried, powdered horse chestnut residue (30 g) was transferred into glass bottles (500ml) and 200ml of organic solvent was added.
b) The mixtures were stored in darkness for 3 days at room temperature without shaking.
c) The extracts were then filtered using a glass funnel with Celite 545. The filtration process allowed separation of solid phase (residue) from solvents. This step was performed under a fume hood.
d) The extracts were transferred to previously weighed round-bottom flask (250ml) and the solvents were removed by rotary vacuum evaporator at 40°C.
e) The residue in the flask, labelled raw extracts, were weighed again and reconstituted in pure solvents to appropriate concentration.
f) The extracts (methanolic- MetHCE, ethanolic- EtHCE, acetone- AcHCE, dichloromethane- DiHCE, chloroform- ChHCE) were placed into small brown bottles (preventing light exposure of active compounds) and kept at -20 C until use.

2.10.2 Preliminary screening of the raw horse chestnut organic extracts by thin layer chromatography

Apparatus

- TLC aluminium sheets 20x20 and 10x20 Silica gel 60 F_{254} (Merck)
- Glass container for TLC plates
- Oven at 110°C (Lab-line Inc., USA)
- Fume hood
- Forceps
- Pencil
- Roller

Reagents

- Methanol (Methanol G CHROMASOLV, Sigma-Aldrich, 34885)
- Chloroform (BDH, HiPerSolv for HPLC, 152834E)
- Ethyl acetate (Sigma-Aldrich, 34858)

- Hexane (n-hexane, 95%, Romil-SA, A9389)
- Anisaldehyde/sulphuric acid spray reagent mixed in order:
  - Ethanol (465ml: J.T. Baker, 2408)
  - Glacial acetic acid (5ml: Merck, 1.00063.2511)
  - p-anisaldehyde (13ml: 4-methoxybenzaldehyd, 99+%, Aeros Organics, 104805000)
  - Sulphuric acid (13ml: Sigma-Aldrich, 258105-2.5L)

Procedure

a) Three different developing mixtures were prepared to determine the best separation of components from raw extracts. The developing mixtures were as follow: chloroform: methanol (90:10) – CM, ethyl acetate: methanol: water (81:11:8) – EMW and ethyl acetate: hexane (1:1) – EH. The developing solvents were differing in relation to polarity.

b) On the TLC (10x20) plates a line was drawn 1.5 cm from the top and from the bottom using a pencil to mark the start and the end of the chromatographic process.

c) Four microlitres of four different extracts (methanolic- MetHCE, ethanolic- EtHCE, acetone - AcHCE, chloroform- ChHCE) were applied at 1.5cm from the base of pre-prepared silica gel TLC plates. Time was allowed for samples to dry. After drying the TLC plates were placed into separate glass containers and developed with CM, EMW and EH under fume hood.

d) When developing solvents reached 1.5 cm from the top of the plates, the TLC plates were removed from the containers using forceps, dried and sprayed with anisaldehyde/sulphuric acid spray reagent. The TLC plates were then heated at 110°C for 10 min. in an oven.

e) The spots were examined and plates developed by different solvents were compared to each other. The developing solvent mixture offering best resolution of bands was chosen for further analysis of the extracts.

f) The same procedure was repeated using larger (20x20) TLC plates and all horse chestnut extracts (including dichloromethane (DiHCE)) were separated on the plates using only EMW developing solvent. The visualisation procedure remained the same.

2.10.3 Determination of antimicrobial activity of the raw horse chestnut organic extracts

Analysis of antimicrobial activity of the extracts was performed using disc diffusion method, bioautography assay and checkerboard techniques.

2.10.3.1 Disc diffusion assay

Apparatus

- Sterile Petri plates (100mm)
- Class II Microbiology Cabinet
- Sterile cotton swabs
- Sterile forceps
- Turbidometer (Biolog, USA)
- Glass Tubes (Biolog, USA) with sterile saline (10ml)
- Incubator
- Sterile discs (6mm: Whatman, Grade AA discs, 2017006)
- Commercial Antibiotic discs (Mastring-S, M-11 and M14, MAST, UK) contained:

Systemic Gram Positive Ring	Systemic Gram Negative Ring
M11 (1-8)	M14 (1-8)
E- erythromycin 5µg	AP- ampicillin 10µg
FC- fusidic acid 10µg	KF- cephalothin 5µg
MT- methicillin 10µg	CO- colistin sulphate 25µg
NO- novobiocin 5µg	GM- gentamicin 10µg
PG- penicillin G 1 unit	S- streptomycin 10µg
S- streptomycin 10µg	ST- sulphatriad 200µg
T- tetracycline 5µg	T- tetracycline 25µg
C- chloramphenicol 25µg	TS- cotrimoxazole 25µg

Reagents

- Mueller-Hinton Agar 2 commercially available (Fluka, 97580-500G-F) contained:

  - Beef heart infusion (2.0g/L)
  - Acid Casein Hydrolisate (17.5g/L)
  - Starch, soluble (1.5g/L)
  - Agar (17.0g/L)
- Bacterial strains:
  - Gram positive (*Staphylococcus aureus* ATCC 9144/NCTC 6571, *Staphylococcus epidermidis* ATCC 12228)
  - Gram negative (*Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048)
- Horse chestnut organic solvent extracts (MetHCE, EtHCE, AcHCE, DiHCE, ChHCE)

Procedure

The susceptibility test of the extracts was based on Kirby-Bauer method and consisted of the following steps:

a) Inoculum preparation

The bacteria to be tested for susceptibility were cultured to log phase. Actively growing on Petri plates (with Nutrient Agar) cultures of *S. aureus*, *E. coli*, *S. epidermidis* and *E. aerogenes* were transferred from plates into glass Biolog tubes using sterile swabs and suspended in sterile saline (10ml) in a Class II microbiology cabinet. The tubes were vortexed to create smooth suspensions. Turbidity of bacterial suspensions was adjusted to 0.5 McFarland standard, which corresponded to 74.5% transmittance. These values were equivalent to bacterial suspension containing between $1x10^8$-$2x10^9$ cfu/ml.

b) Muller-Hinton agar (MHA) preparation

Muller-Hinton agar was prepared (1.7%). Autoclaved agar (121ºC, 15min) was dispensed on sterile Petri plates (100mm diameter) to a depth of 4mm (25ml MHA for each 100mm plate) in a Class II microbiology cabinet.

c) Loading of the extracts on the sterile discs

Fourty and sixty microlitres of the extracts were dispensed onto the sterile discs (6mm diameter) and discs were allowed to dry within the Class II microbiology cabinet. As a

negative control another set of sterile discs were impregnated with the same volume of pure solvents. As positive control commercial discs (MASTRING-S) were used.

d) Inoculation of the plates with MHA

Using sterile swabs the inoculum of bacterial strains (in saline) was streaked on the plates containing MHA. To ensure that the whole plate was inoculated the plates were rotated approximately 60 degrees after each streak.

e) Placing tested discs with extracts and control

Discs with extracts, negative controls (pure solvents) and positive control (commercial discs with impregnated antibiotics) were aseptically placed on the surface of the inoculated MHA.

f) Incubation

Plates were incubated at 30ºC (*S. aureus*, *S. epidermidis*, *E. aerogenes*) and at 37ºC for *E.coli*. The inhibition effect of the extracts was observed after 24 hours of incubation.

2.10.3.2 Bioautography assay

Apparatus

- Turbidometer (Biolog, USA)
- Glass Tubes (Biolog, USA) with sterile saline (10ml)
- Sterile swabs
- Class II Microbiology Cabinet
- Bioassay Dish (sterile, NUNC)
- Incubator

Reagents

- Mueller-Hinton Agar 2 (0.6% (w/v); Fluka, 97580-500G-F)
- Tetrazolium red (10% (w/v); 2,3,5- triphenyl-tetrazolium chloride, BDH, 2568270)
- Bacterial strains on plates

- Gram negative (*Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048)
- Gram positive (*Staphylococcus aureus* ATCC 9144)

- Horse chestnut organic solvent extracts (MetHCE, EtHCE, AcHCE, DiHCE and ChHCE)

Procedure

As an alternative method, bacterial inhibition by organic horse chestnut extracts was investigated by bioautography using all extracts. For bioautography assay *S. aureus* (ATCC 9144), *E. coli* (ATCC 25922) and *E. aerogenes* (ATCC 13048) were used.

Thin layer chromatography (TLC)

a) A set of four plates (G-60, F_{545}, silica gel,20 x 20) were used. The extracts (MetHCE, EtHCE, AcHCE, DiHCE, ChHCE) were placed on TLC plates (4µl each).
b) The TLC plates were developed in ethyl acetate: methanol: water (88:11:8) and dried in fume hood.
c) One of the four plates was inspected under UV lamp (245nm) to determine fluorescent phenolic compound. After this step the same plate was sprayed with anisaldehyde/sulphuric acid spray reagent to visualize all separated constituents.
d) The R_f values of the separated spots was determined using the following formula:

$$R_f = \frac{\text{distance (cm) traveled by the compound}}{\text{distance (cm) traveled by the solvent front}}$$

e) The second, third and fourth plates were used for autobiography assay. The assay was carried out using gram negative *E. coli* (ATCC 25922), *E. aerogenes* (ATCC 13048) and gram positive *S. aureus* (ATCC 9144).

Bioautography

a) The suspension of bacteria (in 0.6% MHA) was prepared in 10ml of sterile saline to 1 McFarland standard (55.6% of transmittance) which is equivalent of $3x10^8$ cfu/ml.

b) Dry TLC plates were covered with a thin layer (1mm) of MHA (0.6%) containing 0.5% tetrazolum red and bacteria (*E. coli*/*S. aureus*/*E. aerogenes*) at a concentration of $1x10^6$ cfu/ml.
c) TLC plates were then placed in NUNC plates and incubated at 37ºC for 24/48 hours.
d) Inhibition zones were determined as clear areas against a pink background.

2.10.3.3 Checkerboard assay

Apparatus

- Sterile 96-Well Plate with Flat Bottom and Lid (Sarstedt, 82.1581.001)
- Multichannel pipette and sterile tips
- Microplate reader, (Varioskan Flash-Thermo Scientific, USA)
- Incubator
- Class II Microbiology Cabinet
- Sterile reservoirs (Reagent Reservoir for multi-channel pipettes, Sigma-Aldrich R1525-100EA)
- Turbidometer (Biolog, USA)
- Glass Tubes (Biolog, USA) with sterile saline (10ml)
- Sterile swabs

Reagents

- Mueller-Hinton Broth (OXOID, CM 0405) - double strength
- Sterile saline
- Ampicillin (200µg/ml: ampicillin sodium salt, Merck, 1.00278.0005)
- Alamar Blue (Resazurin sodium salt, Sigma, R7017-5G)
- PBS buffer (10x) contained:
  - Disodium dihydrogen phosphate dodecahydrate (31.16g: Sigma, 71649-1kg)
  - Monosodium dihydrogen phosphate dihydrate (2.02g: Sigma-Aldrich, 04269)
  - Sodium chloride (85g: Scientific and chemical, 7647-14-5)

The above were dissolved in 800ml deionized water in 1000ml flask. The pH was adjusted to 7.4. The solution was made up with deionized water to 1000ml mark and autoclaved (121ºC/15min/1atm.)

- Bacterial strain on plate
  - Gram negative (*Escherichia coli* ATCC 25922)
- Horse chestnut organic solvent extracts (EtHCE and MetHCE)

Procedure

a) Alamar Blue reagent was prepared in sterile 1X PBS to a concentration of 0.01g/10ml (0.1% (w/v) solution). The reagent was stored at 4ºC until use.
b) Inoculum of bacterial strains (*E. coli*) was prepared in sterile saline (10ml) in similar manner as described in previous section.
c) The assay was done in sterile 96-wells microplate. One hundred microlitres of methanolic (MetHCE)/ethanolic (EtHCE) extract was placed in row A (wells 1,2,3,4) on microtitre plates and 50µl of sterile saline was added to rows B-H (1,2,3,4).
d) Two fold dilutions were performed by transferring 50µl of extracts from row A to B using a multichannel pipette.
e) The action was repeated up to row H. From row H, 50µl of mixtures were discarded to ensure a final volume of 50 µl in each test well.
f) Double strength Muller-Hinton broth was added to wells 1,2 and 3,4 in volumes of 40 and 50µl respectively.
g) A ten microlitre inoculum of *E. coli* was added to wells 1 and 2 whereas wells 3 and 4 remained without bacteria (as background) to ensure that the extracts itself did not exhibit fluorescent activity.
h) As negative control, pure solvents (methanol/ethanol) were applied to wells 5 and 6 on separate microplates and two fold dilution was done in similar manner. This control was carried out to ensure that pure solvents did not exhibit inhibitory effect on the bacteria tested.
i) As antibiotic control, ampicillin solution (200µg/ml) was added in triplicate (rows 7,8,9) and two fold dilutions were prepared up to wells H.
j) Positive controls (in wells 10,11) contained 40µl sterile saline, 50µl 2x Mueller-Hinton broth and 10µl bacterial suspension.

k) Plates were covered with a lid and placed in covered tray with wet tissue on the bottom to provide adequate humidity and prevent condensation of liquid on lids.
l) Incubation time was 24 hours at 37ºC.
m) After that time 10µl of Alamar Blue reagent (0.1% in 1x PBS) was added to all wells and plates were incubated again at 37ºC for 2 hours.
n) Florescence was measured on Varioskan Plate reader (Excitation 540nm/Emission 590nm)
o) Values obtained for the wells containing extracts with bacteria (tested samples) were subtracted from the background (extracts+MHB+no bacteria).
p) Signs of inhibitory effect of the extracts tested were also observed visually. Pink colour indicated presence of bacterial growth in wells whereas blue colour suggested inhibition.

The Minimal Inhibitory Concentration (MIC) was determined as the lowest sample concentration (MetHCE or EtHCE) in the wells that exhibited colour change, or, at which fluorescence approached that of positive control wells.

3 Results

3.1 Chemical composition of horse chestnut waste

3.1.1 Moisture content

Moisture content in horse chestnut waste was established as 48.15% (w/w, SD=0.448, n=3) of fresh sample. The experiment showed that water is rapidly evaporated from the sample. Most of the water content seems to be in the free state and samples do not contain volatile oils, which could retain the water molecules.

The experiment shows that horse chestnut waste contains 48.15% water and 51.85% total solids in fresh residue (Figure 3.1). The total solids are the object of analysis in further parts of the research.

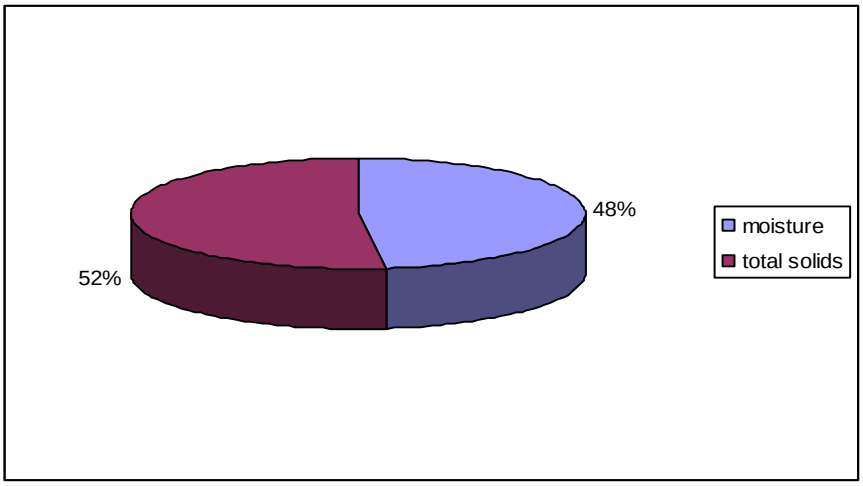


Figure 3.1: Moisture and total solids from horse chestnut residue

3.1.2 Ash content

Dried horse chestnut waste was ashed at 550°C for 5 hours. The average ash content from three samples was 2.97% (w/w, SD=0.176, n=3) of dry matter.

3.1.3 Determination of metal elements by atomic emission/absorption and colorimetric techniques.

The mineral standard curves were plotted and showed the concentration of mineral (mg/L) vs. absorbance (nm) for a range of standard solutions. The correlation coefficient for most of the standard curves, in the case of both atomic absorption and atomic emission, was greater than 0.99 (see Appendix A). This indicates excellent linearity which ensures accurate results.

All values were obtained from atomic absorption or atomic emission (sodium, potassium) except for phosphorus which was determined by colourimetric technique.

The calculation of phosphorus content was done according the procedure included to kit (HACH).

Mineral content of ash is presented in Table 3.1 and shows mineral content calculated for 1 gram of wet sample (horse chestnut waste). The result of this experiment shows that ash from horse chestnut waste mainly consists of potassium. Phosphorus, calcium and magnesium are also considered to be major elements, while iron, nickel, copper, sodium could be considered as trace elements.

Table 3.1: Metal concentration in ash from horse chestnut residue.

Mineral	Horse chestnut waste
Potassium	4.63
Phosphorus	0.87
Calcium	0.35
Magnesium	0.35
Iron	0.024
Nickel	0.013
Sodium	0.013
Copper	0.009

Results expressed as mg/g wet weight of horse chestnut residue.

3.1.4 Protein content

Protein content was measured according to the Kjeldahl method. Horse chestnut waste contains 6.93% (w/w, SD=0.220, n=3) of protein in dry mass.

3.1.5 Lipid content

3.1.5.1 Total lipids content

The lipids fraction of the horse chestnut waste was found to be 1.89% (w/w, SD=0.331, n=3) of the dry mass. Proteins and lipids together comprise 8.8% of horse chestnut waste dry matter.

3.1.6 Analysis of fatty acids by gas chromatography

a) Standard methyl esters of fatty acids

In order to determine the type and concentration of fatty acid, the acid methyl esters of standard fatty acids (FAME) were measured by GC. A typical chromatogram of standard FAME is shown in Figure 3.2.

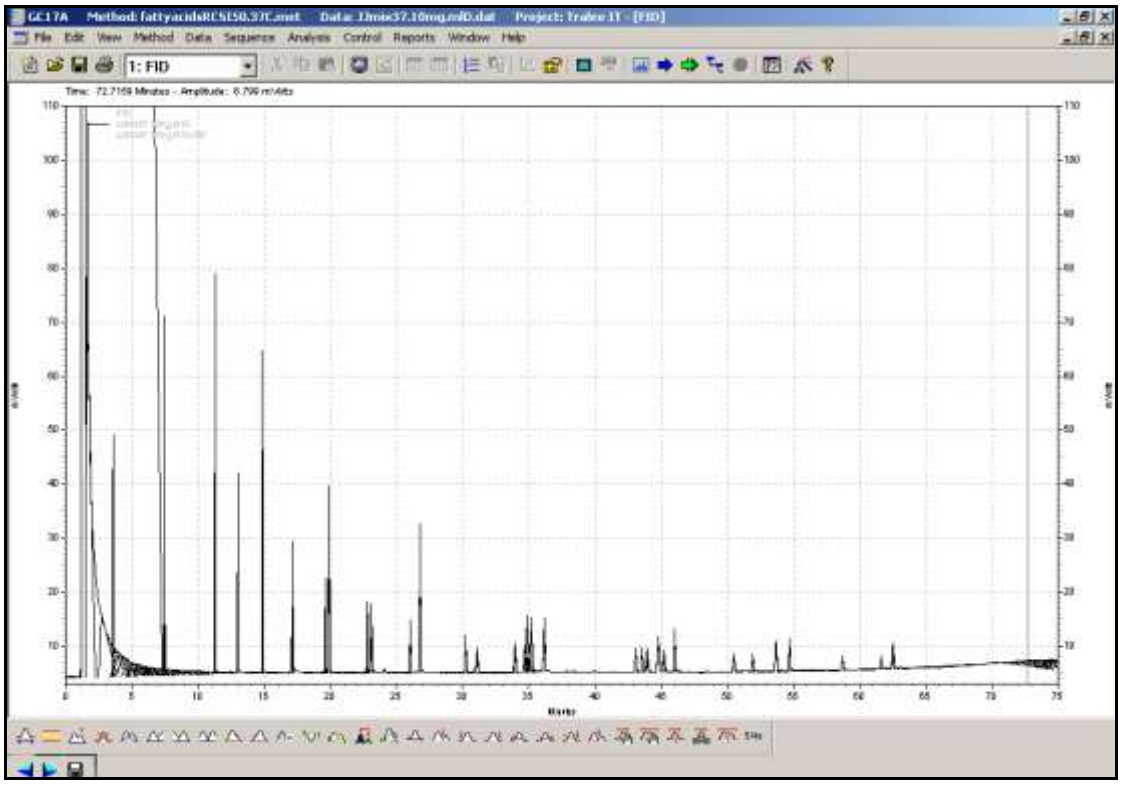


Figure 3.2: Gas chromatography of standard fatty acids (FAME, Supelco).
X-axis as Minutes, Y-axis as mVolts.

Retention time was established as the average of 3 different dilutions of standards (2.5mg, 5mg and 10mg). Table 3.2 shows retention times of all identified standard fatty acids.

Table 3.2: Retention time for standard fatty acids (FAME, Supelco).

Name of methyl esters of fatty acids (FAME, Supelco)	Number of carbons in chain	Retention time (min)			Mean	SD
		2.5mg/ml	5mg/ml	10mg/ml		
Butyric	C4:0	1.552	1.457	1.567	1.525	0.049
Caproic	C6:0	3.553	3.556	3.530	3.546	0.012
Caprylic	C8:0	7.503	7.438	7.402	7.448	0.042
Capric	C10:0	11.429	11.254	11.231	11.305	0.088
Undecanoic	C11:0	13.216	12.983	12.295	12.831	0.391
Lauric	C12:0	15.191	14.847	14.826	14.955	0.167
Tridecanoic	C13:0	17.597	17.124	17.009	17.243	0.254
Myristoleic	C14:1	19.707	19.607	19.582	19.632	0.054
Myristic	C14:0	19.979	19.882	19.860	19.907	0.052
Cis-10-Pentadecanoic	C15:1	22.865	22.795	22.777	22.812	0.038
Pentadecanoic	C15:0	23.172	23.107	23.084	23.121	0.037
Palmitoleic	C16:1	26.077	26.030	26.010	26.039	0.028
Palmitic	C16:0	26.807	26.753	26.738	26.766	0.030
Cis-10-Heptadecanoic	C17:1	30.371	30.206	30.183	30.253	0.084
Heptadecanoic	C17:0	31.131	31.070	31.058	31.086	0.032
Linolenic	C18:3n3	33.903	33.961	33.944	33.936	0.024
γ-Linolenic	C18:3n6	34.643	34.689	34.666	34.666	0.019
Linoleic	C18:2n6c	34.819	34.841	34.833	34.831	0.009
Linolelaidic	C18:2n6t	35.010	35.014	34.999	35.008	0.006
Elaidic	C18:1n9t	35.081	35.014	35.100	35.065	0.037
Oleic	C18:1n9c	35.173	35.169	35.168	35.170	0.002
Stearic	C18:0	36.119	36.164	36.141	36.141	0.018
Cis-5,8,11,14,17-Eicosapentaenoic	C20:5n3	43.076	43.082	43.064	43.074	0.007
Arachidonic	C20:4n6	43.534	43.551	43.539	43.541	0.007
Cis-8,11,14-Eicosatrienoic	C20:3n6	43.919	43.926	43.916	43.920	0.004
Cis-11,14-Eicosadienoic	C20:2	44.820	44.834	44.802	44.819	0.013
Cis-11-Eicosenoic	C20:1	45.199	45.227	45.204	45.210	0.012
Arachidic	C20:0	46.034	46.037	46.019	46.030	0.008
Heneicosanoic	C21:0	50.495	50.503	50.493	50.497	0.004
Cis-4,7,10,13,16,19-Docosahexaenoic	C22:6n3	51.918	51.927	51.922	51.922	0.004
Cis-13,16-Docosadienoic	C22:2	53.670	53.678	53.665	53.671	0.005
Erucic	C22:1n9	54.716	54.725	54.710	54.717	0.006
Behenic	C22:0	58.696	58.723	58.705	58.708	0.011
Tricosanoic	C23:0	61.629	61.653	61.648	61.643	0.010
Nervonic	C24:1	61.931	61.859	61.888	61.893	0.030
Lignoceric	C24:0	62.524	62.539	62.526	62.530	0.007

b) Analysis of sample

Analysis of the fatty acid content of the horse chestnut residue showed 14 peaks (Figure 3.3). The identity of all 14 was determined by comparison of the retention times with those of the standards. Measurement of peak area allowed quantitative analysis.

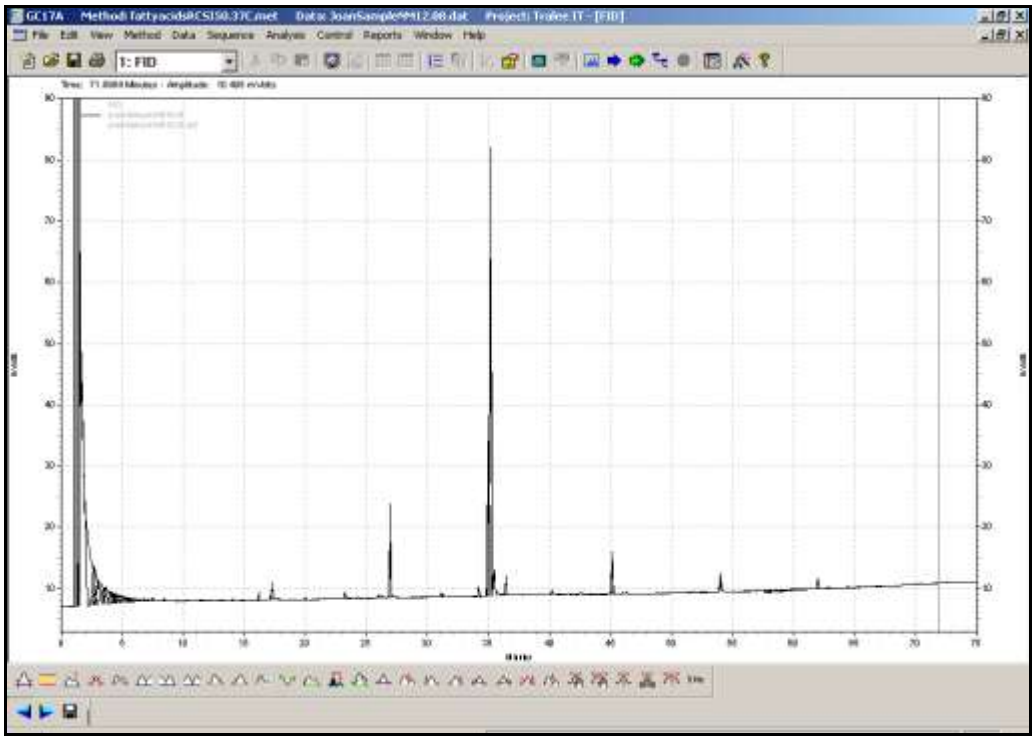


Figure 3.3: Gas chromatography of fatty acids from horse chestnut residue.
X-axis as Minutes, Y-axis as mVolts.

Investigation of fatty acid content led to identification of 12 fatty acids (Table 3.3). A significant fraction and the main component present in horse chestnut waste is oleic acid (74.7%). All other components present in the sample are: palmitic (5.78%), stearic (3.6%), cis-11,14- Eicosadienoic (3.2%), cis- 13,16 –Docosadienoic (2.17%), linolenic (1.04%). The total concentration of the remaining fatty acids is less than 1%.

Table 3.3: Fatty acids content of horse chestnut residue.

Name of identified acid methyl esters in sample	**Number of carbons in chain**	**Retention time**	**Area**	**Amount in sample (mg/ml)**
Myristic	C14:0	19.840	12713	0.042
Pentadecanoic	C15:0	22.987	26982	0.058
Palmitic	C16:0	26.778	848606	4.05
Pamitoleic	C16:1	25.798	23564	0.10
Heptadecanoic	C17:0	31.033	41743	0.29
Cis- 10- Heptadecenoic	C17:1	30.028	18207	0.073
Stearic	C18:0	36.199	385315	2.63
Oleic	C18:1	35.274	8654488	52.30
Linolenic	C18:2n6c	33.948	132683	0.73
unknown	unknown	35.401	686374	uknown
Arachidic	C20:0	45.995	36315	0.24
Cis-11,14- Eicosadienoic	C20:2	44.900	693095	2.24
Unknown	C22:0 ?	61.649	210088	unknown
Cis- 13,16 -Docosadienoic	C22:2	53.703	334069	1.52

3.2 Prebiotic potential of horse chestnut waste

3.2.1 Determination of prebiotic potential of horse chestnut waste

Growth of the *Lactobacillus* and *Bifidibacterium* strains in minimal medium containing 1% (w/v) of FOS or 1% (w/v) of horse chestnut extract (HCE) is presented.

3.2.1.1 Determination of the prebiotic potential of HCE for *Lactobacillus delbrueckii subsp. lactis*

All figures show the changes in optical density and pH profile during 8 hours of incubation in minimal medium containing 1% of FOS or 1% horse chestnut extract (HCE) prepared by stirring at 20°C for 1hour.

As indicated by the blue line (Figure 3.4) cultivation of *Lactobacillus delbrueckii subsp. lactis* in minimal medium (MM) showed very low levels of growth. The addition of horse chestnut extract (HCE) greatly improved the growth of bacteria (yellow line). This suggests that this strain was able to ferment HCE. Fermentation of HCE leads to production of lactic acid which corresponds to the decreasing pH level as shown in Figure 3.5. Cultivation of this probiotic strain in minimal medium in the presence of 1% FOS produced a slightly higher OD at 6 and 8 hours (Figure 3.4). There was a corresponding decrease in pH levels (Figure 3.5). However there is no significant difference between growth of *L.lactis* in medium with 1% FOS or HCE ($p>0.05$).

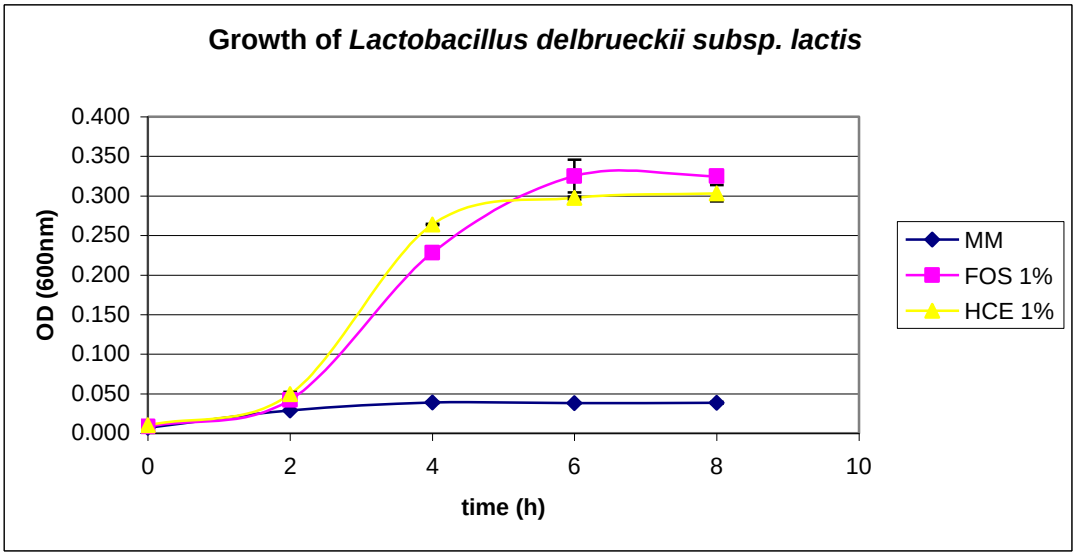


Figure 3.4: Growth curve of *Lactobacillus delbrueckii subsp. lactis* in unsupplemented minimal medium (MM) and minimal medium supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

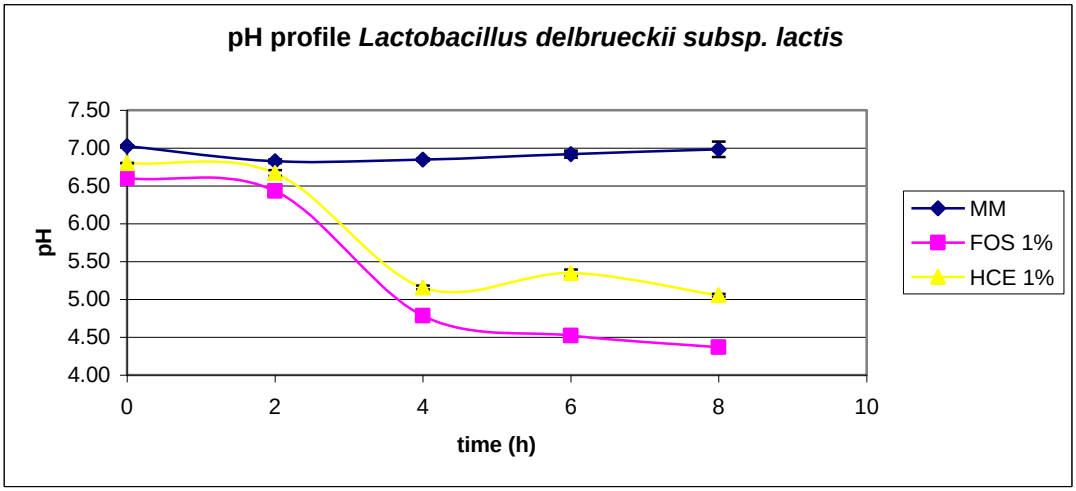


Figure 3.5: The pH profile of *Lactobacillus delbrueckii subsp. lactis* when grown in unsupplemented minimal medium (MM) and minimal medium supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

The effect of extraction of horse chestnut residue under different condition was investigated. The horse chestnut waste extract was prepared by:

a) stirring at 20ºC for 1 hour
b) stirring at 90ºC for 1 hour
c) autoclaving at 121ºC for 15 minutes (as described in section 2.2.2.).

Growth curves obtained for all three extracts showed no significant difference in growth rates (Figure 3.6). Similarly, the pH profile for all 3 extracts showed no significant difference (Figure 3.7).

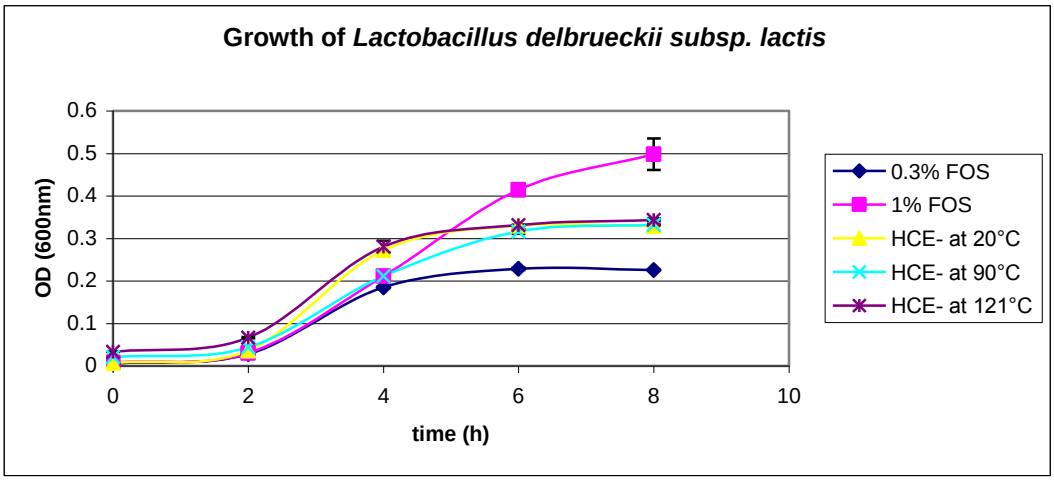


Figure 3.6: Influence of different horse chestnut aqueous extracts (each 1% (w/v)) on growth of *Lactobacillus delbrueckii subsp. lactis.*
Each value shown mean value with the upper and lower values.

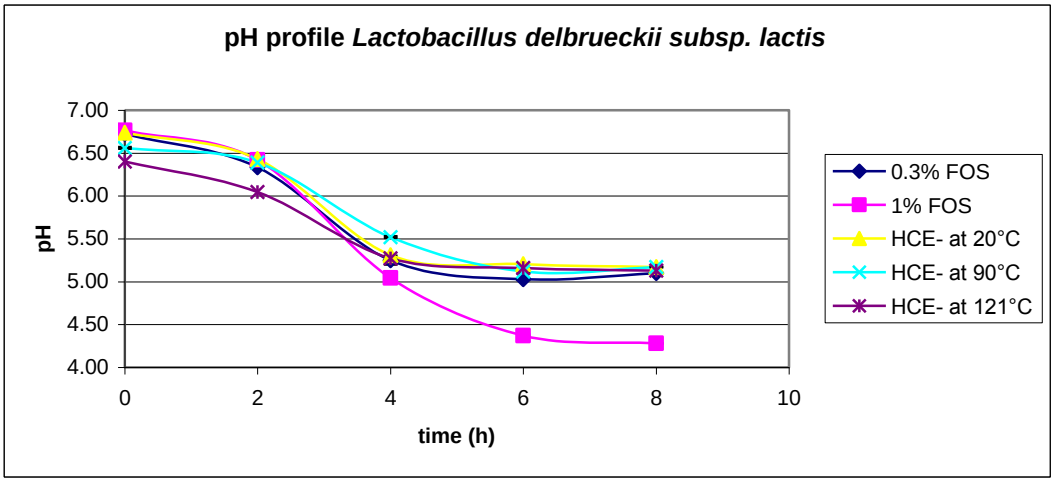


Figure 3.7: Acidity of minimal medium during incubation of *Lactobacillus delbrueckii subsp. lactis* in the presence of different horse chestnut aqueous extract preparations (each 1% (w/v)).
Each value shown mean value with the upper and lower values.

In order to provide a more direct comparison between FOS and HCE, 0.3% (w/v) FOS in MM was also used as a substrate. A concentation of FOS of 0.3% (w/v) was used to compare to the maximal concentration of HCE (0.3% (w/v) achieved, when the extract was prepared by using conditions of high temperature (121ºC) and pressure (1atm) as described in section 3.2.2. Figure 3.6 shows that the growth of *Lactobacillus delbrueckii*

subsp. lactis obtained using 0.3% FOS was significantly less than that of any of the horse chestnut residue extracts. The pH profile (see Figure 3.7) is similar for all three HCE at 4, 6, and 8 hours. The 0.3% FOS as substrate shows the same level of acidity as 1% HCE while 1% FOS has significantly lower pH profile at 6 and 8 hours which would correspond to higher yields of lactic acid. Following the previous experiment, which showed no significant difference in growth of *Lactobacillus delbrueckii subsp. lactis* and pH profile obtained for all of the three horse chestnut extracts used, it was decided to carry out further extractions by stirring at 20°C as this was the most economical method.

Figure 3.8 presents a growth curve of *Lactobacillus delbrueckii subsp. lactis* in minimal medium with 1% (w/v) of FOS compared to 1% (w/v) horse chestnut extract (HCE). Two extractions were prepared by stirring at 20°C for 0.5 hour and 1 hour. As indicated by the yellow line horse chestnut extract has very similar effect on the growth of *Lactobacillus delbrueckii subsp. lactis* even if was made in half the time. This suggests that the components of the water soluble fraction of horse chestnut residue were highly soluble. Figure 3.9 shows pH profile for *L.lactis* in medium with FOS and HCE (both 1% w/v).

This experiment clearly showed that all active compounds which stimulate the growth of *L. delbrueckii subsp. lactis* were highly soluble in water based medium and that all components which have prebiotic potential are rapidly solubilised.

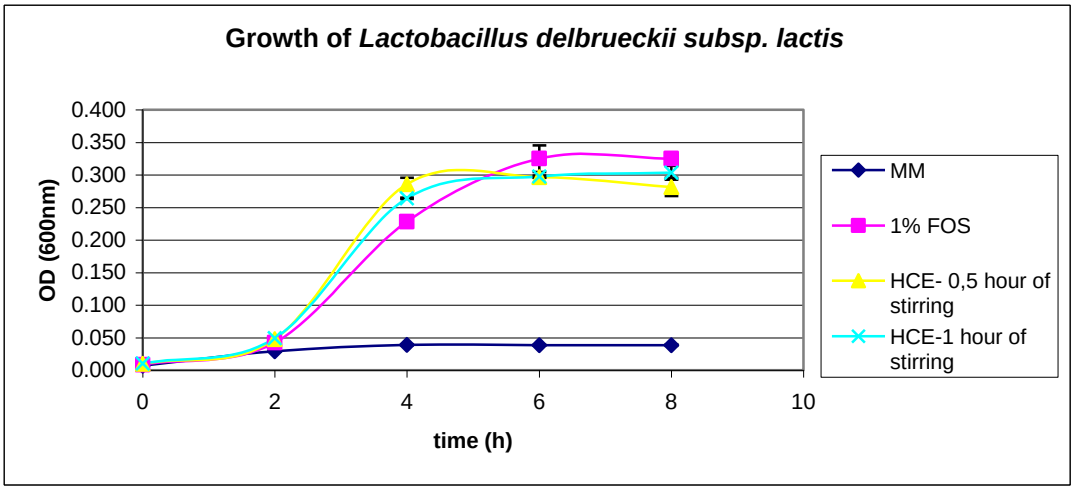


Figure 3.8: Growth curve of *Lactobacillus delbrueckii subsp. lactis* on minimal medium (MM) with different time preparation of HCE (both 1% (w/v)).
Each value shown mean ± standard deviation for n=3.

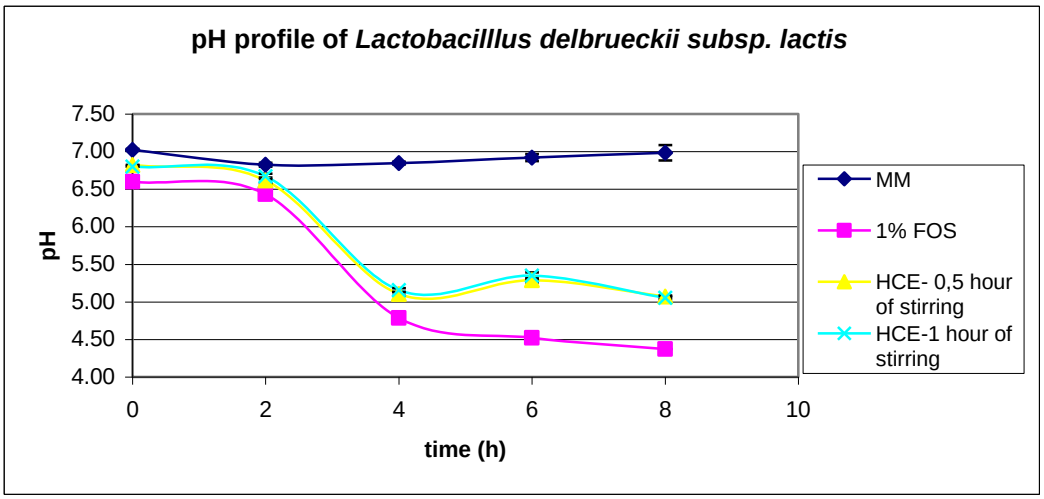


Figure 3.9: The pH profile of *Lactobacillus delbrueckii subsp. lactis* on minimal medium (MM) with different time preparation of HCE (both 1% (w/v)).
Each value shown mean ± standard deviation for n=3.

Following an initial extraction of horse chestnut residue (1% (w/v) HCE) by stirring at 20°C for 30 minutes, the soluble fraction was removed by centrifugation at 11,000rpm for 20 minutes. Supernatant (soluble fraction from first stirring) was then separated from the pellet and minimal medium was added to the pellet in the same volume as removed supernatant. A second extraction (30 minutes at 20°C) then took place. Figure 3.10 shows that the growth of *L. delbrueckii subsp. lactis* was negligible on this second extract. It can also be seen from the pH profile (Figure 3.11) that there is no significant difference between the acid production by this bacterial strain grown on unsupplemented minimal media and when supplemented with the second extract. The growth and pH profile obtained from first extract was the same as that found in previous experiments.

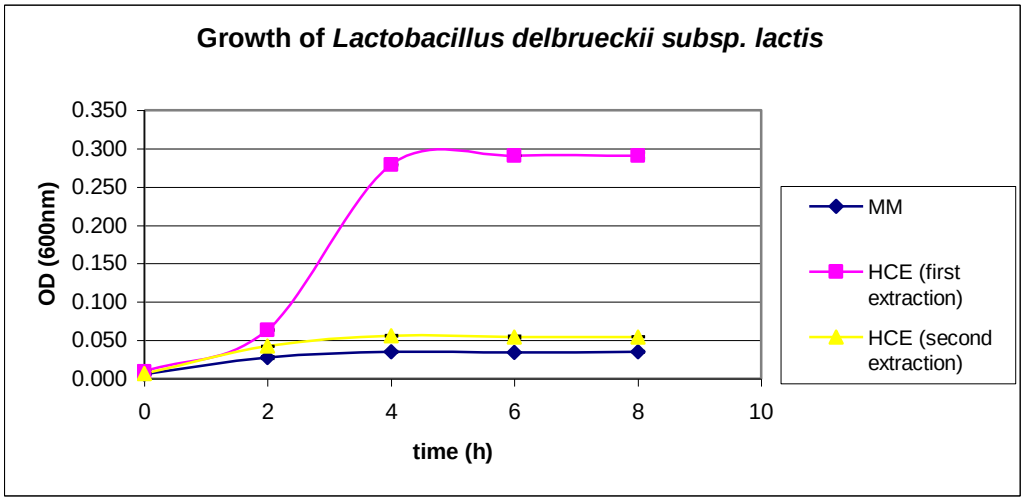


Figure 3.10: Growth of *Lactobacillus delbruecki subsp. lactis* in unsupplemented minimal medium (MM) and minimal medium supplemented with 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

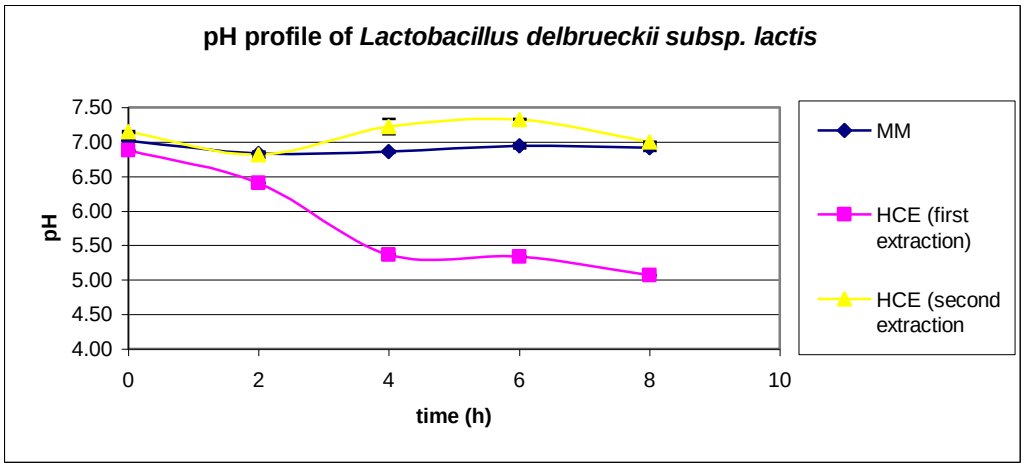


Figure 3.11: The pH profile of *Lactobacillus delbruecki subsp. lactis* in unsupplemented minimal medium (MM) and minimal medium supplemented with 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

In all further experiments the HCE was prepared by a single extraction of the horse chestnut waste stirring for 30 minutes at 20°C.

3.2.1.2 Determination of the prebiotic potential of HCE for *Lactobacillus plantarum* ATCC 8014

Growth of *Lactobacillus plantarum* ATCC 8014 strain stimulated by 1% (w/v) FOS was weak (pink line Figure 3.12). Minimal medium which contained 1% (w/v) HCE improved the growth significantly (p=0.004) by comparison with 1% FOS. *Lactobacillus plantarum* seems to ferment HCE better than the commercial prebiotic

FOS. The influence of HCE on the acidifying activity was also noticed (Figure 3.13). The pH in culture media declined at a lower rate after 12 hours of incubation, moreover it seems to remain stable up to 24 hours.

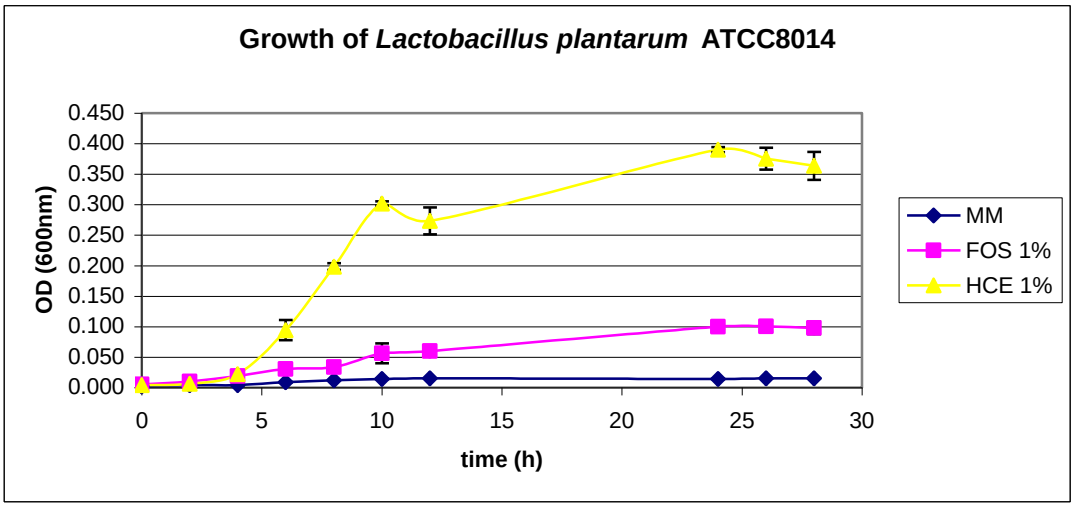


Figure 3.12: Growth curve of *Lactobacillus plantarum* ATCC 8014 in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

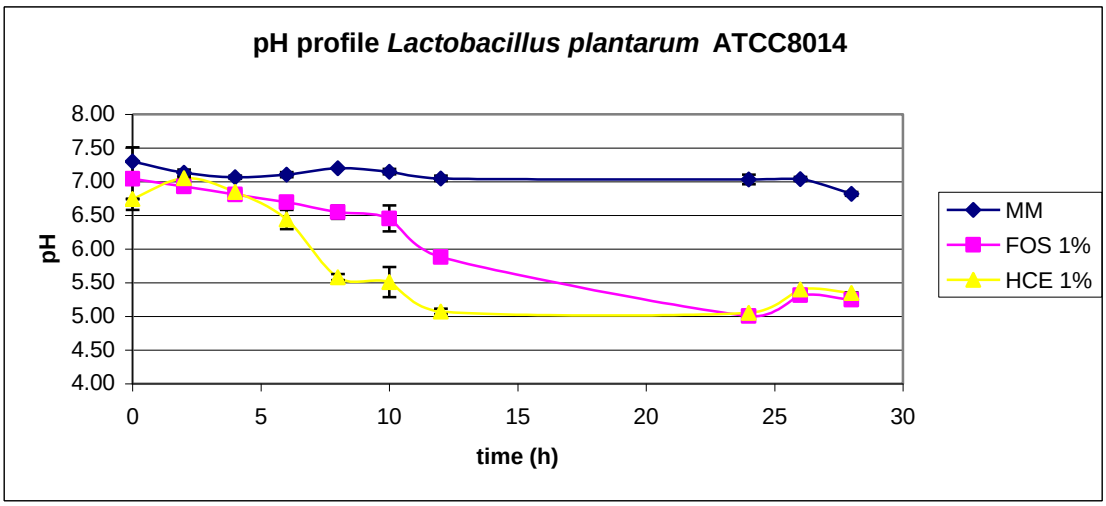


Figure 3.13: Acidification of culture medium *Lactobacillus plantarum* ATCC 8014 during 24 hours incubation in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

3.2.1.3 Determination of the prebiotic potential of HCE for *Lactobacillus rhamnosus* ATCC 7469

Incubation of *Lactobacillus rhamnosus* ATCC 7469 in minimal medium with 1% (w/v) HCE gave better stimulation effect than 1% (w/v) FOS after 6 hours (Figure 3.14). The pH profile shows that *Lactobacillus rhamnosus* produced lactic acid at the same level when grown on either on FOS or HCE medium (Figure 3.15).

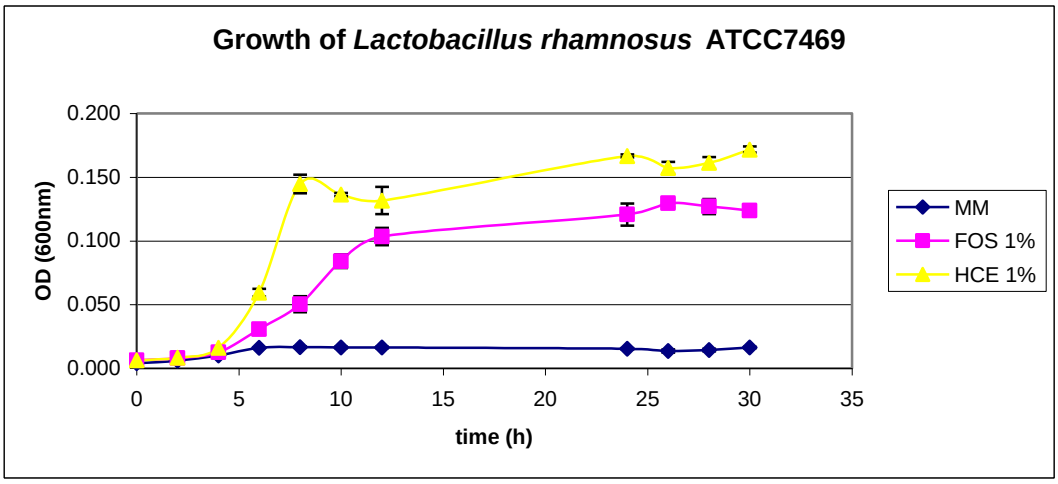


Figure 3.14: Growth curve of *Lactobacillus rhamnosus* ATCC 7469 in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

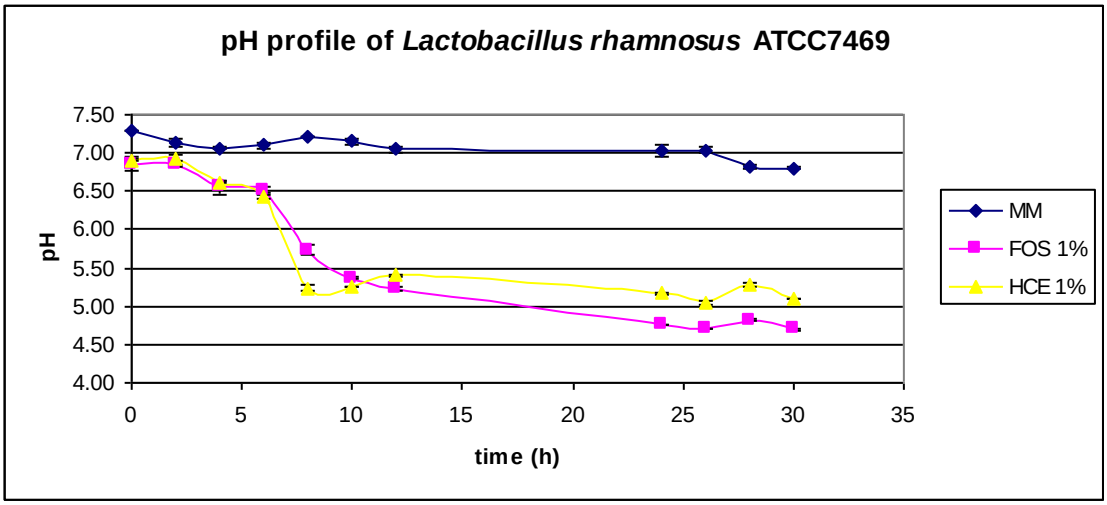


Figure 3.15: The pH profile of *Lactobacillus rhamnosus* ATCC 7469 grown in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

3.2.1.4 Determination of the prebiotic potential of HCE for *Lactobacillus acidophilus* ATCC 4356

The growth stimulating effect of HCE was observed also in *Lactobacillus acidophilus* ATCC 4356 strain. The number of bacteria was significantly higher (p=0.007) in medium containing 1% (w/v) HCE than in medium containing 1% (w/v) FOS (Figure 3.16). The degree of acidification was the same for both substrates (1% FOS or HCE). Figure 3.19 shows the pH profiles.

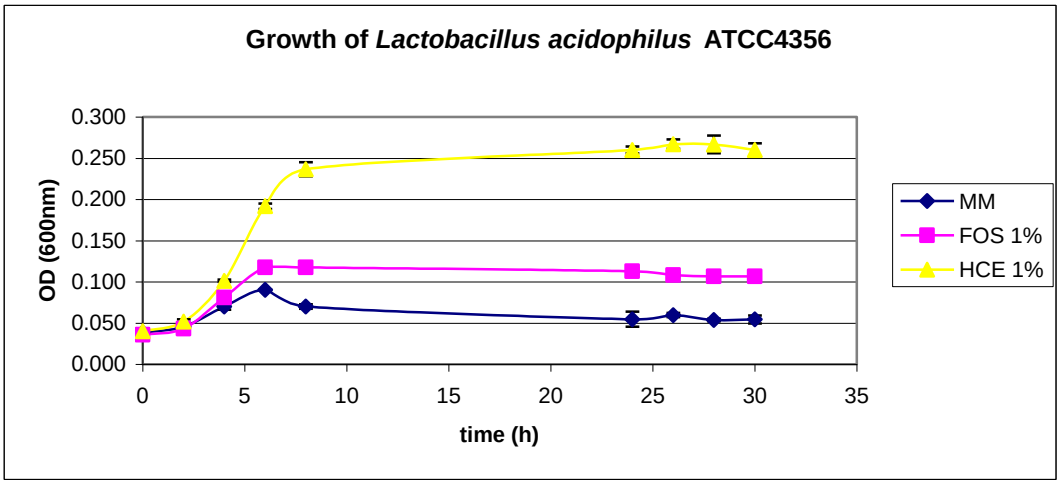


Figure 3.16: Growth curve of *Lactobacillus acidophilus* ATCC 4356 in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

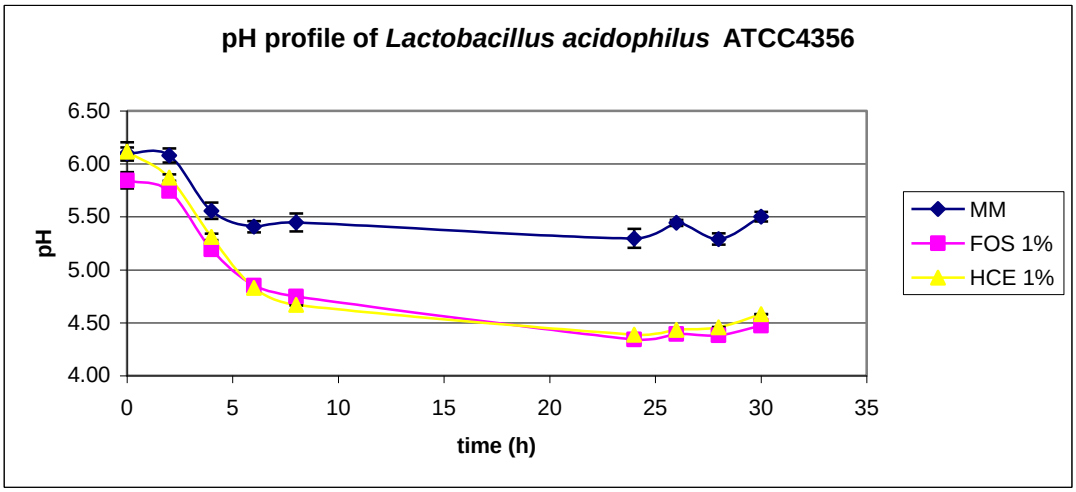


Figure 3.17: The pH profile of *Lactobacillus acidophilus* ATCC 4356 grown in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

3.2.1.5 Determination of the prebiotic potential of HCE for *Bifidobacterium infantis* ATCC 15697

The growth rates of *Bifidobacterium infantis* ATCC 15697 in minimal medium with 1% (w/v) FOS and 1% (w/v) HCE were similar with rapid growth from 2-8 hours. By 8 hours on HCE the culture appeared to have reached stationary phase and had a greater number of cells than the culture grown on FOS. However the OD of the culture growing on FOS continued to increase (Figure 3.18) so that ODs between 24 and 30 hours were similar. The pH profiles of the culture on both media showed no significant statistical difference (Figure 3.19).

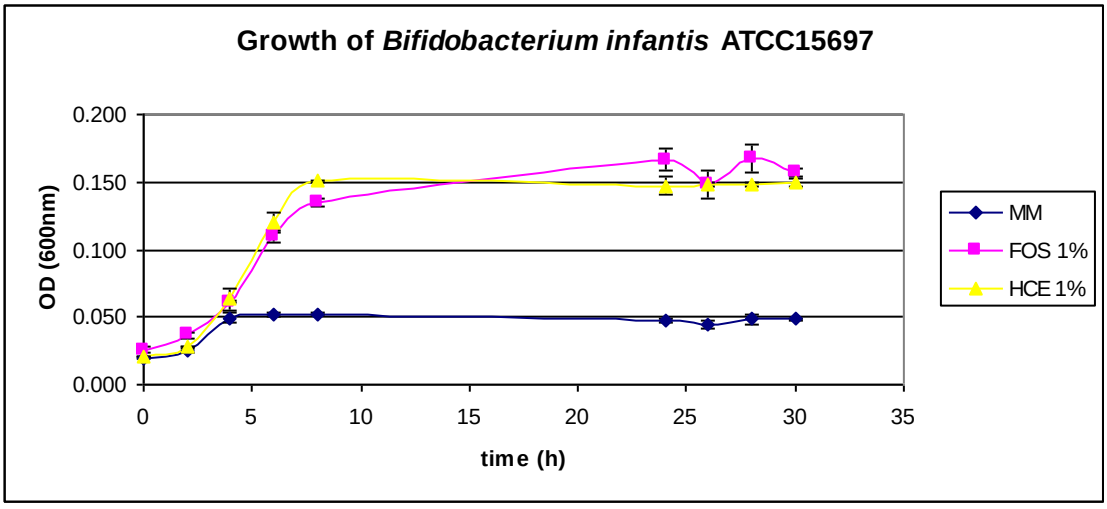


Figure 3.18: Growth curve of *Bifidobacterium infantis* ATCC 15697 in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

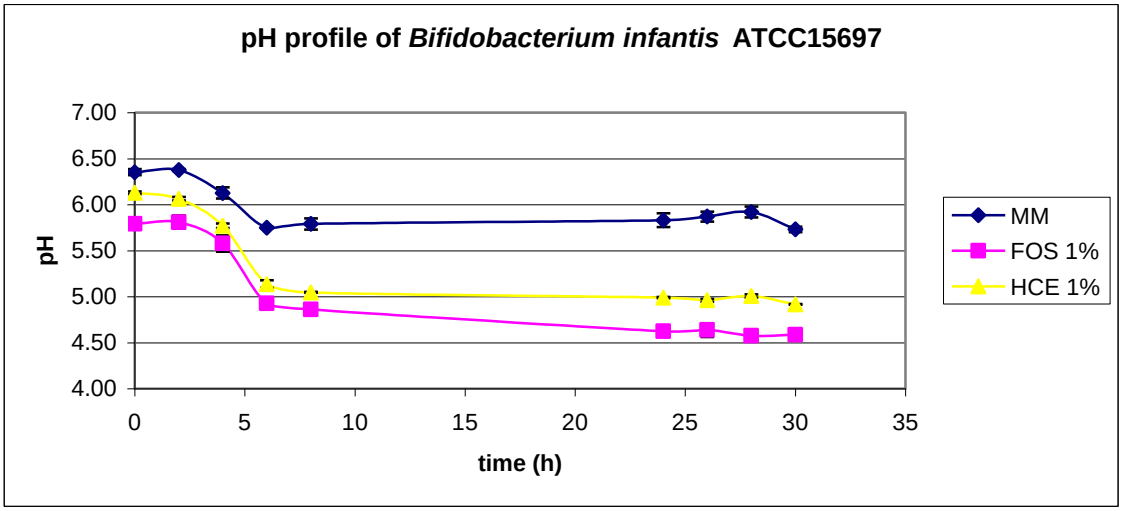


Figure 3.19: The pH profile of *Bifidobacterium infantis* ATCC 15697 grown in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

3.2.1.6 Determination of the prebiotic potential of HCE for *Bifidobacterium angulatum* ATCC 27535

Stimulating effect of 1% (w/v) HCE on the growth *Bifidobacterium angulatum* ATCC 27535 was also observed (Figure 3.20) although in this case it was higher than 1% (w/v) FOS. After 8 hours of incubation with HCE, bacterial cells continued to proliferate in comparison to the use of FOS. There was slightly higher acidification for growth on the medium containing HCE in this case (Figure 3.21).

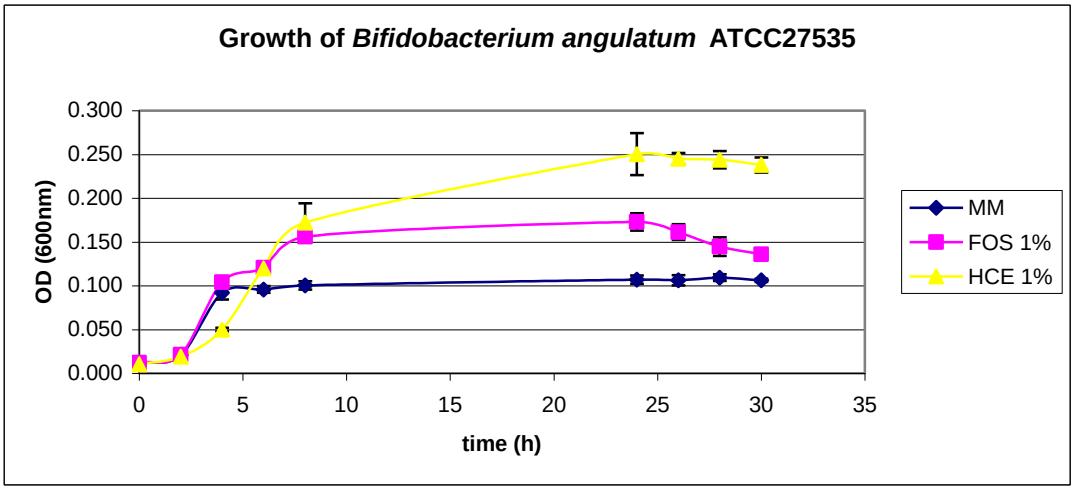


Figure 3.20: Growth curve of *Bifidobacterium angulatum* ATCC 27535 in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3

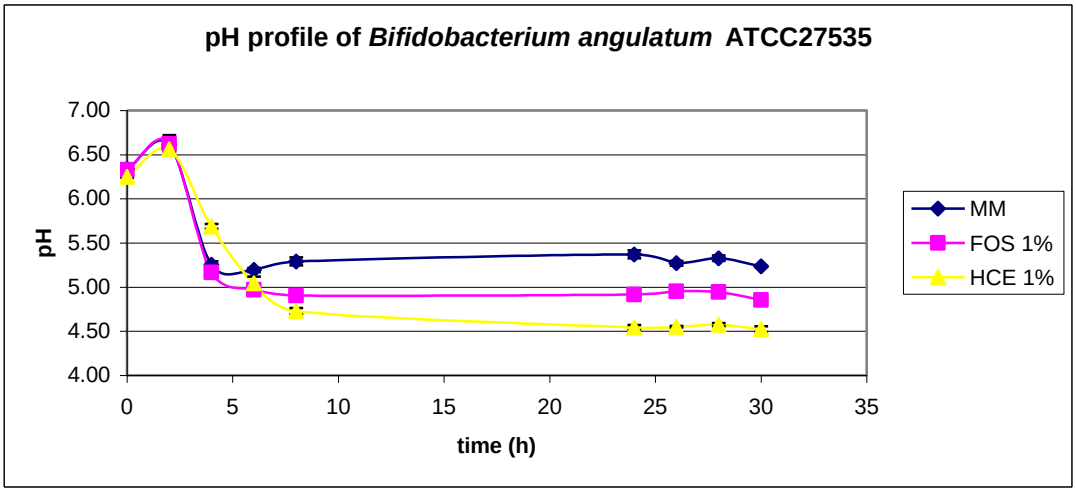


Figure 3.21: The pH profile of *Bifidobacterium angulatum* ATCC 27535 grown in unsupplemented minimal medium (MM) and supplemented with 1% (w/v) FOS or 1% (w/v) HCE.
Each value shown mean ± standard deviation for n=3.

3.2.2 Estimation of the quantity of the water soluble fraction obtained from horse chestnut waste

The water soluble fraction of powdered horse chestnut residue was determined gravimetrically. Two gram samples of horse chestnut residue were used for all experiments. The following results were obtained:

a) Sample stirred at 20ºC - 0.16% (w/v) of water soluble fraction was obtained

b) Sample stirred at 90ºC - 0.17% (w/v) water soluble fraction

c) Sample autoclaved at 121°C yielded 0.30% (w/v) water soluble fraction

As shown above, only a small proportion of horse chestnut residue was soluble in water and the degree of solubility depended on the temperature of stirring mixture. Of course raw material (pieces of crushed seeds) contained mostly fragments of fruity skin and nut. Prior determination of chemical analysis of crude waste showed that main component of crude material was carbohydrates. The water soluble fraction should not, in theory, contain lipids, therefore carbohydrates could be a significant amount of this fraction.

3.3 Analysis of lyophilised horse chestnut aqueous extract

3.3.1 Moisture content

The moisture content was established using the method described in part 2.1. The experiment showed that the moisture content of lyophilised horse chestnut aqueous extract (lHCE) is 8% (w/w, SD=0.165, n=3) of fresh sample.

3.3.2 Ash content

The ash content was established as 13.72% (w/w, SD=0.069, n=3) of dried sample.

3.3.3 Metal elements

The experiment was carried out as described in part 2.1.3. The amount of ash which was used for preparation of ash solution was 17.2mg. The content of each mineral was calculated separately and is presented in Table 3.4.

Table 3.4: Metal concentration in ash from lHCE

Mineral	**Horse chestnut aqueous extract (lyophilised form)**
Potassium	41.13
Phosphorus	5.15
Magnesium	2.76
Sodium	2.03
Calcium	1.99
Iron	0.26
Nickel	0.12
Copper	0.06

Results expressed as mg/g wet weight of horse chestnut aqueous extract.

A significant mineral element present in ash from lHCE is potassium (41.13mg/1g wet sample). Phosphorus, magnesium, sodium and calcium are also considered to be major elements.

3.3.4 Protein content

Using the formula (y = 0.5791x + 0.0195), which was obtained from standard curve of BSA (Appendix E) the protein content was calculated for 5mg of lHCE and result showed 0.141 mg/ml (w/w, SD=0.014, n=3) of protein content in fresh sample.
A 5mg sample of lHCE was used what gave 2.82% of protein content in fresh sample (lyophilised horse chestnut aqueous extract).

3.3.5 Reducing sugars content

The glucose standard curve was used to calculate the concentration of reducing sugars in the fresh samples (lHCE and FOS). The absorbance of the samples fell within the standard curve range, therefore, using formula y = 0.1048x + 0.0402 (Appendix F), the reducing sugars content was established as indicated in Table 3.5.

Table 3.5: Sugar content in FOS and lHCE.

Number of samples n=3	**Sugar content (µmol/10ml)**	
	MEAN	**SD**
lHCE	11.00	0.15
FOS	7.42	0.02

Results given as percentage (w/w)
fresh sample ± standard deviation (n=3).

The value for lHCE (11µmol/10ml) was calculated as 2mg of reducing sugars in 10mg of the fresh sample which is equivalent to 20% reducing sugars. The value (7.42 µmol/10ml) obtained for FOS, gave 1.33mg of reducing sugar in 15mg of the fresh FOS sample, corresponding to 8.86% of reducing sugar.

3.3.6 Phenolic content

Absorbance for concentration of lHCE 50 and 25 mg/ml (rows F1,F2 and G1,G2) was too high therefore determination of phenolic content was calculated using formula obtained from standard curve of phloroglucinol (y = 6.2785x + 0.0396; Appendix G) only for these lHCE solution that fell within the range of the phenolic standard curve.

Figure 3.22 shows the concentration of phenolic content in relation to lHCE concentration. The total phenolic content in each sample was plotted against the lHCE content resulting in linear graph (y=0.0705+0.0721) and a regression value R^2=0.9909. The phenolic content of the lHCE is comprises 2.06% or 20.56 mg/g dry matter.

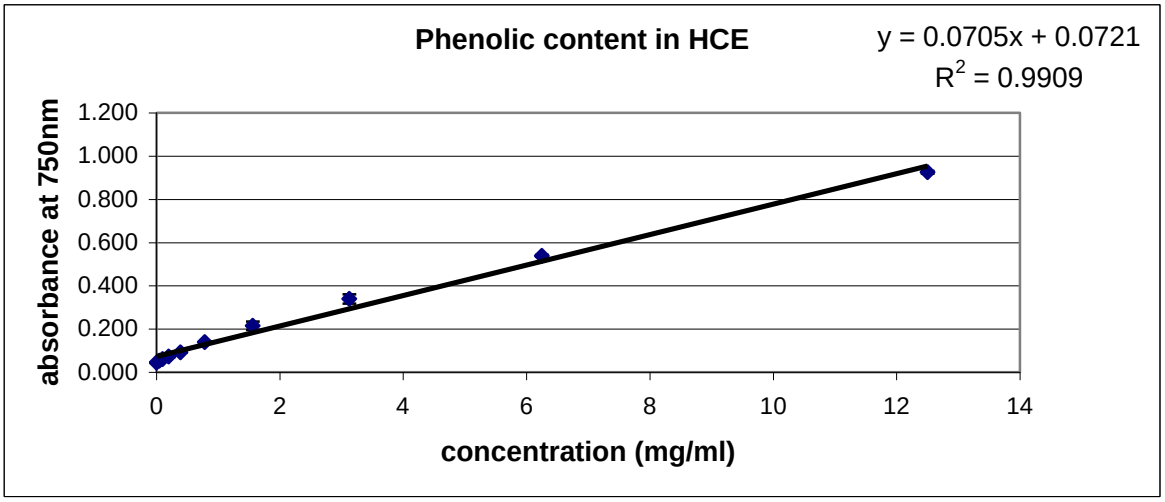


Figure 3.22: Linear correlation between phenolic content and absorbance at 750nm in different concentrations of lHCE (mg/ml).
Values for each point of measure reported as mean value with the upper and lower values.

3.3.7 Analysis of lyophilised horse chestnut aqueous extract by Anion/Cation Exchange Chromatography

Anion Exchange Chromatography

Monosaccharides- glucose (t_R= 6.3min.), fructose (t_R=6.0min.) and galactose (t_R=6.5min.) as well as disaccharide- maltose (t_R=8.4min.), presented in Appendix N, were readily detected on IR detector. Each of these sugars gave a single peak. The oligosaccharides FOS and GOS (Appendix O) gave rise to several peaks for each sample when subjected to anion exchange chromatography. GOS (Vivinal GOS DOMO®) according to the specification sheet (Appendix C) contains glucose (22%) galactose (0.8%), lactose (23%) and galactooligosaccharides (57%) in dry mass.
Appendix O shows separation by anion exchange chromatography of the components of GOS. A single peak at t_R=6.6min. may be the combination of both glucose and

galactose peaks (t_R=6.34 and 6.58min. respectively). Lactose is represented by a peak at t_R=9.86min, while there are 2 peaks representing different galactooligosaccharides, t_R=14.49min and 20.41min.

The separation achieved with FOS (Appendix O) suggests incomplete resolution but did show a peak at t_R=6.0 min. representing fructose.

Anion exchange chromatography of lyophilised horse chestnut aqueous extract (lHCE) showed one major peak and several poorly defined peaks (Appendix M). The retention times of these peaks were compared to sugar standards. The chromatogram for lHCE appears to consist of peaks corresponding to monosaccharides (4.7-7.1 minutes) and oligosaccharides (7.1-10 minutes), although it was not possible to establish the precise identity of the components from the chromatograms obtained. In this experiment only qualitative determination of carbohydrate content in the sample was concluded.

Cation Exchange Chromatography

Cation Exchange Chromatography of various carbohydrates (mono,- di,- and oligosaccharides), prebiotics and lyophilised horse chestnut aqueous extract (lHCE) was performed on SUPELCOSIL C160-H. The chromatographs of separated monosaccharides and disaccharides are presented in Appendix P and Appendix Q, respectively. In contrast to anion exchange, monosaccharides were retained by the column and were eluted last whereas oligosaccharides were eluted from the column in much shorter time. The monosaccharides glucose (t_R=15.965), galactose (t_R=17.172) and fructose (t_R=17.362) were poorly resolved and tended to elute as a single peak as did the disaccharides sucrose (t_R=13.867) and lactose (t_R=14.229). Maltose (t_R=13.773) gave rise to a major peak and some unidentified peaks probably related to the solvents used. The standard oligosaccharide dextrin 15 gave major peak at t_R=11.298 and two minor peaks (Appendix R). One of them corresponded to sucrose (t_R=13.853) and the other (t_R=12.707) is probably due to some degradation of dextrin 15 which, with this retention time, seemed to correspond to an oligosaccharide component. The oligosaccharide raffinose was eluted from the column at t_R=12.627 (Appendix R).

The separation of the components of GOS, FOS and inulin is presented in Appendix S.

In the specification of GOS product reported by DOMO® (see Appendix C), Vivinal GOS contains galactooligosaccharides, lactose, glucose and galactose (57%, 23%, 22%

and 0.8% respectively). The cation exchange chromatography of GOS (Appendix S) clearly confirmed the presence of all those components and their qualitative analysis was performed by comparison of the retention times for standard mono- and di-saccharides. The following peaks corresponded to oligosaccharides (t_R=12.257 and t_R=12.937), t_R=14.117 for lactose, t_R=15.927 for glucose and t_R=17.135 for galactose.

Qualitative analysis of separated FOS components (Appendix S) showed one major peak (t_R=11.038) for oligosaccharides, and two peaks with t_R=17.320 corresponding to fructose and t_R=13.755 for an unidentified disaccharide (possible maltose).

Inulin exhibited one single peak at t_R=11.063 (Appendix S).

Cation exchange chromatography of lHCE exhibited several peaks (Appendix T). These peaks were compared to retention times of mono,- di,- and oligosaccharides. Peaks with t_R=11.162, t_R=11.922 and t_R=12.498 appeared to correspond to oligosaccharides because their retention times are very close to those obtained for dextrin 15, GOS and FOS. The peak with t_R=15.512 and with t_R=16.01 seemed to be associated with glucose. Fructose was also found as a minor component of lHCE with t_R=17.482. Finally, the peak with t_R=13.908 obtained in cation exchange chromatography, with sharp symmetry, was not identify conclusively, but seemed to correspond to a disaccharide (possible sucrose).

Table 3.6 presents retention times of all separated standard carbohydrates, commercial prebiotics and lHCE by anion/cation exchange chromatography.

Table 3.6: Carbohydrate analysis of mono,- di,- oligosaccharides, commercial prebiotics and lHCE by anion and cation exchange chromatography.

Analysed compounds	**Number of peaks**	**Retention time (t_R) in minutes**	**Indentified carbohydrate**
Anion exchange chromatography			
glucose	1	6.3	.
fructose	1	6.0	.
galactose	1	6.5	.
maltose	1	8.4	.
GOS	4	6.6	glucose+galactose
		9.8	lactose
		14.4	galactooligosaccharides
		20.4	galactooligosaccharides
FOS	incomplete resolution	6.0	fructose
lHCE	incomplete resolution	4.7-7.1	monosaccharides
		7.1-10	oligosaccharides
Cation exchange chromatography			
glucose	1	15.965	.
galactose	1	17.172	.
fructose	1	17.362	.
sucrose	1	13.867	.
lactose	1	14.229	.
maltose	1	13.773	.
dextrin 15	3	13.853	sucrose
		12.707	oligosaccharides
		11.298	oligosaccharides
raffinose	1	12.627	.
GOS	5	12.257-12.937	oligosaccharides
		14.117	lactose
		15.927	glucose
		17.135	galactose
FOS	3	11.038	oligosaccharides
		17.32	fructose
		13.755	maltose
inulin	1	11.063	.
lHCE	6	11.162-12.498	oligosaccharides
		13.908	sucrose
		15.512-16.01	glucose
		17.482	fructose

3.3.8 Analysis of lyophilised horse chestnut aqueous extract by Size Exclusion Chromatography using FPLC system

3.3.8.1 Fractionation of lyophilised horse chestnut aqueous extract

Figure 3.23 shows the size exclusion chromatograph of lyophilised horse chestnut aqueous extract (lHCE). Fractions (91 fractions 0.5ml volume) were collected on a fractionator containing Eppendorf tubes.

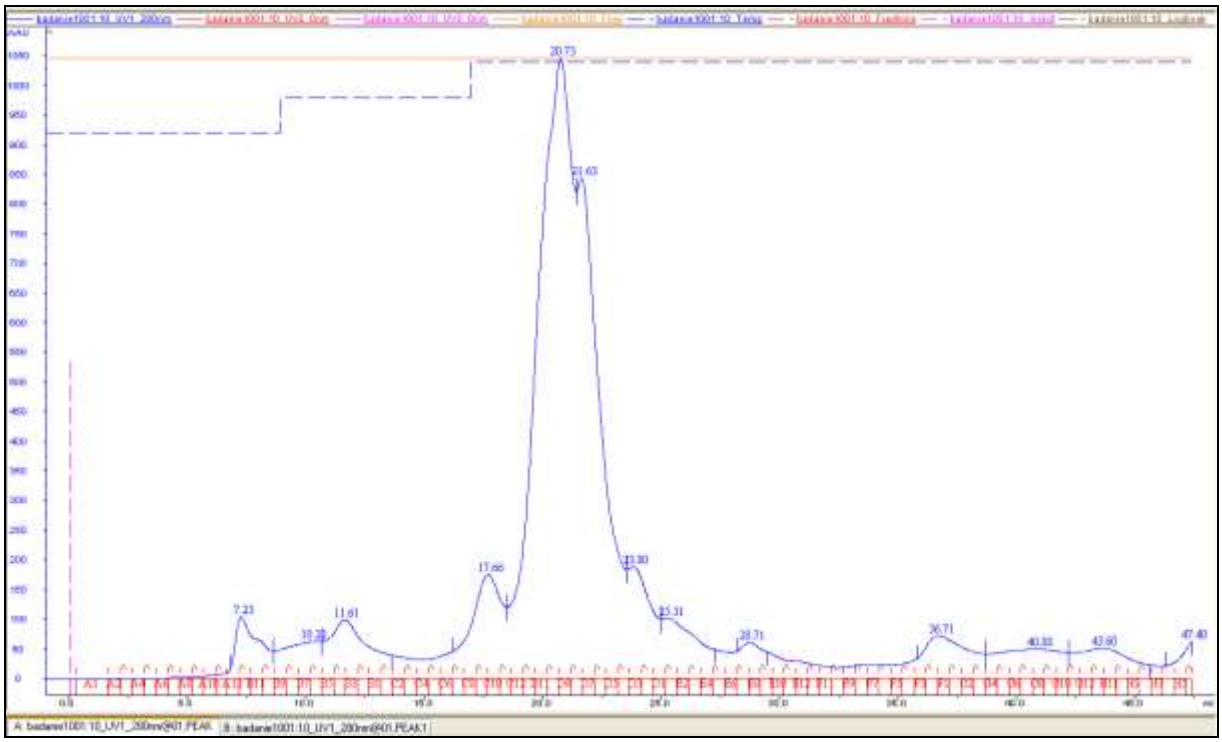


Figure 3.23: Size exclusion chromatography of lyophilised horse chestnut aqueous extract (lHCE).

The chromatograph shows the absorbance of the fractions at 280nm. The number value on each peak refers to the volume of eluent from the column up to that point. Two significant peaks emerge. The highest peak was obtained after 20.73ml of eluent was collected with a second incompletely resolved peak at 21.63ml. The chromatograph presents only the response signal (absorbance at 280nm) and the molecular sizes were not established. The fractions were retained for analysis of protein and sugar content.

3.3.8.2 Determination of sugar content in fractions of lyophilised horse chestnut aqueous extract by Dubois assay

The formula of the line, y=27.614+0.0463 (Appendix H)) was used in the determination of total sugar content in the lHCE fractions In fractions 13,15,17,29,31,33,35,37,39,41 dilutions were carried out due to high absorbance in the original fractions. Absorbances of the diluted fractions were recorded, then the sugar content was calculated taking into account the appropriate dilution factor.

Figure 3.24 shows that highest concentration of total sugar was detected in fractions 37 and 39.

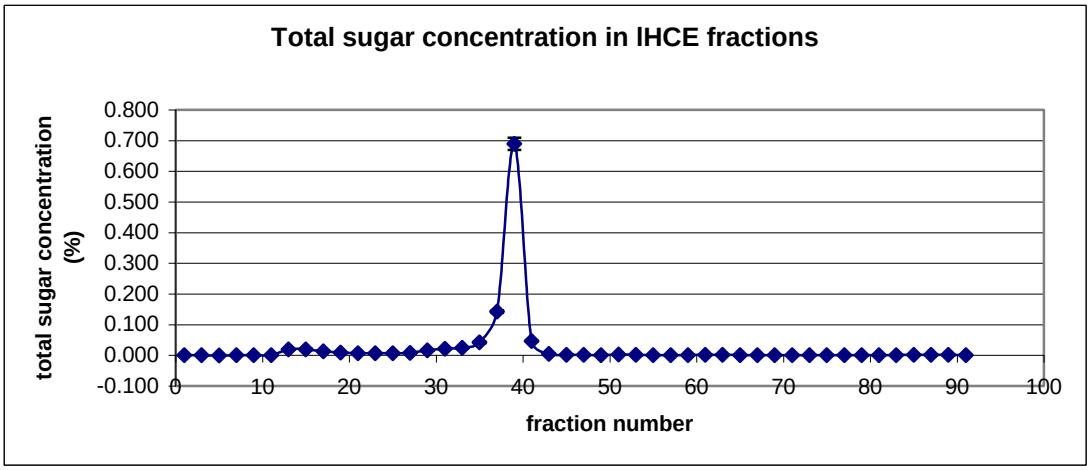


Figure 3.24: Concentration of total sugar in lHCE fractions.
Values for each point of measurement reported as mean ± standard deviation (n=3)

3.3.8.3 Determination of protein content in fractions of lyophilised horse chestnut aqueous extract by Bradford assay

The results show that all fractions have low protein content. Significantly fractions 37 and 39 show only a trace of protein. Figure 3.25 shows both protein and sugar content of the fractions.

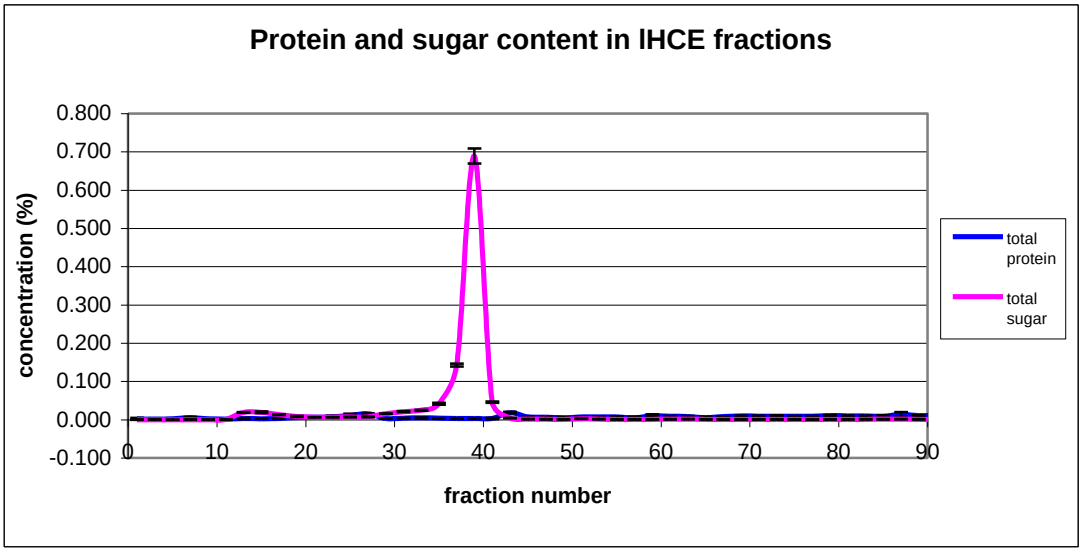


Figure 3.25: Concentration of total protein and total sugar in lHCE fractions.
Values for each point of measurement reported as mean ± standard deviation (n=3).

3.3.8.4 Estimation of molecular mass of sugar fraction of lHCE by size exclusion chromatography

To estimate the molecular mass of a sample of interest (lHCE):

1. K_{av} values for all dextran standards (MW 5000, 12000 and 80000) were calculated using formula below.

$$K_{av} = \frac{V_R - V_O}{V_C - V_O}$$

V_O-void volume
V_C-bed volume
V_R-retention (elution) volume of separated sample

Void volume (V_O) of the column was determined with dextran blue. Dextran blue has a molecular weight of 2,000,000 which is above the fractionation range of the Superdex 200 10/300GL (fractionation range of separated dextrin is $1x10^3$-$1x10^5$) therefore dextran blue is not retained by the column and is eluted in the void volume (V_O).

Values for V_R for each dextran standards were derived from the graphs (Figure 3.26, 3.27 and 3.28). The highest peak obtained from Dubois assay and expressed as fraction

number represents in practice the volume of eluted dextran. Because 0.5ml was collected per fraction, peak value was divided by two for determination of retention (elution) volume (V_R). The bed volume (V_C) of the column is 24ml (according to the specification GE Healthcare).

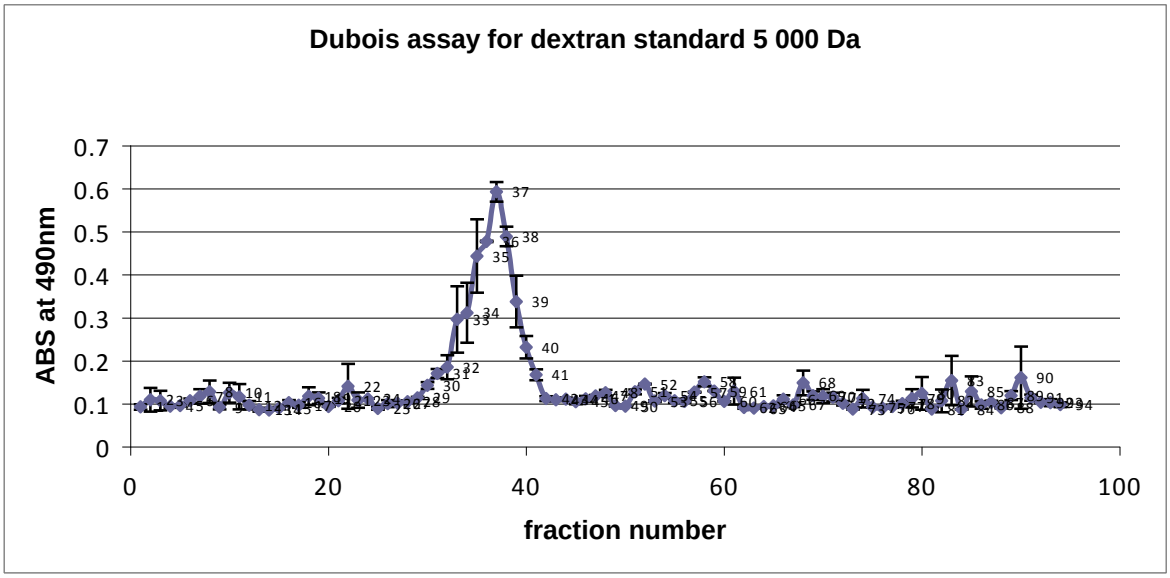


Figure 3.26: Dubois assay for dextran standard MW=5,000 Da (10mg/ml) fractionated on Superdex 200 10/300GL.
Parameters: flow 0.4ml/min., 0.5ml per fraction.
Values for each point of measurement reported as mean value with the upper and lower values.

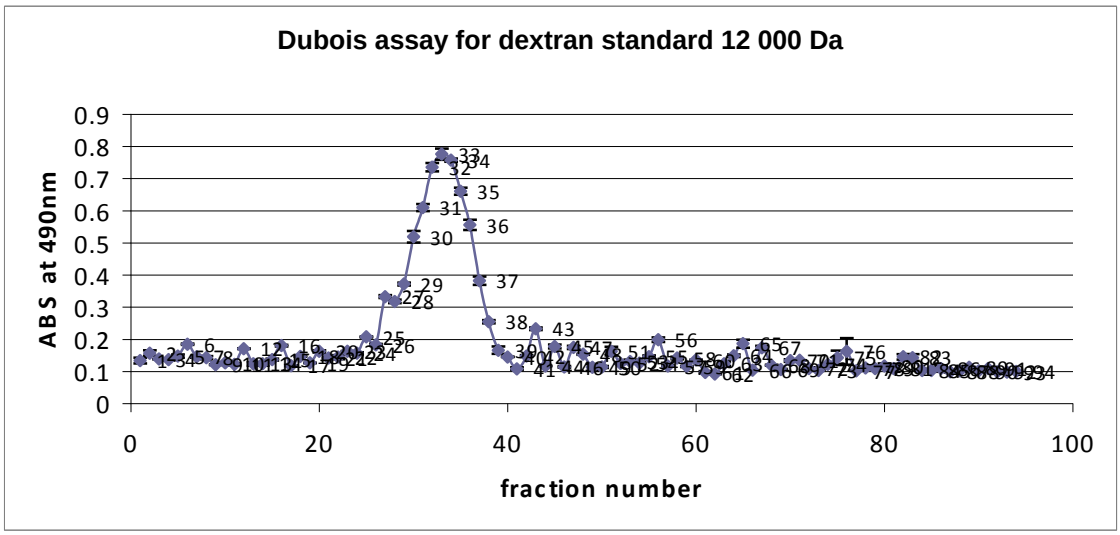


Figure 3.27: Dubois assay for dextran standard MW=12,000 Da (10mg/ml) fractionated on Superdex 200 10/300GL.
Parameters: flow 0.4ml/min., 0.5ml per fraction.
Values for each point of measurement reported as mean value with the upper and lower values.

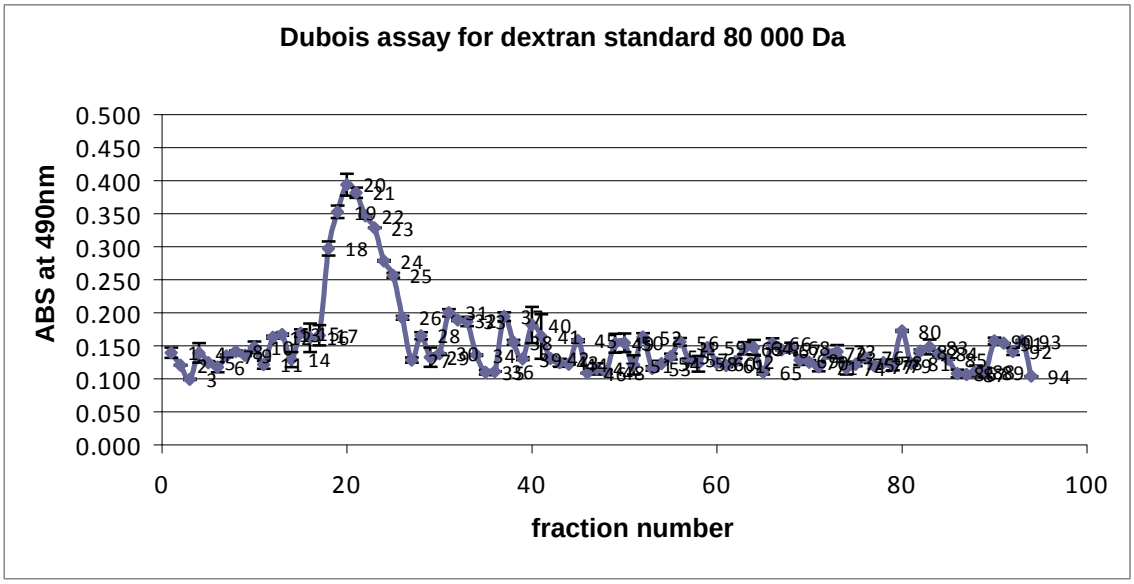


Figure 3.28: Dubois assay for dextran standard MW=80,000Da (50mg/ml) fractionated on Superdex 200 10/300GL.
Parameters: flow 0.4ml/min., 0.5ml per fraction.
Values for each point of measurement reported as mean value with the upper and lower values.

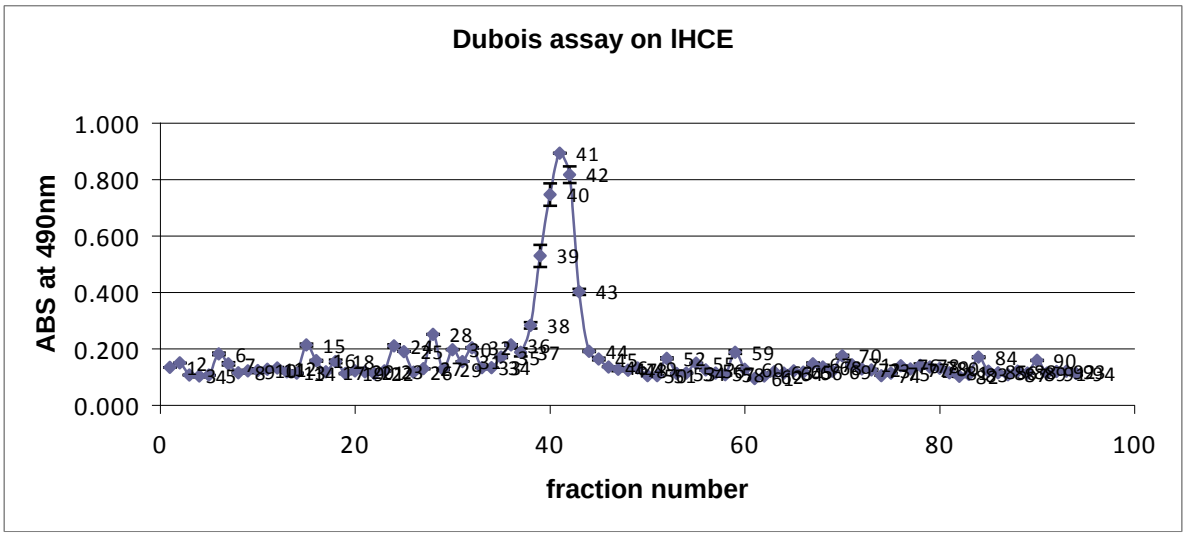


Figure 3.29: Dubois assay for lHCE (10mg/ml) fractionated on Superdex 200 10/300GL.
Parameters: flow 0.4ml/min., 0.5ml per fraction.
Values for each point of measurement reported as mean value with the upper and lower values.

2. A standard curve of dextran standards was plotted (Appendix J) showing elution volume parameters vs. logarithm of molecular weight of dextran standards.

3. The highest peak obtained by Dubois assay was established as 41st fraction (Figure 3.29) of lHCE, therefore retention volume (V_R) of lHCE fraction is 20.5ml

4. Using the straight line formula from the standard curve for dextran, the average molecular weight of Dubois assay-positive (carbohydrate) components of the one major peak from the lHCE fraction was determined as 2735 Da. Tables 3.7 shows all values (V_R, V_C, V_O, K_{av}) for fractions of dextran standards and lHCE.

Table 3.7: Values (V_R, V_C, V_O and K_{av}) obtained for dextran standards (5, 12 and 80kDa) and for lHCE fractionated on Superdex 200 10/300GL.
Ln(x) and EXP (antilog) values are also presented.

Dextran standard	**V_R(ml)**	**V_C(ml)**	**V_O(ml)**	**K_{av}**	**Ln(x)**	**EXP (antilog)**
80000	10.5	24	8	0.15625	11.32742	83068
12000	16.5	24	8	0.53125	9.279356	10715
5000	18.5	24	8	0.65625	8.596668	5414
lHCE	20.5	24	8	0.78125	**7.913981**	**2735**

3.4 Determination of the prebiotic index (PI) for horse chestnut aqueous extract (HCE)

3.4.1 Changes in bacterial biomass of *Lactobacillus*, *Bifidobacterium* and *E. coli* grown on commercial prebiotics and HCE.

The growth of five *Lactobacillus* and four *Bifidobacterium* strains after 24 hours of incubation on 1% (w/v) glucose, commercial prebiotics (GOS, FOS, inulin) or 1% (w/v; 0.16% soluble solids) horse chestnut extract (HCE) is shown in Table 3.8. Changes in cell biomass of probiotic and enteric strains were also monitored in unsupplemented medium (MRS/M9). For most of the probiotic strains tested the growth (as optical density at 600nm) on the commercial prebiotics or HCE was less than that with glucose (positive control). Of the 45 different possible combinations (9 probiotic strains and 5 different media: MRS only, MRS+GOS, MRS+FOS, MRS+Inulin and MRS+HCE), 32 exhibited significantly lower ($p \leq 0.05$) increase in cell density when cultured with a prebiotic (GOS, FOS and inulin) and HCE in comparison to growth on glucose.

Table 3.8: Increase in cell density between time 0 and time 24h[b] for probiotic strains grown with various carbohydrates and horse chestnut aqueous extract (HCE).

Bacterial Culture	MRS/M9 only	Glucose	GOS	FOS	Inulin	HCE
*L. plantarum*ATCC 8014	0.64±0.03[a]	2.47±0.01	2.23±0.00[a]	1.00±0.02[a]	0.62±0.01[a]	0.83±0.03[a]
L. rhamnosus ATCC 7469	0.60±0.01[a]	2.29±0.01	2.06±0.01[a]	0.71±0.01[a]	0.55±0.01[a]	0.63±0.03[a]
L. casei ATCC 393	0.47±0.00[a]	2.37±0.00	1.96±0.02[a]	0.57±0.01[a]	0.47±0.01[a]	0.51±0.01[a]
L. acidophilus ATCC 4356	0.61±0.01[a]	2.26±0.00	2.05±0.00[a]	0.70±0.01[a]	0.57±0.00[a]	0.65±0.01[a]
L. fermentum ATCC 9338	0.21±0.01[a]	0.85±0.02	1.24±0.01[a]	0.38±0.02[a]	0.25±0.01[a]	0.30±0.01[a]
B. infantis DSM 20088	0.17±0.02[a]	1.72±0.01	0.74±0.06[a]	0.30±0.01[a]	0.17±0.00[a]	0.22±0.03[a]
B. angulatum DSM 20098	0.13±0.01[a]	1.75±0.16	0.64±0.04[a]	0.95±0.18[a]	1.51±0.01	0.36±0.07[a]
B. longum DSM 20219	0.10±0.00[a]	0.64±0.06	0.43±0.02[a]	0.26±0.04[a]	0.08±0.01[a]	0.16±0.00[a]
B. breve DSM 20091	0.03±0.00[a]	0.30±0.01	0.34±0.02	0.39±0.01[a]	0.10±0.03[a]	0.29±0.02
E. coli ECOR 1 ATCC 35320	0.02±0.00[a]	0.96±0.05	0.81±0.06	0.24±0.02[a]	0.03±0.00[a]	0.21±0.02[a]

Values reported as OD (at 600nm) ± standard deviation (n=3)

[a] Mean value (± standard deviation) for the probiotic grown on commercial prebiotics and HCE differ significantly ($p \leq 0.05$) from glucose grown cells.

[b] Increase in cell density during 7 days reported for *B. breve* DSM 20091

In the case of the *Bifidobacterium* strain, *B. breve* DSM 20091, the growth on GOS and HCE is similar to that on glucose (positive control) with no significance difference

($p>0.05$). *B. breve* grown on FOS obtained significantly higher ($p\leq0.05$) growth than on glucose. Table 3.8 also presents the change in cell biomass of the enteric strain (*E. coli* ECOR 1 ATCC 35320) grown on unsupplemented M9 medium as well as on M9 supplemented with 1% (w/v) glucose (used as a positive control), commercial prebiotics (GOS, FOS, inulin) and HCE.

The data shows that among all prebiotics and HCE tested, FOS, inulin and HCE did not support the growth of enteric strain as well as glucose; however the growth of *E. coli* on FOS, inulin and HCE was significantly less ($p\leq0.05$) compared to positive control.

Statistical analysis

The values, as shown in Tables 3.8, derived from the prebiotic assay, are reported as an average of 3 replicates. The data was analyzed using the SPSS program (IBM SPSS Statistics, Version 19). All data obtained for each strain was subjected to analysis for normality of distribution and then, depending on the results, comparison, between the groups (MRS/M9 only, glucose, GOS, FOS, inulin and HCE) for each strain. This was carried out using Kruskal-Wallis or ANOVA One-Way tests.

In the case of analysis of bacterial growth with non-normally distributed data (*L. acidophilus, L. rhamnosus, B. longum* and *E. coli*) the Kruskal-Wallis test was used to determine whether or not there was a significant difference in the increase of cell densities between all groups. This analysis showed that a significant difference ($P=0.05$) existed between data from each microorganism tested.

Following on from this the Mann-Whitney U test was used between two chosen groups (e.g. glucose vs. other sample) where at least one of them indicated not normally distributed data. Between those groups, where the data were both normally distributed, the Independent Sample T-test was performed. Mann-Whitney U test and Independent Sample T-test were used to determine whether or not there was a significant difference in change of cell biomass between the control (glucose) and the commercial prebiotics (FOS, GOS, inulin), HCE and unsupplemented MRS/M9.

In the case of the remaining strains (*L. plantarum, L. rhamnosus, L. casei, B. infantis, B. angulatum, B. breve*), the data obtained was found to be normally distributed therefore ANOVA One-way (Tukey) test was used to determine whether there was a significant difference between the control (glucose) and other samples tested. The Tukey test allowed multiple comparison of all samples tested. The significant differences (as p value) in growth of probiotics on glucose compared to growth on test samples are

presented and summarized in Table 3.9. In most cases the cell density of bacteria on glucose supplemented broths (MRS/M9) was significantly higher ($p \leq 0.001$). P-values for the differences observed for glucose versus each prebiotic are presented in table 3.9.

Table 3.9: The p values obtained from SPSS analysis (glucose vs. other samples).

GLUCOSE vs.	*L.plantarum* ATCC 8014	*L.rhamnosus* ATCC 7469	*L.casei* ATCC 393	*L.acidophilus* ATCC 4356	*L.fermentum* ATCC 9338	*B.infantis* DSM 20088	*B.angulatum* DSM 20098	*B.longum* DSM 20219	*B.breve* DSM 20091	*E.coli ECOR1* ATCC 35320
MRS/M9 only	$p \leq 0.001$	p=0.046	$p \leq 0.001$	p=0.046	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	p=0.046	$p \leq 0.001$	$p \leq 0.001$
GOS	$p \leq 0.001$	p=0.046	$p \leq 0.001$	p=0.043	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	p=0.030	p=0.326	p=0.066
FOS	$p \leq 0.001$	p=0.046	$p \leq 0.001$	p=0.046	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	p=0.003	p=0.004	$p \leq 0.001$
INULIN	$p \leq 0.001$	p=0.046	$p \leq 0.001$	p=0.046	$p \leq 0.001$	$p \leq 0.001$	p=0.283	$p \leq 0.001$	$p \leq 0.001$	p=0.046
HCE	$p \leq 0.001$	p=0.046	$p \leq 0.001$	p=0.046	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	p=0.994	p=0.001

Significance level at 0.05 (n=3).

SPSS analysis was also carried out to determine whether or not there was a significant difference in the increase in cell densities between the strains grown on HCE and commercial prebiotics (FOS, GOS, inulin). There results (p values) are presented in Table 3.10.

Table 3.10: The p values (as significant difference) obtained for probiotics growth on HCE compared to GOS, FOS and inulin.

HCE vs.	*L.plantarum* ATCC 8014	*L.rhamnosus* ATCC 7469	*L.casei* ATCC 393	*L.acidophilus* ATCC 4356	*L.fermentum* ATCC 9338	*B.infantis* DSM 20088	*B.angulatum* DSM 20098	*B.longum* DSM 20219	*B.breve* DSM 20091
GOS	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$	p=0.046	$p \leq 0.001$	$p \leq 0.001$	p=0.142	$p \leq 0.001$	p=0.153
FOS	$p \leq 0.001$	p=0.014	p=0.003	p=0.003	p=0.002	p=0.134	p=0.001	p=0.069	p=0.002
INULIN	$p \leq 0.001$[a]	p=0.035[a]	p=0.10	$p \leq 0.001$[a]	p=0.014[a]	p=0.609	$p \leq 0.001$	$p \leq 0.001$[a]	$p \leq 0.001$[a]

A significance level at p=0.05 (n=3)

[a] Significantly higher growth of probiotics on HCE in comparison to GOS, FOS and inulin.

In most cases the increase in cell biomass for those strains grown on HCE differ significantly ($p \leq 0.05$) from those grown on commercial prebiotics. Some probiotics indicated significantly higher growth ($p \leq 0.05$) on HCE compared to commercial prebiotics. These are: *L. plantarum, L. fermentum, L. rhamnosus, L. acidophilus, B. longum* and *B. breve* if paired with inulin.

3.4.2 Prebiotic index (PI)

The prebiotic index (PI), know also as prebiotic activity scores, obtained for all probiotics grown on prebiotics and HCE are presented in Table 3.11. The modified

formula (section 2.4) provided a potential method for calculating PI scores for each probiotic. The formula determined the growth of *Lactobacillus* and *Bifidobacterium* species using commercial prebiotics (GOS, FOS and inulin) and HCE relative to growth of a non-probiotic *E. coli*. Calculations took into account the potential growth of bacteria on medium, not supplemented with any sugars (control MRS/M9).

Table 3.11: Prebiotic Index (PI) of *Lactobacillus* and *Bifidobacterium* species grown on commercial prebiotics (GOS, FOS, inulin) and horse chestnut extract (HCE) after 24 hours (* for *B. breve* 7 days).

PI				
Bacterial Culture	**GOS**	**FOS**	**INULIN**	**HCE**
*L. plantarum*ATCC 8014	1.03±0.09	0.82±0.03	-0.58±1.32	0.52±0.11
L. rhamnosus ATCC 7469	1.02±0.08[a]	0.27±0.05[a]	-1.81±0.12[a]	0.08±0.06
L. casei ATCC 393	0.93±0.09[a]	0.22±0.02	0.16±0.18	0.13±0.01
L .acidophilus ATCC 4356	1.04±0.09[a]	0.24±0.03	-1.38±0.15[a]	0.13±0.06
L. fermentum ATCC 9338	1.90±0.19	1.09±0.14	4.19±1.99[a]	0.73±0.05
B. infantis DSM 20088	0.45±0.09	0.36±0.07	0.04±0.77	0.16±0.06
B. angulatum DSM 20098	0.38±0.05	2.05±0.11	53.33±6.21[a]	0.69±0.17
B. longum DSM 20219	0.74±0.13	1.27±0.47	-2.3±0.42[a]	0.48±0.10
B. breve DSM 20091*	1.37±0.23	5.53±0.64	13.65±6.58	4.64±0.45

[a] Mean value ± standard deviation (n=3) for the PI scores of probiotics grown on HCE differ significantly ($p \leq 0.05$) from the cells grown on commercial prebiotics (GOS, FOS, Inulin).

With the modified formula (section 2.4), glucose yielded a score of 1.00 and the growth of probiotic and enteric strains on known prebiotics or HCE was compared to growth on glucose. PI scores obtained for GOS were the highest in the case of seven of the strains tested. Growth of all probiotics on HCE indicated positive PI scores; additionally *B. breve* DSM 20091 paired with HCE obtained the highest PI (4.64) among all bacteria tested with HCE. However this value was not significantly different ($p>0.05$) from GOS, FOS and inulin. In contrast the lowest PI scores observed were for *L. rhamnosus* ATCC 7469, *L. casei* ATCC 393 and *L. acidop*hilus ATCC 4356 grown on HCE (0.08, 0.013 and 0.013, respectively). It has to be mentioned that concentration of HCE of 1% is referred to the amount of horse chestnut residue added directly into the growth medium (MRS/M9). As established in section 3.2.2 soluble matter of HCE was 0.16%.
The PI wer also calculated according to the original formula established by Huebner *et al*. (2008) are shown in Table 3.12. These calculations have also used the same data as presented in Table 3.8.

Table 3.12: Prebiotic Index (PI) according to the original formula established by Huebner *et al.* (2008) for *Lactobacillus* and *Bifidobacterium* species grown on commercial prebiotics (GOS, FOS, inulin) and horse chestnut extract (HCE) after 24 hours (* for *B. breve* 7 days).

PI				
Bacterial Culture	**GOS**	**FOS**	**INULIN**	**HCE**
L. plantarum ATCC 8014	0.03±0.06	0.13±0.04	0.41±0.02	0.11±0.01
L. rhamnosus ATCC 7469	0.01±0.02	0.06±0.02	0.42±0.01	0.06±0.01
L. casei ATCC 393	-0.01±0.03	-0.07±0.03	0.22±0.01	-0.02±0.02
L. acidophilus ATCC 4356	0.01±0.03	-0.01±0.03	0.31±0.01	0.05±0.01
L. fermentum ATCC 9338	0.15±0.04	0.09±0.04	0.39±0.01	0.04±0.02
B. infantis DSM 20088	-0.18±0.07	-0.11±0.03	0.17±0.02	-0.14±0.02
B. angulatum DSM 20098	-0.22±0.04	0.16±0.05	0.72±0.02	-0.03±0.04
B. longum DSM 20219	-0.11±0.03	0.05±0.05	0.15±0.03	0.00±0.02
B. breve DSM 20091*	0.08±0.06	0.47±0.03	0.29±0.13	0.37±0.02

Values reported as mean ± standard deviation (n=3)

3.4.3 Prebiotic effect of HCE with different concentration (1%-5% w/v)

Increases in cell densities for *Lactobacillus plantarum* ATCC 8014 and *Escherichia coli* ATCC 35320 strains grown in MRS/M9 supplemented with HCE ranging from 1-5% (w/v) are presented in Table 3.13.

Table 3.13: Increases in cell densities (OD at 600nm) of *Lactobacillus plantarum* ATCC 8014 and E*scherichia coli* ATCC 35320 between time 0 and 24 hours grown on HCE ranging 1%-5% (w/v).

Bacterial strain	MRS/M9+ HCE 1%	MRS/M9+ HCE 2%	MRS/M9+ HCE 3%	MRS/M9+ HCE 4%	MRS/M9+ HCE 5%
Lactobacillus plantarum ATCC 8014	0.706±0.057	0.869±0.026	0.930±0.078	0.983±0.029	1.160±0.021
Escherichia coli ECOR 1 ATCC 35320	0.151±0.014	0.205±0.010	0.320±0.020	0.362±0.012	0.403±0.019

Values reported as mean ± standard deviation (n=3)

Graphical presentation of the increase in cell densities of both *L.plantarum* and *E.coli* during 24 hours of incubation in medium enriched with 1%-5% HCE provides the Figure 3.30.

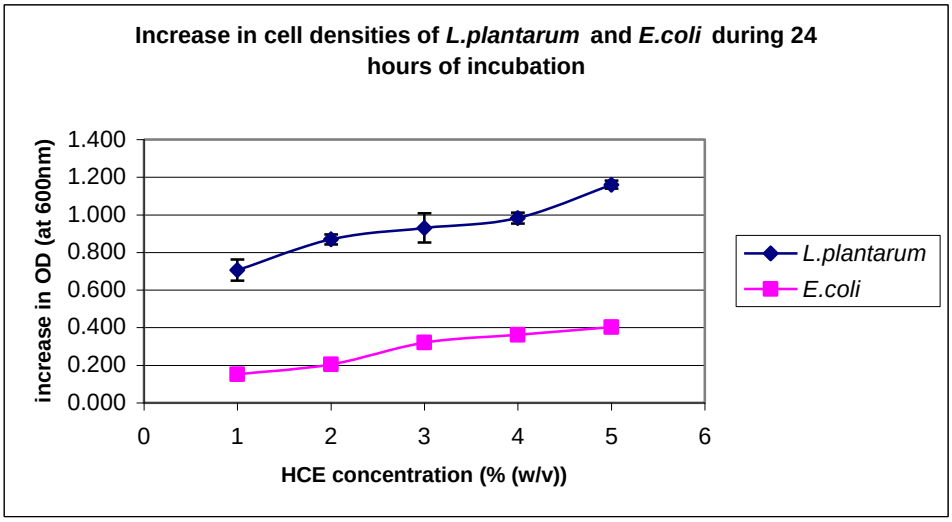


Figure 3.30: Graphical presentation of increases in optical densities (at 600nm) of *Lactobacillus plantarum* ATCC 8014 and *Escherichia coli* ATCC 35320 after 24 hours of incubation with 1%-5% (w/v) HCE.
Each value represents mean ± standard deviation (n=3).

Table 3.14 presents PI (according to the modified formula) obtained for *Lactobacillus plantarum* ATCC 8014 grown on HCE in concentration ranging from 1% to 5% (w/v) and comparing to PI using commercial prebiotics (GOS, FOS and inulin).

Table 3.14: Prebiotic Index (PI) of *Lactobacillus plantarum* ATCC 8014 grown on commercial prebiotics (GOS, FOS, inulin) and horse chestnut extract (HCE 1%-5%) after 24 hours.

	PI							
Bacterial Culture	1% GOS	1% FOS	1% INULIN	1% HCE	2% HCE	3% HCE	4% HCE	5% HCE
Lactobacillus plantarum ATCC 8014	1.01±0.09	1.19±0.16	-10.35±7.94	0.59±018	0.89±0.15	0.63±0.12	0.63±0.09	0.78±0.05

Values reported as mean value ± standard deviation (n=3)

3.5 Development of a novel method for determination of prebiotic efficacy

Table 3.15 compares the increases in cell densities of *Lactobacillus plantarum* ATCC 8014 after 24 hours in 96-well microplate vs. universal bottles.

There is a significant difference between ODs obtained in the two test situations. However the difference appears to be reasonably consistent with the OD obtained in microtitre plate 50.82±2.86 % of that obtained in universal glass bottles.

Table 3.15: Increase in cell density between time 0 and 24 hours for *Lactobaicllus plantarum* ATCC 8014 assayed in different incubation conditions.

Conditions of prebiotic assay						
Incubation equipments	Incubation volume	MRS	Glucose	GOS	FOS	HCE
In microplate 96-wells [a]	200µl	0.324±0.034	1.316±0.094	1.066±0.064	0.421±0.074	0.399±0.033
In glass bottles [b]	10ml	0.596±0.046	2.488±0.018	2.215±0.012	0.899±0.107	0.769±0.077

[a] Values expressed as mean ± standard deviation for n=16
[b] Values expressed as mean ± standard deviation for n=6

3.6 Determination of the effect of different processing conditions on prebiotic activity of lyophilised horse chestnut aqueous extract (lHCE)

3.6.1 Effect of exposure to low pH on prebiotic index

The prebiotic index (PI) scores for *Lactobacillus plantarum* ATCC 8014 grown on the prebiotic (GOS, FOS) and lHCE incubated at pH 3.0, 4.0, 5.0 or 6.0 are presented in Table 3.16. Control scores were also determined for each prebiotic and lHCE. Treated GOS and lHCE retained similar prebiotic activity compared to non-treated sample (control); moreover these samples were not significantly different from their controls (untreated GOS and lHCE). In contrast FOS after exposure to low pH levels between 3.0 and 6.0, had a significantly lower PI ($p \leq 0.05$) in comparison with the control.

Table 3.16: Effect of low pH for 24h on prebiotic index [a].

	Treated sample (pH at)				**Untreated sample (control)**
	3.0	**4.0**	**5.0**	**6.0**	
GOS	0.76±0.03	0.77±0.14	0.67±0.02	0.75±0.10	0.63±0.14
FOS	0.22±0.05[b]	0.25±0.04[b]	0.30±0.03[b]	0.29±0.10[b]	0.67±0.18
lHCE	0.28±0.01	0.30±0.02	0.29±0.02	0.28±0.01	0.30±0.02

[a] PI determined for commercial prebiotics (GOS, FOS) and lHCE with *Lactobacillus plantarum* ATCC 8014 and *E.coli* ECOR 1 as assay strains.
[b] Mean value ± standard deviation (n=3) for low pH treatment differs significantly ($p \leq 0.05$) from control (untreated sample) for the specific prebiotic and lHCE.

3.6.2 Effect of low pH with high temperature on prebiotic index

The prebiotic index (PI) scores for *Lactobacillus plantarum* ATCC 8014 grown on the prebiotic (GOS, FOS) and lHCE treated with heat at 85ºC and low pH at 3.0, 4.0, 5.0 or

6.0 are presented in Table 3.17. Control scores were also determined for each prebiotic and lHCE. Scores for treated GOS and FOS did not differ significantly from their controls, however a difference in PI scores for FOS treated samples were observed especially at pH 4.0. For lHCE samples after heating at 85ºC and at pH 3.0 and 4.0 the PI scores decreased significantly ($p \leq 0.05$) from the controls but remained the same after treatment at pH 5.0 and 6.0 (PI= 0.28 and 0.29, respectively) in comparison to non-treated lHCE (PI=0.29).

Table 3.17: Effect of heat at 85°C and low pH on prebiotic index [a].

	Treated sample (heat at 85ºC and pH at)				**Untreated sample (control)**
	3.0	**4.0**	**5.0**	**6.0**	
GOS	0.65±0.06	0.64±0.05	0.70±0.07	0.54±0.09	0.70±0.06
FOS	0.20±0.01	0.08±0.01	0.11±0.03	0.11±0.03	0.16±0.07
lHCE	0.21±0.01[b]	0.24±0.01[b]	0.28±0.00	0.29±0.01	0.29±0.01

[a] PI determined for commercial prebiotics (GOS, FOS) and lHCE with *Lactobacillus plantarum* ATCC 8014 and *E.coli* ECOR 1 as assay strains.
[b] Mean value ± standard deviation (n=3) for heat and low pH treatment differs significantly ($p \leq 0.05$) from control (untreated sample) for the specific prebiotic and lHCE.

3.6.3 Effect of Maillard reaction on prebiotic index

The PI scores for FOS and lHCE before and after modification by Maillard reaction conditions are presented in Tables 3.18. The controls represent those samples where FOS or lHCE were added directly to growth medium containing glycine without heating. For FOS samples PI scores are negative and differ significantly ($p \leq 0.05$) from the control. In contrast, treated lHCE samples obtain higher PI scores in comparison to control (untreated lHCE), additionally the difference is significant ($p \leq 0.05$).

Table 3.18: Effect of Mailard reaction on prebiotic index [a].

	Treated sample (Maillard reaction) for			**Control sample**
	1h	**2h**	**3h**	
FOS	-0.06±0.04[b]	-0.09±0.00[b]	-0.14±0.00[b]	0.14±0.06
lHCE	0.21±0.03[b]	0.24±0.05[b]	0.21±0.03[b]	0.15±0.01

[a] PI determined for commercial prebiotic FOS and lHCE with *Lactobacillus plantarum* ATCC 8014 and *E.coli* ECOR 1 as assay strains.
[b] Mean value ± standard deviation (n=3) for Maillard reaction treatment differs significantly ($p \leq 0.05$) from control (untreated sample) for FOS and lHCE.

The percentage relative browning is shown in Table 3.19. Due to high absorbance of lHCE samples at the beginning of Maillard reaction the values were corrected. The same procedure was employed for FOS and glucose.

Table 3.19: Percent relative browning products of FOS, glucose and lHCE.

Percent relative browning products			
time of incubation (h)	**FOS**	**lHCE**	**GLUCOSE**
1	3.09±0.00	85.19±2.47	2.47±0.00
2	5.25±0.31	144.14±15.74	3.09±0.00
3	7.72±0.31	166.05±3.09	98.15±0.62

Values are reported as mean ± standard deviation (n=3)

As shown in Table 3.19, lHCE obtained very high percentage of browning products (166.05±3.09). FOS indicated only 7.72±0.31% final percent browning.

3.7 Qualitative and quantitative analysis of organic acids in fermentation broths

Qualitative analysis of organic acid content in the bacterial medium during 48 hours of incubation revealed that the major organic acids present were lactic acid (LA) and acetic acid (AA). Using the equation of the line from the standards curves of LA (Appendix K) and AA (Appendix L) the quantity of these organic acids was calculated and presented in Table 3.20. Additionally pH measurements were taken at the end of incubation (48h) for all probiotic strains grown on MRS medium supplemented in 1% (w/v) of commercial prebiotic, 1% (w/v) HCE or 1% (w/v) glucose. Table 3.21 shows the degree of acidification of medium by *Lactobacillus* and *Bifidobacterium* strains.

Table 3.20: Organic acids produced by different probiotic strains after 48 hours of incubation (*7 days incubation for *B. breve*) in MRS medium supplemented with commercial prebiotics, HCE and glucose.

Production of lactic acid (LA) by probiotic strains						
Probiotic strains	**MRS**	**GLUCOSE**	**GOS**	**FOS**	**INULIN**	**HCE**
*L. plantarum*ATCC 8014	16.17±0.97	157.41±4.79	102.86±1.38	26.71±0.99	13.78±2.59	18.68±0.21
L. acidophilus ATCC 4356	12.75±2.55	127.18±9.71	83.79±8.22	13.53±7.86	25.52±5.34	10.24±0.62
L. casei ATCC 393	12.88±0.13	141.01±0.68	75.47±4.15	17.62±2.39	11.93±1.14	13.11±3.53
L. rhamnosus ATCC 7469	9.19±0.04	144.62±1.72	85.83±1.53	15.92±1.93	12.84±1.77	14.71±0.95
L. fermentum ATCC 9338	0.00	36.87±1.13	35.15±3.87	2.07±0.02	0.00	0.00
B. angulatum DSM 20098	0.00	53.98±0.26	41.60±0.32	45.34±0.48	50.78±0.87	2.27±0.27
B. infantis DSM 20088	9.91±2.9	88.76±4.24	61.16±0.20	35.86±7.08	36.16±1.98	37.19±0.07
B. longum DSM 20219	0.00	62.20±1.50	52.27±1.66	61.52±5.84	29.25±1.60	0.00
B. breve DSM 20091*	0.00	31.07±0.45	42.66±0.50	49.21±0.75	0.00	6.09±0.03

Production of acetic acid (AA) by probiotic strains						
Probiotic strains	**MRS**	**GLUCOSE**	**GOS**	**FOS**	**INULIN**	**HCE**
*L .plantarum*ATCC 8014	8.39±1.56	4.58±1.87	102.97±0.48	5.60±1.17	3.96±0.85	3.8±0.05
L .acidophilus ATCC 4356	0.00	0.00	0.59±0.14	0.00	10.55±1.68	0.00
L .casei ATCC 393	0.00	2.47±1.96	8.07±0.50	0.00	0.00	0.54±0.09
L .rhamnosus ATCC 7469	48.48±1.18	6.56±2.75	20.61±0.46	13.24±0.24	11.59±1.20	83.96±3.58
L .fermentum ATCC 9338	0.00	7.09±3.48	9.11±1.78	2.42±0.86	8.72±0.11	1.34±0.71
B .angulatum DSM 20098	26.10±2.87	7.59±2.09	55.21±3.70	65.95±2.37	102.21±3.35	48.31±0.78
B .infantis DSM 20088	17.36±5.84	42.38±6.15	24.15±1.61	6.60±0.22	29.00±0.64	24.69±1.91
B .longum DSM 20219	38.45±1.65	86.79±1.66	79.37±7.78	68.87±0.23	48.73±1.42	44.62±0.22
B .breve DSM 20091*	0.00	21.13±1.86	20.99±0.24	19.90±1.01	0.13±0.42	20.05±1.36

Values are expressed as mmol/L of broth and reported as mean value (± standard deviation) for n=3.

Table 3.21: pH after 48 hours of incubation (* for *B. breve* 7 days of incubation) in MRS medium with various prebiotics, glucose or HCE.

Probiotics	MRS only	MRS+glucose 1%	MRS+GOS 1%	MRS+FOS 1%	MRS+INULIN 1%	MRS+HCE 1%
L.plantarum	6.37±0.02	4.22±0.01	4.56±0.01	5.82±0.01	6.37±0.02	6.16±0.01
L.acidophilus	6.46±0.01	4.15±0.01	4.52±0.02	6.18±0.02	6.74±0.11	6.26±0.01
L.casei	6.50±0.01	4.10±0.01	4.64±0.00	6.24±0.01	6.50±0.11	6.34±0.01
L.rhamnosus	6.56±0.02	4.23±0.01	4.64±0.01	6.22±0.02	6.54±0.03	6.36±0.02
L.fermentum	7.20±0.10	5.64±0.05	5.70±0.01	7.01±0.05	7.25±0.09	7.06±0.04
B.angulatum	6.53±0.06	4.42±0.04	4.55±0.01	4.54±0.01	4.61±0.02	6.38±0.12
B.infantis	6.56±0.01	4.79±0.02	5.32±0.05	6.32±0.02	6.64±0.07	6.48±0.03
B.longum	6.68±0.02	4.70±0.03	4.86±0.01	5.01±0.05	5.62±0.03	6.36±0.01
*B.breve**	6.40±0.02	5.10±0.06	4.80±0.05	4.99±0.18	6.34±0.02	5.90±0.02

Values reported as mean value (± standard deviation) for n=3.

For most probiotics the lowest pH in medium was obtained where the strains were grown on 1% glucose, which was the positive control. Only *B. breve* indicated lower pH on 1% GOS than on 1% glucose.

Among all *Lactobacillus* strains grown on 1% HCE, the lowest pH was obtained by *L.plantarum* (6.16±0.01) and was correlated with the highest production of lactic acid (LA=18.67±0.21mM). In the case of *Bifidobacterium* strains the highest acidification was observed for *B. breve* (5.90±0.02) grown on HCE. Some strains grown on the extract did not produce organic acids, for example *L. fermentum* and *B. longum* did not produce lactic acid and *L.acidophilus* did not produce acetic acid. The highest pH for HCE grown cells was obtained for *L. fermentum* (7.06±0.04) and this strain also produced a very low amount of acetic acid (AA=1.34±0.71mM), moreover lactic acid was not identified in medium of *L. fermentum* supplemented by HCE.

3.8 Determination of the effect of lyophilised horse chestnut aqueous extract on hyaluronidase activity

Hyaluronidase (HA-ase) as previously mentioned (section 1.4.1.7) is an enzyme described in nomenclature as EC 3.2.1.35 and belongs to the group of hydrolases which cleave β 1,4 glycosidic linkage between N-acetyloglucosamine and D-glucuronate residues in hyaluronic acid (HA). HA-ase also hydrolyses 4-S and 6-S chondroitin sulphate and catalyses transglycosylation. HA-ase is mostly present in testes, liver lysosomes and serum (Garg and Hales, 2004).

Inhibitory effects of heparin and lHCE on hyaluronidase activity were observed by way of decreased absorbance at 585nm as shown in Figure 3.31. The degree of inhibition was strictly correlated with the concentration of heparin and lHCE. In contrast FOS did not exhibit any inhibitory effect on HA-ase.

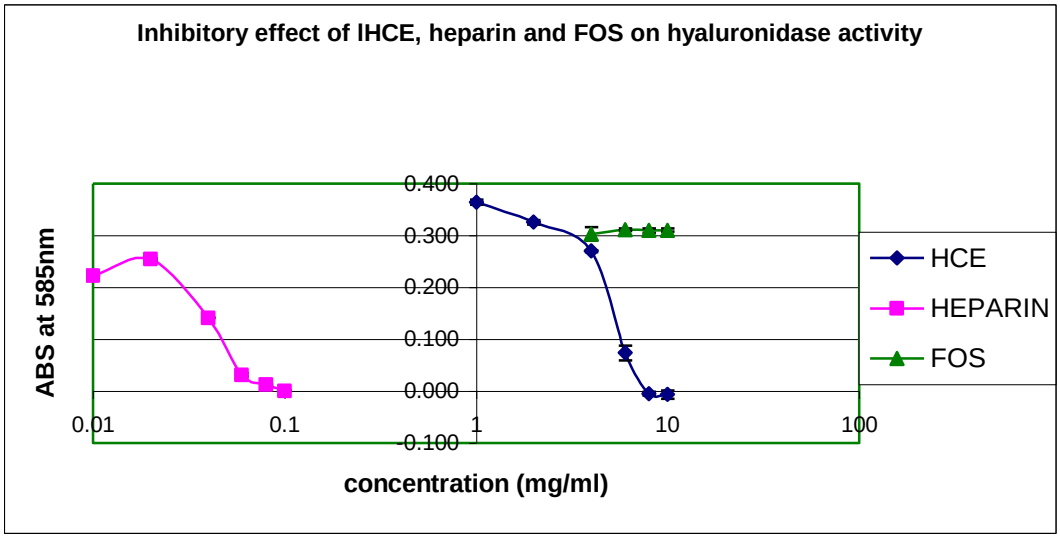


Figure 3.31: Inhibitory effect of heparin, lHCE and FOS on hyaluronidase activity.
Data are indicated as mean value with the upper and lower values.

The percentage inhibition of HA-ase by tested samples (inulin, lHCE and FOS) is presented in Figure 3.32. To show the difference between the samples the axis with inhibitor concentration (mg/ml) was applied in logarithmic scale. The IC_{50} for lHCE and heparin were also determined and estimated as 4.8mg and 0.032mg/ml respectively.

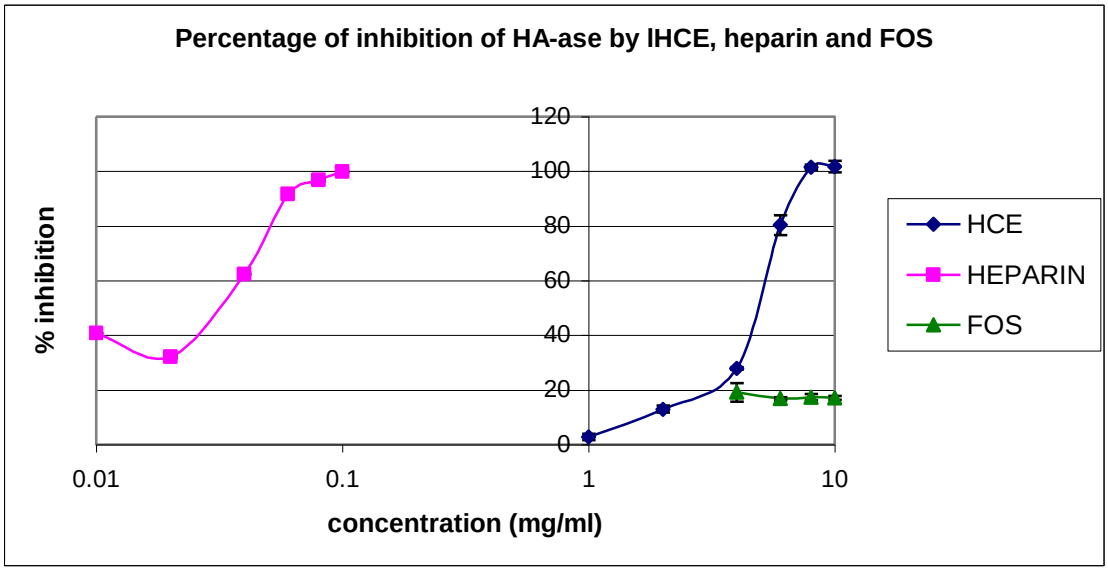


Figure 3.32: Percentage of hyaluronidase inhibition by lHCE, heparin and FOS.
Data are indicated as mean value with the upper and lower values.

The next step of the research was to determine whether a mixture of heparin and lHCE would give rise to a different inhibitory effect on HA-ase than when the samples were applied separately. Figure 3.33 shows the absorbance obtained at the end point of enzymatic reaction of hyaluronidase (HA-ase) with hyaluronic acid (HA) in the presence of lHCE, heparin and a mixture of both. Positive control (enzymatic reaction with no inhibitors) is also included.

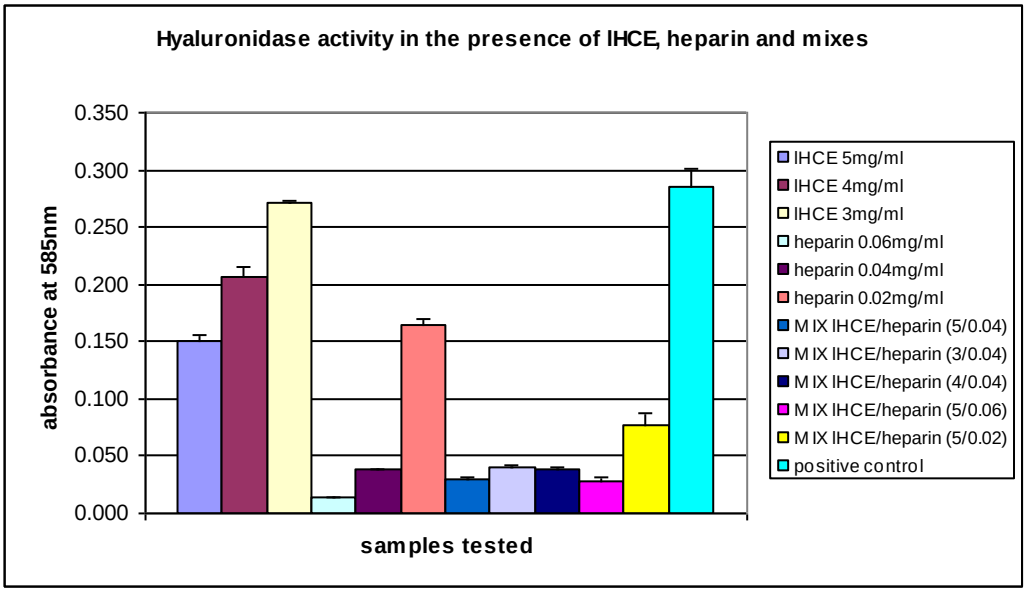


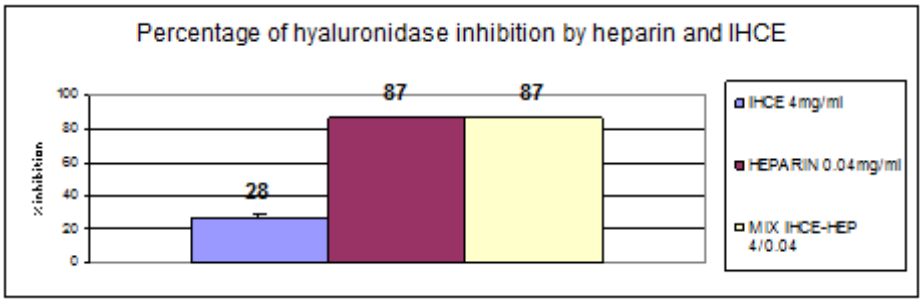
Figure 3.33: Decrease in absorbance at 585nm as effect of hyaluronidase inhibition by lHCE, heparin and their mixtures.
Data are indicated as mean value with the upper and lower values.

The inhibitory effect of lHCE on HA-ase activity was observed and was manifested with decreased absorbance (at 585nm) as a result of less hydrolyzed product production. The effect of lHCE on HA-ase was greater as more lHCE was applied. Using 3mg/ml of lHCE showed absorbance of 0.272 which was similar to positive control (no inhibition) whereas application 5mg/ml of lHCE resulted with absorbance 0.151 (or 47% inhibition).

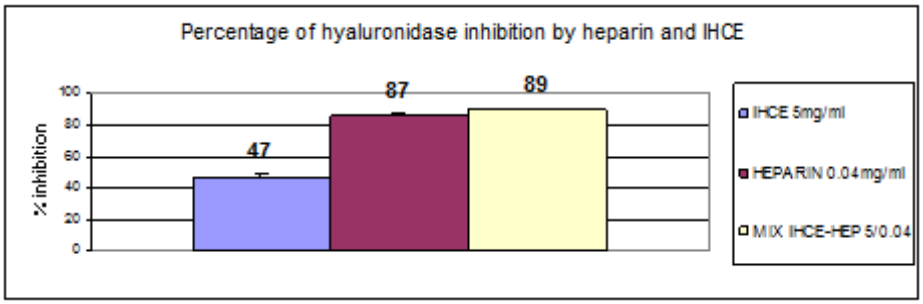
In Figure 3.34 the percent inhibition of HA-ase by lHCE, heparin and their mixtures is presented.

Analysing the data obtained for the Figures 3.34 A,B,C, and D inhibitory effect on hyaluronidase inhibition was greater in the mixture of 5mg/mL lHCE and 0.02mg/mL heparin than when either concentration alone was administered. Figure 3.42 D showed that using 5mg/ml of lHCE with 0.02mg/ml of heparin resulted in an increase in inhibition by 26%, in comparison to 5mg/ml lHCE and by 30% in comparison to 0.02mg/ml heparin. Heparin exhibited much stronger inhibitory effect on the enzyme even in small quantities.

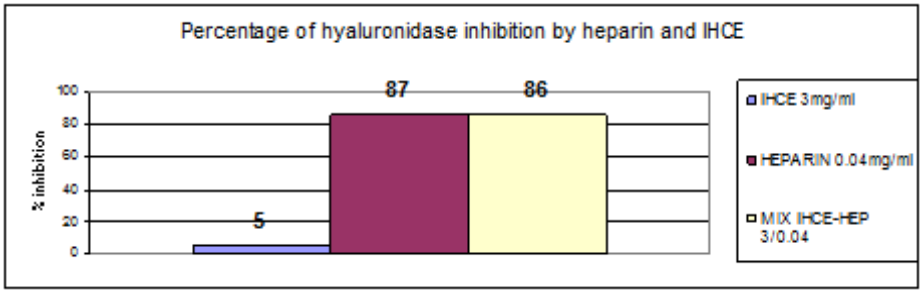
A.



B.



C.



D.

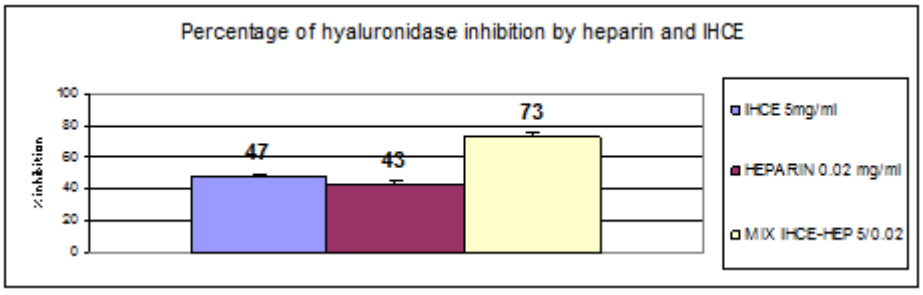


Figure 3.34: Dose-dependent inhibition on hyaluronidase activity by lHCE, inulin and their mixtures. Data are indicated as mean value with the upper and lower values.

3.9 Determination of inhibitory effect of lyophilised horse chestnut aqueous extract on elastase activity

3.9.1 Assay of elastase in the presence of commercial inhibitor (*N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone).

Enzymatic assay of elastase is presented in Figure 3.35 and Figure 3.36. Figure 3.35 shows elastase concentration ranging between 0.5-0.008 U/ml graphed against fluorescence obtained during enzymatic reaction with constant amount of elastin substrate (DQ substrate) 25µg/ml. The reaction was continuous therefore the fluorescence was measured at multiple time points for period of 120 min. As shown on the graphs the higher the amount of digested products produced, the greater the fluorescence. The reaction is continuous and more digestion products are obtained if more elastase is used. A low concentration of elastase (0.008-0.03 U/ml) yielded low fluorescence which did not exceed 100 (Figure 3.36).

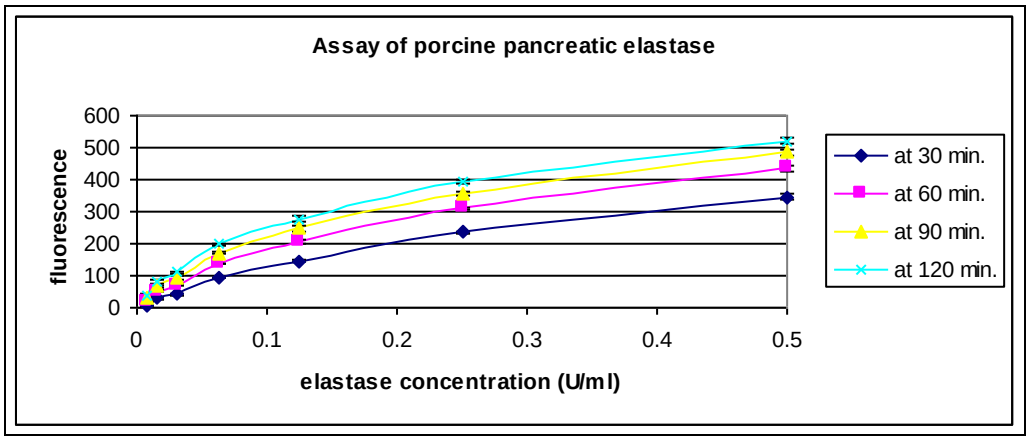


Figure 3.35: Assay of porcine pancreatic elastase using EnzChek Elastase Assay Kit. The substrate (DQ elastin) concentration used was 25µg/ml. Elastase was assayed at different concentrations (0.5-0.008 U/ml) at 30, 60, 90 and 120 minutes of incubation time. Values for each point of measurement represent mean value with the upper and lower values.

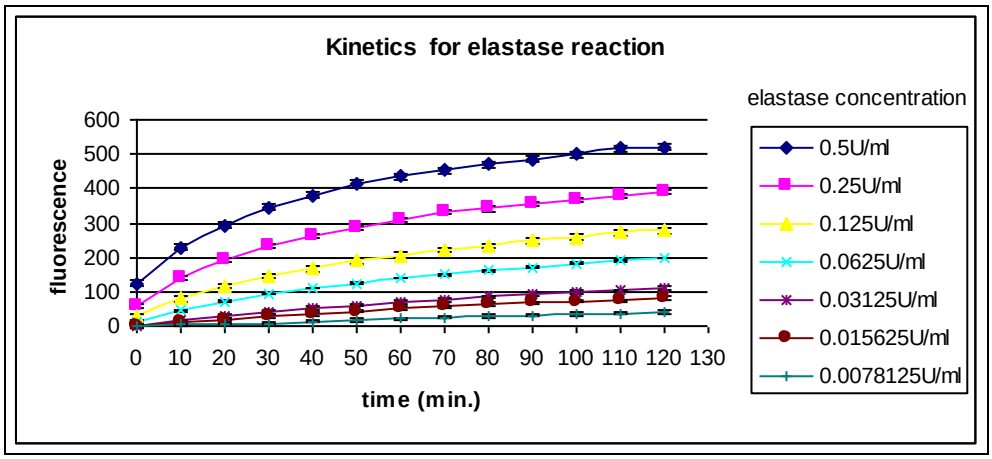


Figure 3.36: Kinetics of elastase using EnzChek.
The substrate (DQ elastin) concentration used 25µg/ml. Elastase at different concentration (0.5-0.008 U/ml) was incubated for 120 minutes while fluorescence was measured every 10 minutes. Values for each point of measurement represent mean value with the upper and lower values.

3.9.2 Assay of elastase in the presence of commercial inhibitor (*N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) and the lyophilised horse chestnut aqueous extract

The assay of elastase was also performed using elastase inhibitor *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone. Figure 3.37 shows the inhibitory effect on elastase by different concentrations (ranging from 0.0503 to 0.0004 mg/ml) of *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone. The substrate elastin (DQ elastin) was used in this assay and its concentration was 25µg/ml. Concentrations of substrate between 0.0503 and 0.001mg/ml inhibited elastase (0.5U/ml) activity, as confirmed by low fluorescence. Therefore it was decided to use *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone as model inhibitor for screening of lyophilised horse chestnut aqueous extract (lHCE) for potential elastase inhibition.

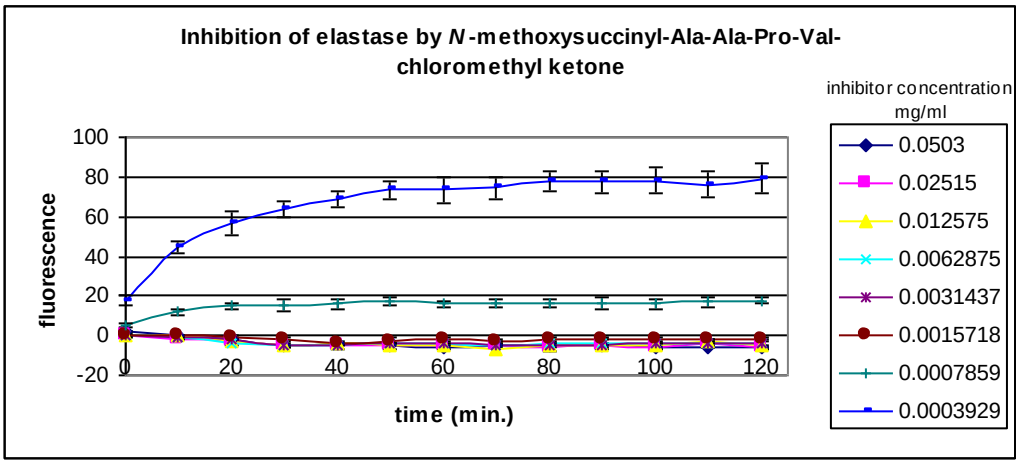


Figure 3.37: Inhibitory effect of *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone on elastase. The substrate (DQ elastin) concentration used was 25µg/ml. Elastase at concentration 0.2U/ml was incubated at different concentration of *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (0.0503-0.0004 mg/ml). Fluorescence was measured every 10min for 120 min. Values for each point of measurement represent mean value with the upper and lower values.

The inhibitory effect of lHCE on elastase activity is shown in Figure 3.38. The control inhibitor *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was used at a concentration of 0.0503 mg/ml which corresponded to 0.1M. The concentration of elastin was 25µg/ml. The activity of this elastase enzyme preparation was 0.2U/ml. lHCE solution of 12.5 to 50 mg/ml actively inhibited elastase. The fluorescence obtained for those samples falls below fluorescence for positive control (no-inhibitor control) which means that lHCE had an inhibitory effect on elastase and this effect continued for the whole incubation time (120min.). Low concentrations of lHCE (3.125mg/ml and 6.5mg/ml) appeared to cause a slight stimulation of elastase activity.

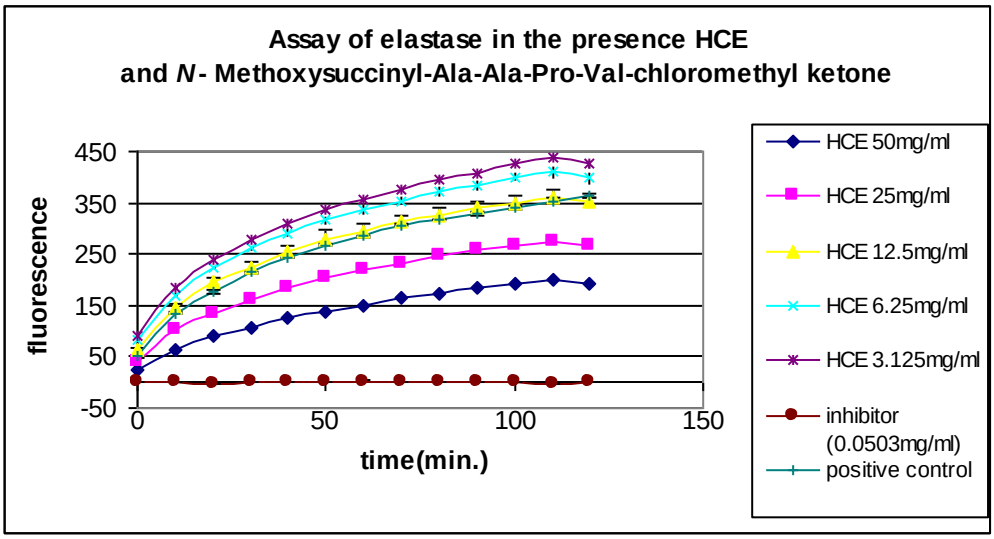


Figure 3.38: Inhibitory effect of lHCE and *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone on elastase.
The substrate (DQ elastin) concentration used was 25µg/ml. Elastase at concentration 0.2U/ml was incubated at different concentrations of lHCE (50-3.125mg/ml) and *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (0.0503 mg/ml). Positive control represents a no-inhibitor reaction. Fluorescence was measured every 10min for 120 min. Values for each point of measurement represent mean value with the upper and lower values.

The Figure 3.39 shows the percentage of inhibition of elastase by lyophilised horse chestnut aqueous extract (lHCE). Different concentrations of lHCE (50-3.125mg/ml) and incubation periods (30, 90, 120 min.) were tested. The results obtained showed that in all incubation times the percent inhibition was the same. However, at lower concentrations, there was a significant stimulatory effect. IC_{50} was estimated to be approximately 50mg/ml of lHCE but a biphasic activity is evident.

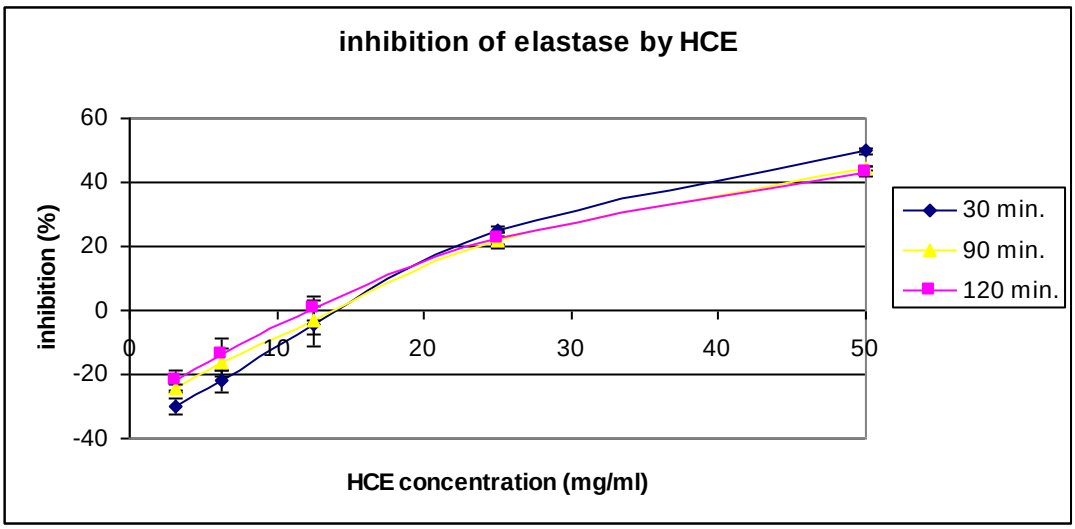


Figure 3.39: Percentage of elastase inhibition by lHCE.
The substrate (DQ elastin) concentration used was 25µg/ml. Elastase at concentration 0.2U/ml was incubated at different concentration of lHCE (50-3.125mg/ml). Fluorescence was measured after 30, 90 and 120 minute of incubation. Values for each point of measurement represent mean ± standard deviation (n=2).

3.10 Preparation of crude organic extracts of horse chestnut residue and assessment of antimicrobial activity

3.10.1 Extraction of raw compounds from horse chestnut residue

Direct extraction with methanol, ethanol, acetone, chloroform and dichloromethane was carried out to prepare crude extracts from the horse chestnut residue. The quantity of material extracted from 30g samples is shown in Table 3.22.

Table 3.22: Quantity of raw extracts obtained from horse chestnut residue.

Extractant	Quantity of raw extract
methanol	0.8511
ethanol	1.1963
acetone	1.3468
chloroform	1.3650
dichloromethane	1.3958

Values are reported in g per 30g of the residue

The percentage extracted from the residue, as presented in Figure 3.40 did not differ substantially. The highest quantity of raw extract from the residue was obtained by using dichloromethane (1.3958g/30g, 4.65%) as extractant. Methanol extracted the lowest quantity of material (0.8511g/30g, 2.84%).

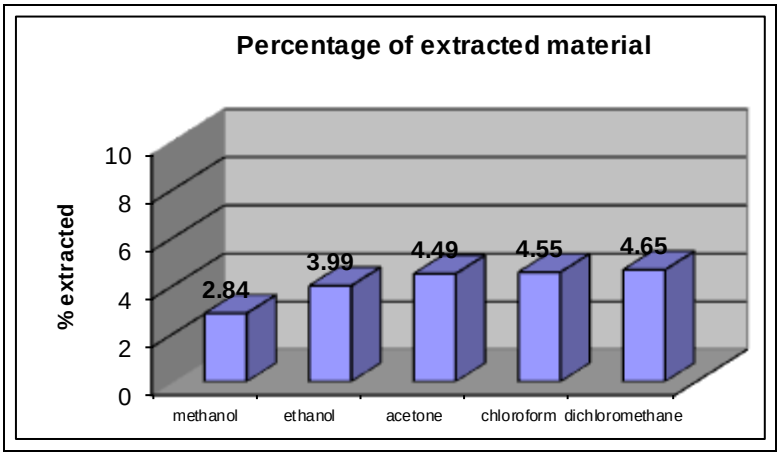


Figure 3.40: Percentage of material extracted from the horse chestnut residue by each solvent.

3.10.2 Preliminary screening of the raw horse chestnut organic extracts by thin layer chromatography

The solvent system was optimized to separate components of each extract of horse chestnut residue. Four microlitres of samples: EtHCE, MetHCE, AcHCE, DiHCE and ChHCE were spotted on TLC plate. The mobile phases used for TLC chromatography were chloroform:methanol (CM) 90:10, ethyl acetate:methanol:water (EMW) 88:11:8 and ethyl acetate:hexane (EH) 1:1. Developed chromatographs were stained with anisaldehyde/sulphuric acid spray reagent. Separation was more effective in ethyl acetate:methanol:water (EMW) solvent system because more active UV and anisaldehyde spots were obtained. The chemical profile of the chromatograms obtained using EMW solvent system is presented in Figure 3.41. All UV active spots were also indicated.

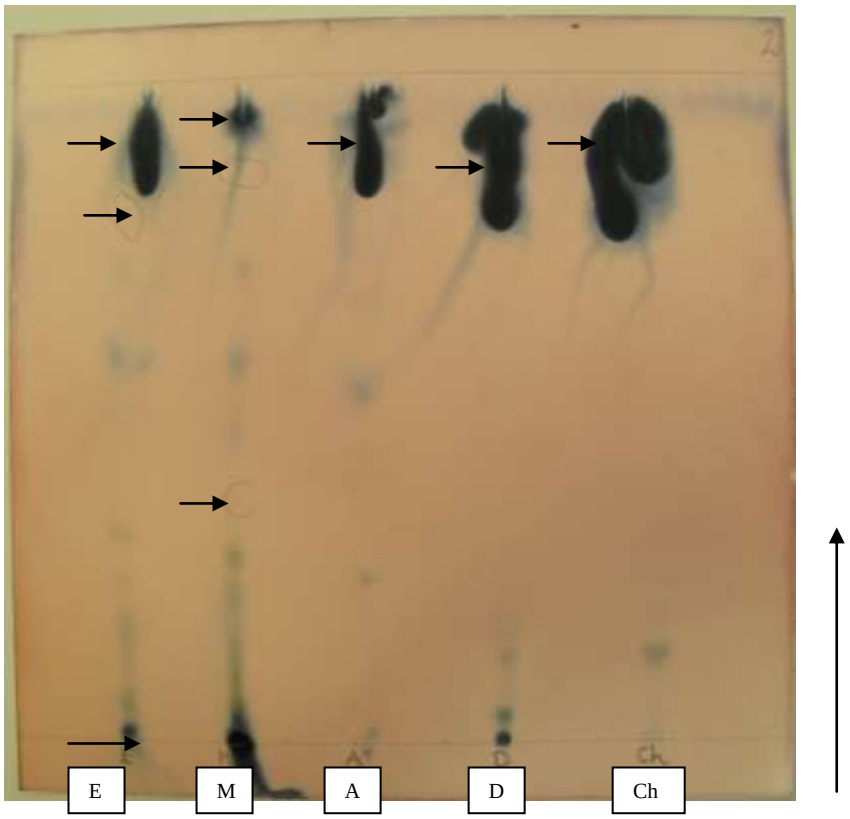


Figure 3.41: TLC chromatogram 20x20 (silica gel F_{545}) of extracts from horse chestnut residue developed in ethyl acetate:methanol:water (88:11:8).
Developed chromatogram was stained with anisaldehyde/sulphuric acid spray reagent. E- EtHCE, M- MetHCE, A- AcHCE, D-DiHCE and Ch- ChHCE. Arrows show UV active compounds.

The TLC chemical profile of both methanolic and ethanolic extracts indicated that these solvents extracted the highest number of visible compounds (7 and 8 respectively). In contrast TLC of chloroform extract showed only 3 distinct spots. Retention factors (R_f) of all separated compounds obtained from the organic extracts of horse chestnut residue are presented in Table 3.23.

Ethanolic and methanolic extracts of the residue had the highest number of UV active compounds (2 and 4 respectively) whereas acetone, dichloromethane and chloroform extracts had only one large active UV spot each with R_f ranging 0.86-0.97, 0.75-0.94 and 0.74-0.95 respectively.

Table 3.23: R_f of separated spots obtained after TLC of various horse chestnut organic extracts.

Number of spots	R_f				
	EtHCE	MetHCE	AcHCE	DiHCE	ChHCE
1	0.00	0.00	0.00	0.00	0.00
2	0.06	0.06	0.25	0.04	0.14
3	0.18	0.18	0.52	0.14	0.74-0.95
4	0.23	0.28	0.86-0.97	0.75-0.94	-
5	0.31	0.56	-	-	-
6	0.56	0.71	-	-	-
7	0.71	0.90-0.96	-	-	-
8	0.82-0.95	-	-	-	-

3.10.3 Determination of antimicrobial activity of the raw horse chestnut organic extracts

3.10.3.1 Disc diffusion assay

The inhibition zones formed by standard antibiotic discs for Gram-positive bacteria (*S. aureus* ATCC 9144, *S. epidermidis* ATCC 12228) and Gram-negative bacteria (*E. coli* ATCC 25922, *E. aerogenes* ATCC 13048) are presented in Figure 3.42. Positive controls of all antibiotic discs clearly showed that bacteria were sensitive to some standard antibiotics.

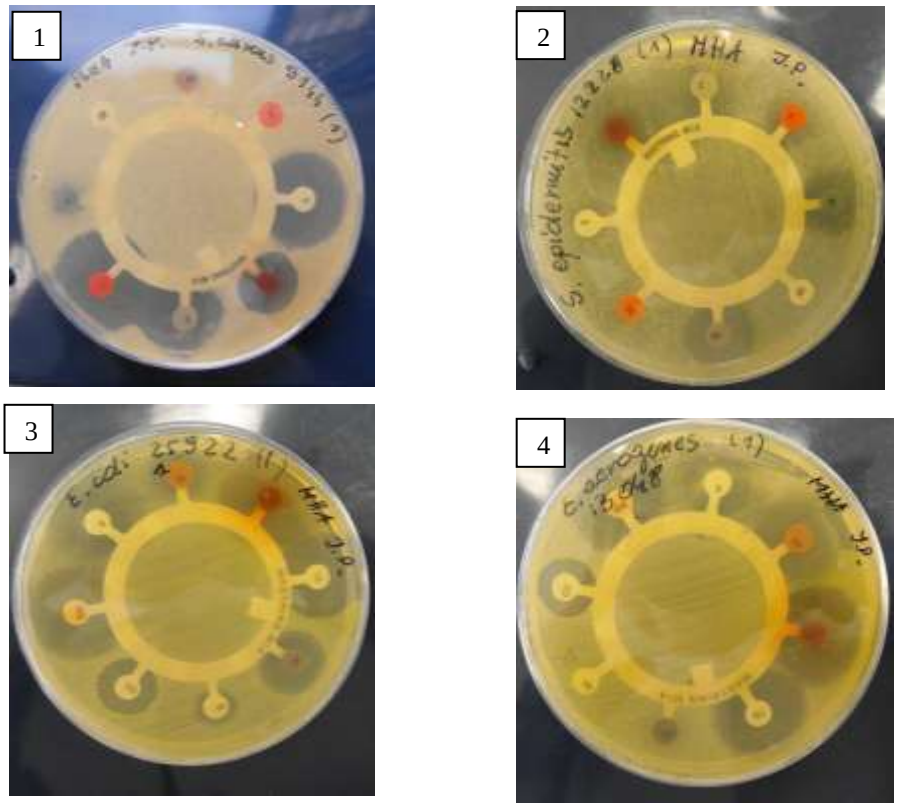


Figure 3.42: Effect of inhibition of gram positive and gram negative bacteria by antibiotics impregnated on Mastring S (M11) for 1- *S. aureus* ATCC 9144, 2-*S. epidermidis* ATCC 12228 and Mastring S (M14) for 3-*E .coli* ATCC 25922, 3-*E. aerogenes* ATCC 13048.

Inhibition zone sizes were determined for all bacteria grown in Muller-Hinton agar in the presence of standard antibiotic discs (Mastring-S) and the results are presented in Table 3.24 for Gram-positive bacteria and in Table 3.25 for Gram-negative bacteria. The weights and units of antibiotic impregnated into Mastring-S discs are not compatible with the recommended values for the Bauer-Kirby test. Further there are no standards available from the manufacturing company or in the literature (MAST, United Kingdom, 2011, personal communication). The lack of interpretative standards for bacteria inhibited by antibiotics impregnated on Mastring-S did not allow determination of the susceptibility of bacteria (such as resistant, intermediate, susceptible).

Table 3.24: Zones of inhibition (mm)[a] obtained for gram positive bacteria by agar diffusion assay using standard antibiotic discs (Mastring-S).

Antybiotic discs	Bacteria	
Mastring S, M13	*Staphylococcus aureus* ATCC 9144	*Staphylococcus epidermidis* ATCC 12228
T	15	10
C	20	10
E	26	10
FC	0	12
MT	0	0
NO	0	16
PG	0	0
S	24	0

[a] Means determined for Mastring discs by calculating the average of two replicates (n=2).
T- Tetracycline 5µg , C- Chloramphenicol 25µg,
E- Erythromycin 5µg, FC- Fusidic acid 10µg,
MT-methicillin 10µg, NO- Novobiocin 5µg,
PG-Penicillin G 1 unit, S- Streptomycin 10µg.

Table 3.25: Zones of inhibition (mm)[a] obtained for gram negative bacteria by agar diffusion assay using standard antibiotic discs (Mastring-S).

Antibiotic discs	Bacteria	
Mastring S, M14	*Escherichia coli* ATCC 25922	*Enterobacter aerogenes* ATCC 13048
AP	14	0
KF	8	0
CO	14	13
GM	23	19
S	17	15
ST	0	0
T	25	22
TS	21	19

[a] Means determined for Mastring discs by calculating the average of two replicates (n=2).
AP- Ampicillin 10µg, KF- Cephalothin 5µg,
CO- Colistin Sulphate 25µg, GM- Gentamicin 10µg,
S- Streptomycin 10µg, ST- Sulphatriad 200µg,
T- Tetracycline 25µg, TS- Cotrimoxazole 25µg.

The dry extracts from an original 30g of horse chestnut residue (Table 3.22) were dissolved in 2ml solvents giving the following concentrations: MetHCE-425.55mg/ml,

EtHCE-598.15mg/ml, AcHCE-673.15mg/ml, ChHCE-682.5mg/ml and 697.mg/ml. The total volumes of extracts added to discs were 40µl or 60µl. Negative controls were also tested applying pure solvents on disc in the same volume as extracts tested. The controls did not show any inhibitory effect on bacteria due to their quick evaporation from the discs. The results obtained from agar diffusion assay for all horse chestnut extracts are presented in Figure 3.43. This method did not show significant inhibition by the extracts of any bacterial strain tested. Methanolic extract (MetHCE) seemed to have some inhibitory effect on all Gram-negative and Gram-positive bacteria; moreover some stimulatory effect of MetHCE was also observed. Yellow coloured zones appeared around perimeter of the zone of clearing of the discs containing MetHCE and a higher density of bacterial cells was observed.

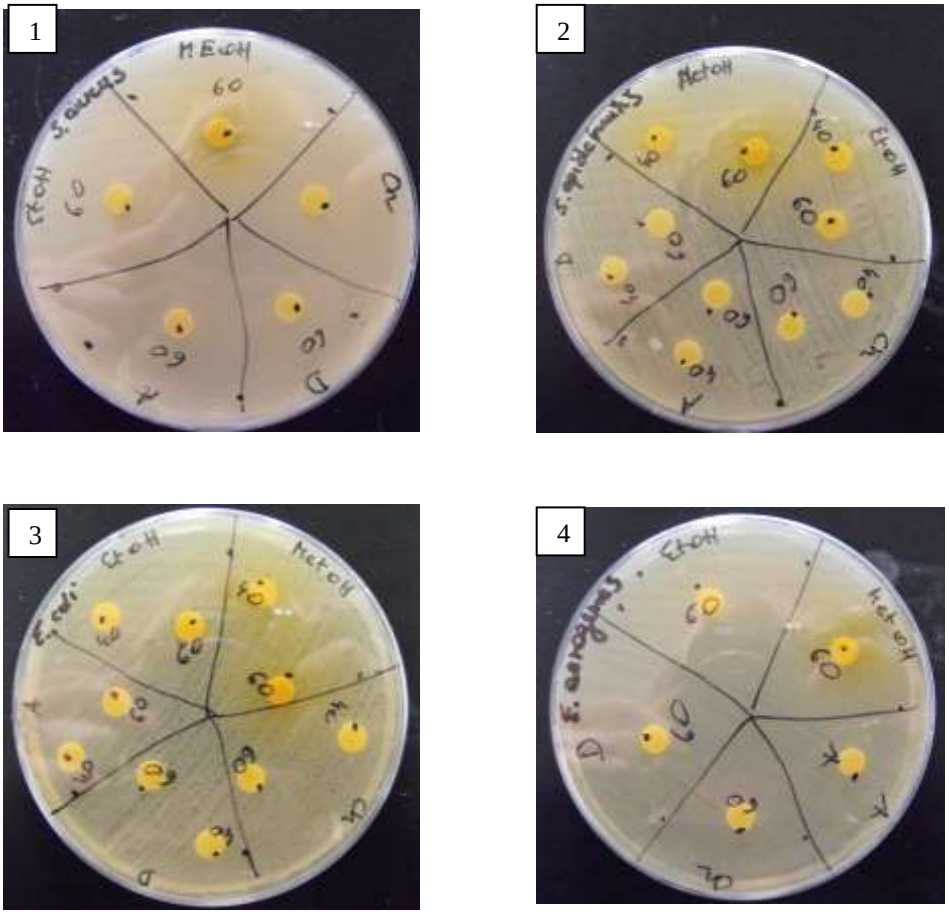


Figure 3.43: Effect of inhibition of Gram-positive and Gram-negative bacteria by horse chestnut organic extracts.
Extracts (MetOH-MetHCE, EtOH-EtHCE, A-AcHCE, D-DiHCE, Ch-ChHCE) impregnated on discs with volume 40 and/or 60µl. 1- *S. aureus* ATCC 9144, 2-*S. epidermidis* ATCC 12228, 3-*E. coli* ATCC 25922, 3-*E. aerogenes* ATCC 13048.

3.10.3.2 Bioautography assay

Results obtained for bioautography assay demonstrated that separated components of all crude extracts derived from horse chestnut residue possessed antimicrobial effect against *S. aureus* ATCC 9144, *E. coli* ATCC 25922 and *E. aerogenes* ATCC 13048. The Figure 3.44 shows the inhibition zones obtained on TLC plates. These zones of inhibition corresponded to the retention factor (R_f) of separated compounds are also summarized in Table 3.26.

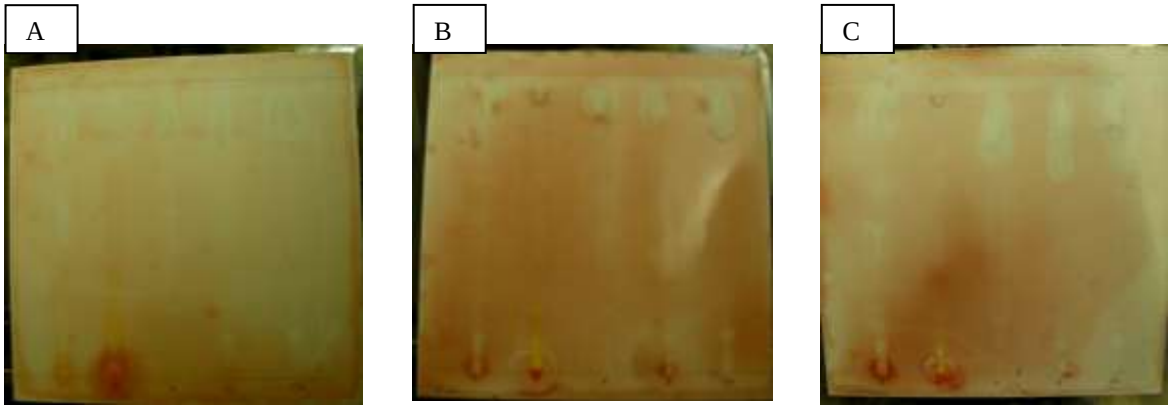


Figure 3.44: Bioautography of horse chestnut organic extracts.
In order: EtHCE, MetHCE, AcHCE, DiHCE, ChHCE, A- *S. aureus* ATCC 9144, B-*E. coli* ATCC 25922, C-*E. aerogenes* ATCC 13048.

Table 3.26: Inhibition zones obtained after bioautography of horse chestnut organic extracts.

Bacteria	Inhibition zones (R_f) for the organic extracts from horse chestnut residue				
	EtHCE	MetHCE	AcHCE	DiHCE	ChHCE
S. aureus ATCC 9144	0.18 0.23 0.56 0.82-0.95	0.9-0.96	0.13 0.25 0.52 0.86-0.97	0.04 0.14 0.75-0.94	0.14 0.74-0.95
E. coli ATCC 25922	0.18 0.82-0.95	0.9-0.96	0.13 0.86-0.97	0 0.14 0.75-0.94	0 0.14 0.74-0.95
E. aerogenes ATCC 13048	0.18 0.82-0.95	0 0.9-0.96	0.13 0.86-0.97	0 0.14 0.75-0.94	0 0.14 0.74-0.95

The most active compounds from all extracts were located at the top of chromatograms and were shown as large inhibition zones. In case of AcHCE, DiHCE and ChHCE the separated compounds to R_f=0.14 had antimicrobial activity against all strains tested.

The inhibitory effect of specified constituents from the extracts on Gram-negative bacteria (*E. coli*, *E. aerogenes*) was the same; whereas Gram-positive *S. aureus* was inhibited by many more of the separated components of the horse chestnut extracts e.g. EtHCE and AcHCE indicated four different inhibition zones.

3.10.3.3 Checkerboard assay

Minimum inhibitory concentrations (MIC) of the organic extracts (MetHCE and EtHCE) were examined by the checkerboard assay method. The organism tested was *Escherichia coli* ATCC 25922. The MetHCE extract was dissolved in methanol (two fold-dilutions) to the following concentration 426, 213, 106.5, 53.25, 26.625 and 13.313mg of raw material per millilitre, EtHCE was tested with concentration of 598, 299, 149.5, 74.75, 37.375, 18.688, 9.344 and 4.672 mg of raw material per millilitre. As positive control ampicillin was used in concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml. As negative control pure solvents were also tested and their concentration started from 100% and then two-fold dilution in sterile saline was performed. The inhibitory effect was monitored by fluorescence measurement. Figure 3.45 presents inhibitory effect of ampicillin on *E. coli* ATCC 25922. The MIC of ampicillin was determined to be 25µg/ml.

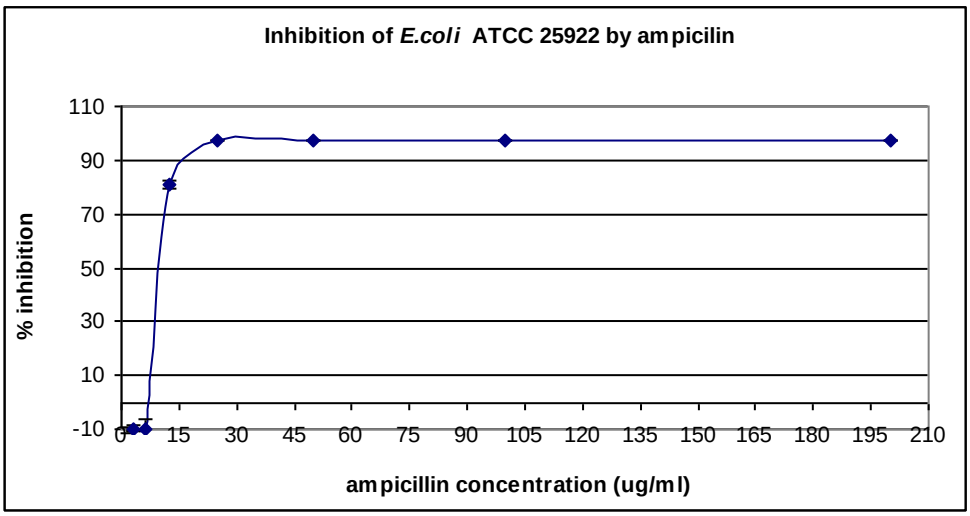


Figure 3.45: Percent of inhibition of *Escherichia coli* ATCC 25922 by ampicillin.
Values for each point of measure reported as mean value with the upper and lower values.

Figure 3.46 shows inhibitory effect of EtHCE as well as pure ethanol (EtOH) on *E. coli* ATCC 25922.

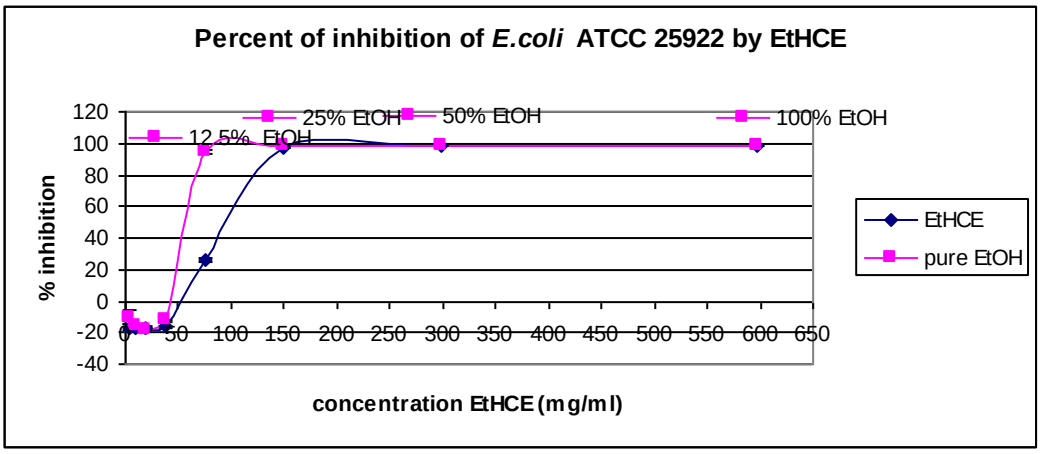


Figure 3.46: Percent of inhibition of *Escherichia coli* ATCC 25922 by ethanolic extract from horse chestnut residue (EtHCE) compared to pure solvent (EtOH).
Values for each point of measure reported as mean value with the upper and lower values.

In Figure 3.46 each point of EtHCE concentration is related to the appropriate negative control (pure ethanol) dilution. As seen on the plot pure solvents had an inhibitory effect on *E. coli* and it was not possible to determine whether the extract (EtHCE) itself is responsible for any bacterial inhibition. The MIC was not established.

Figure 3.47 presents percent of inhibition of MetHCE on *E. coli* ATCC 25922. Similar to previous extract, each point of MetHCE concentration is related to the appropriate negative control (pure methanol) dilution.

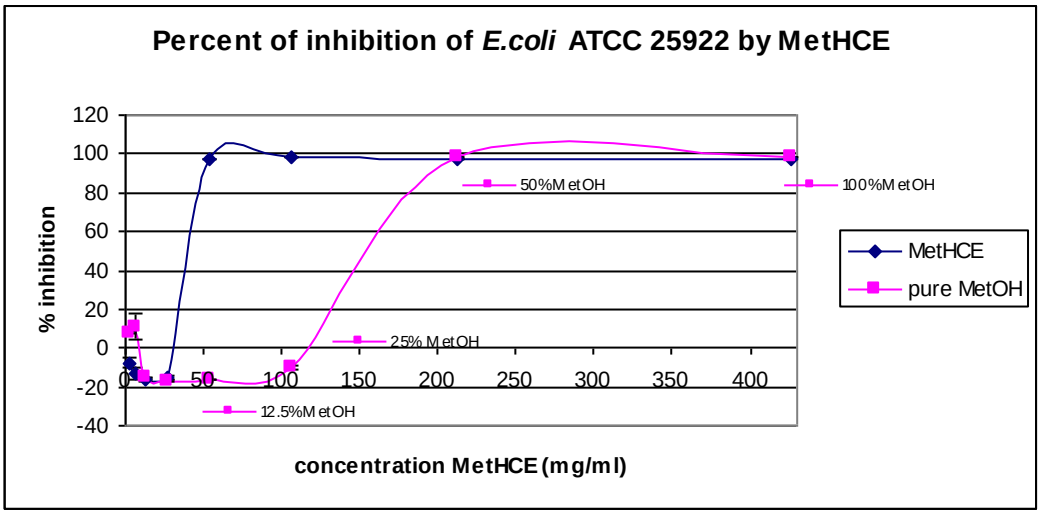


Figure 3.47: Percent of inhibition of *Escherichia coli* ATCC 25922 by methanolic extract from horse chestnut residue (MetHCE) compared to pure solvent (MetOH).
Values for each point of measure reported as mean value with the upper and lower values.

Pure methanol had also inhibitory effects on bacterial growth: 100% inhibition was observed with 100% and 50% pure methanol. It has to be emphasized that the same concentration of methanol solvent was present in the methanolic extract (MetHCE) for each specific point of measurement. The plot clearly shows 100% inhibition by MetHCE at concentration of pure methanol which had <0% inhibition (or a stimulatory effect) on the *E. coli*. The plot also clearly shows 100% inhibition at concentration of 426, 213, 106.5 and 53mg/ml of MetHCE. At concentration of 106.5 and 53 mg/ml 100% inhibition was demonstrated despide the fact that at the equivalent concentration of pure methanol (12.5%) a stimulatory effect was observed. MIC of MetHCE was determined as 53mg/ml.

4 Discussion

4.1 Horse chestnut residue analysis and potential prebiotic activity

4.1.1 Compositional analysis of the horse chestnut residue

Analysis of the composition of horse chestnut residue has shown a mean of 48.15% moisture (section 3.1.1) in fresh material and 51.85% total solids (w/w). These values are similar to the results (49.2% dry matter (Table 1.3)) reported by Baraldi and colleagues (2005) for fresh seeds from *Aesculus hippocastanum*. There is therefore, only a small difference (3.7%) in the total solids component of fresh seeds and the residue despite ethanolic extraction. The ash content of the residue (2.97% w/w; section 3.1.2) was 18% higher than that of fresh seeds (2.51%). Potassium was the major mineral of ash from horse chestnut waste and its quantity was determined as 4.6mg/g fresh residue. Phosphorus, calcium and magnesium are also present in the ash but at low concentrations of less than 1mg/g of wet weight of horse chestnut residue (Table 3.1).

A proportion of the ash components remains unaccounted for in this study. Analysis carried out by Peňa-Méndez *et al*. (Peña-Méndez *et al.*, 2008) on chestnut showed similar results with potassium present in greatest concentration followed by phosphorus and magnesium. In their analysis the full ash composition was not determined.

Analysis of the horse chestnut residue showed much a higher protein proportion (6.93%; section 3.1.4) than that found in fresh seeds (2.64%) by Baraldi *et al.* A lower lipid content was determined in the residue (1.89%; section 3.1.5.1) then in fresh seeds (4.13%) These two main differences appear to be due to processing and ethanolic extraction which was carried out in the company where the horse chestnut residue was produced from fresh seeds. Certainly the lower level of lipid can readily be attributed to the repeated ethanolic extraction of the seeds.

Determination of fatty acid content led to identification of 12 fatty acids in horse chestnut residue (Table 3.3). The fatty acid composition of lipid fraction of horse chestnut residue seemed similar to that of fresh seeds. The fatty acids: myristic, pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic, linoleic and arachidic

were found in the residue. These have also been identified in fresh seeds, although their proportions differ. Oleic acid is the main fatty acid present in the residue (74.4%) and fresh seeds (43.2%) and may play an important role in energy storage as well as protection of horse chestnut seeds against freezing environmental conditions. It has been established that a high percentage of oleic acid may also occur in those seeds which are the storage organs for developing embryos (Simin *et al.* 1996). Palmitic acid is present in horse chestnut residue at a concentration of 5.78%, whereas fresh seeds contain 7.1% of this fatty acid. High content of linoleic acid is present in fresh seeds (35.2%). Linoleic acid was not identified in the lipid fraction from horse chestnut residue. It remains unclear why this is the case, however the variations seen in organic acid content between seeds and residue may be attributed to different solubilities of the fatty acids in absolute alcohol.

4.1.2 Prebiotic potential of the horse chestnut residue

Research was also carried out to establish the prebiotic activity of the horse chestnut residue. This research was initiated on the basis of the presence of complex carbohydrates and anecdotal evidence of digestive improvement in farmed animals following consumption of chestnuts. Prebiotics are defined as food ingredients that support the growth of probiotic bacteria and are utilized by these probiotic organisms in a process called fermentation (Cho and Finocchiaro, 2010). It is an important feature of prebiotics that they do not similarly stimulate the growth of competing enteric flora.

4.1.2.1 Preliminary tests with minimal medium: Information on growth

The preliminary test for prebiotic activity of aqueous extracts of horse chestnut residue was carried out using minimal medium for cultivation of probiotic strains. A simple extraction process was carried out initially by stirring 1% (w/v) horse chestnut residue in minimal medium at ambient temperature for 1 hour. Subsequent trials were also carried out under different conditions varying temperature and extraction time. The

prebiotic potential of 1% (w/v) FOS was also tested and compared to the growth supporting effect of HCE.

The experiment (section 3.2.1) confirmed a potential prebiotic activity in aqueous extracts from horse chestnut residue *in vitro*. The *Lactobacilllus* and *Bifidobacterium* strains were able to metabolise the extract; however the capacity to digest it depended on the individual strains. *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus acidophilus* ATCC 4356 exhibited the best growth in minimal medium containing horse chestnut extract (1% HCE). Moreover their growth was significantly higher ($p \leq 0.05$) than in medium with commercial prebiotic 1% FOS, e.g. after 24 hours of growth, the OD of *L. plantarum* grown on HCE (0.376) was 337% greater than the OD of the same bacteria when grown on FOS (OD of 0.086; Figure 3.12) when corrected for growth on unsupplemented medium and similarly the OD of *L. acidophilus* when grown on HCE (0.205) was 253% greater than the OD of the same probiotic strain when grown on FOS (OD of 0.05; Figure 3.16). For other strains, differences in growth were observed but these were not statistically significant at $p \leq 0.05$, e.g. *Bifidobacterium angulatum* ATCC 27535 on HCE had 117% greater OD than on FOS after 24 hours (Figure 3.20).

4.1.2.2 Minimal medium with prebiotic supplementation: Information on metabolism

Probiotic strains used in this research were evidently able to utilize the horse chestnut extract such that fermentation products including short chain fatty acids (SCFA) and lactic acid were produced. The presence of these acids was generally manifested by decreasing pH in the medium. In most cases acidification was greater when bacteria were grown in medium with 1% FOS. The pH typically decreased to a constant level, which, in the case of *Lactobacillus* strains grown on HCE (mean pH 4.92) was less acidic than the same organism grown on FOS (mean pH 4.62).The average final pH of the *Bifidobacterium* strains (*B. infantis, B. angulatum*) was 4.77 in the case of both HCE and FOS.

The effect of lowering pH during fermentation often correlates with a slow down in bacterial growth. This can be seen when growth curves are compared with pH profiles, e.g. growth of *Lactobacillus delbrueckii subsp.lactis* (Figure 3.4) with pH (Figure 3.5).

The pH dropped to near pH 5.0 after 4 hours and the growth is seen to plateau subsequently, e.g. OD of 0.325±0.002 for cells growing on FOS and OD of 0.300±0.005 for growth on HCE at 6 and 8 hours of incubation (Figure 3.5).

It is important to note that the solid horse chestnut residue added to the minimal medium did not dissolve completely. The residue was added at a concentration of 1% (w/v) but the solubilised material was determined to be 0.16% (see section 3.2.2). The insoluble matter was collected by filtration and discarded, therefore prebiotic power of the extract (known as 1% (w/v) HCE) corresponds to 0.16% solubilized matter, whereas the concentration of commercial FOS used in all experiment was 1% (w/v) all of which solubilised.

The preliminary study also showed that HCE retained the prebiotic-like properties after heat treatments (90ºC and autoclaving). In a study using *Lactobacillus delbrueckii subsp. lactis* maximal OD readings obtained after 8 hours of growth were 0.329±0.004 for HCE extracted at 20ºC, 0.332±0.000 at 90ºC and 0.344±0.003 autoclaved at 121ºC (no significant difference in readings; Figure 3.6). This clearly shows that the active prebiotic components remained stable when subjected to both heat and pressure. Heating and autoclaving did appear to influence solubility, however. As previously mentioned the actual solubilised proportion yielded by the addition of 1% (w/w) of HCE in all 20ºC extractions was equivalent to 0.16%, dry matter. This concentration changed when extraction took place at higher temperatures increasing to 0.17% soluble material with HCE heated to 90ºC and to 0.3% when autoclaved material was used (section 3.2.2).

A trial was carried out to examine the effect of prolonged extraction at 20ºC. Two extracts were prepared, the first being 1% (w/v) HCE stirred for 30 minutes while the second was 1% (w/v) HCE stirred for 1 hour. The results (Figure 3.8) showed that there was no significant gain achieved by the prolonged extraction.

In order to more directly compare the effect of HCE as a prebiotic with FOS an experiment was carried out using 0.3% FOS, 1% FOS and 1% (actual, 0.16%) HCE with *Lactobacillus delbrueckii subsp. lactis* (Figure 3.6). The growth of *Lactobacillus delbrueckii subsp. lactis* was significantly less on 0.3% FOS than on the horse chestnut extract. After 8 hours of incubation the strain obtained OD 0.226±0.000 and 0.329±0.004 if grown on 0.3% FOS and 1% HCE, respectively. These values represent 45% better growth of *Lactobacillus delbrueckii subsp. lactis* on 1% HCE than on 0.3% FOS. Furthermore there was a significant difference ($p \leq 0.05$) in the growth on 1% FOS

when compared with 0.3% FOS with the final readings 0.498±0.037 and 0.226±0.000 respectively.

4.1.3 Preparation and analysis of lyophilised horse chestnut aqueous extract

In order to provide consistent material for analysis, it was decided to use lyophilisation as method of preparing a powdered form of the solubilised extract. Chemical analysis revealed that the main component (81%) of the powder is carbohydrate and that reducing sugars constitute 20% of the total (section 3.3.5). While there is reasonable protein content (2.82%; section 3.3.4), it is hypothesized that the growth stimulation and prebiotic effect of the water soluble fraction of the horse chestnut residue are due to the carbohydrate content. The presence of the minerals potassium, phosphorus and magnesium in lyophilized horse chestnut extract (lHCE; Table 3.4) may have enhancing effects, promoting the growth of probiotic strains.

4.1.4 Calculation of a prebiotic efficacy score

It is important to be able to quantify and validate the prebiotic effect demonstrated by the HCE. Determination of a quantitative score for the prebiotic activity of the aqueous HCE would allow direct comparison with known prebiotics. The procedure carried out followed a method established by Huebner *et al*. (2007). The medium used for cultivation of probiotic bacteria was the deMan, Rogosa and Sharpe medium (MRS). The experiment included a selection of species of intestinal bacteria (*Lactobacillus* and *Bifidobacterium* species) cultured *in vitro* with HCE and other existing commercial prebiotic products; GOS, FOS and inulin.

4.1.4.1 Changes in bacterial biomass of probiotics and enteric strains cultured on 1% (w/v) prebiotics (GOS, FOS, inulin) and 1% (w/v) HCE

The growth of bacteria (*Lactobacillus, Bifidobacterium and Escherichia coli*) was measured after 24 hours of incubation with 1% (w/v) of glucose, GOS, FOS, inulin and

1% (w/v; 0.16% soluble solids) horse chestnut extract (HCE). Optical densities at 600nm obtained after growth at 24h were subtracted from the values from time 0h to obtain the actual increase in cell densities.

In order to compare the efficacy of the commercial prebiotics with that of the HCE, a large number of trials were performed (with each sugar and HCE tested in triplicate for every strain). Ten different strains of bacteria were grown on each of three commercially available prebiotics (GOS, FOS and inulin) as well as HCE and glucose.

As expected, the growth of probiotic and enteric strains on glucose (positive control) was significantly higher ($p \leq 0.05$) than on the commercial prebiotics and HCE (Table 3.8) with some exceptions. In the case of *Bifidobacterium angulatum* DSM 20098 while the growth was higher at 1.75±0.16 on glucose than at 1.51±0.01 on inulin, the difference was not significant ($p=0.283$; Table 3.9). *Bifidobacterium breve* DSM 20091 exhibited a higher growth (0.34±0.02) on GOS and (0.39±0.01) on FOS compared with glucose (0.30±0.01). In addition growth of *Bifidobacterium breve* on HCE (0.29±0.02) was very similar to that on glucose. While growth of *Escherichia coli* ATCC 35320 was higher on glucose (0.96±0.05) than on GOS (0.81±0.06), it was not significant at p ($p=0.066$; Table 3.9).

Growth promoting effects of commercial prebiotic products such as GOS on probiotic strains were observed. GOS demonstrated the best stimulatory effect on all bacteria studied (including the enteric strain). The following probiotics: *L. plantarum*, *L. acidophilus*, *L. fermentum*, *L. rhamnosus*, *L. casei*, *B. longum* and *B. infantis* indicated significantly better ($p \leq 0.05$) growth on GOS enriched MRS medium than on the MRS supplemented with other prebiotics (FOS, inulin) and HCE. Controversially, *E. coli* also grew significantly ($p \leq 0.05$) better in GOS-enriched M9 medium than in M9 medium supplemented with FOS, inulin or HCE. The growth supporting effect of GOS on both probiotic and enteric strains may be due to the high percentage of glucose in GOS product. Typical analysis of VIVINAL GOS showed that glucose comprises 20.6% of dry matter (see Appendix D- Certificate of Analysis of VIVINAL® GOS).

In contrast, inulin indicated the weakest stimulatory effect on almost all probiotic strains with the exception of *B. angulatum* DSM 20098. Inulin did not, however, enhance the growth of the enteric strain, which is of significance.

Table 3.10 displays the statistical values for differences in growth of probiotics on HCE vs. GOS, FOS and inulin. It can be seen that the OD values obtained from growth on

FOS were significantly higher ($p \leq 0.05$) than the values obtained for growth on HCE for all strains with the exception of *B. infantis* ($p=0.134$) and *B. longum* ($p=0.069$).

The appearance of probiotic growth stimulation was also monitored in comparison to the growth of the culture in pure MRS medium. This measurement was treated as blank control (as a measure of growth in unsupplemented medium).

After 24 hours of incubation, for all of the test strains (five *Lactobacillus* and four *Bifidobacterium* species), the growth on the MRS with 1% HCE was better than on unsupplemented MRS. Additionally, six probiotic strains exhibited significantly better ($p \leq 0.05$) growth on 1% HCE than on unsupplemented MRS medium as evidenced by measurement of optical density (at 600nm). Growth of *E. coli* on M9 with 1% HCE was also significantly higher ($p \leq 0.05$) than growth on unsupplemented M9. This growth-supporting effect of the horse chestnut aqueous extract was also compared to commercial products such as GOS, FOS and inulin. In six out of nine cases the growth of probiotics in medium supplemented with 1% HCE was significantly better ($p \leq 0.05$) than with 1% inulin (Table 3.10). These were *L. plantarum* ATCC 8014, *L. rhamnosus* ATCC 7469, *L. acidophilus* ATCC 4356, *L. fermentum* ATCC 9338, *B. longum* DSM 20219 and *B. breve* DSM 20091. An important characteristic of a prebiotic is that is should inhibit or significantly reduce the growth of enteric strain *E. coli*, while stimulating growth of probiotic strains. Table 3.8 clearly shows that the growth of *E. coli* is significantly reduced on 1% HCE (OD 0.21±0.02) when compared with growth on glucose (OD 0.96±0.05).

It has to be underlined that the preparation of the extract in MRS medium was similar to that described in previous experiment (see section 2.2.2) and the value of 1% (w/v) for the extract concentration refers to the amount of horse chestnut residue added directly into MRS medium. However this was equivalent to 0.16% soluble matter content for HCE (section 3.2.2).

The term "prebiotic" according to Gibson and Roberfroid refers to "food ingredients that beneficially effect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health" (Gibson and Roberfroid, 1995). The methods for determination of prebiotic efficacy based on Huebner's work showed that the horse chestnut aqueous extract exhibits potential prebiotic activity. The extract represents a

potential probiotic-growth promoting supplement because of its supporting effect on large spectrum of intestinal bacteria.

4.1.4.2 Difference in growth in MRS and minimal medium

It was noticed that the growth of probiotic strains in MRS medium and minimal medium (MM) differed substantially. The growth of probiotic in MRS medium enriched with HCE demonstrated different increases in cell density when compared to minimal medium supplemented with the same amount of HCE. For example, after 24 hours, growth of *Lactobacillus plantarum* ATCC 8014 in MM supplemented with 1% FOS and 1% HCE (as OD at 600nm) was 0.094±0.004 and 0.386±0.004 respectively (Figure 3.12), while growth on unsupplemented MM was only OD 0.012±0.000. The same strain grown in MRS medium enriched with 1% FOS and 1% HCE showed growth (as OD at 600nm) of 1.001±0.018 and 0.831±0.028, respectively (Table 3.8). However growth on unsupplemented MRS at 24 hours had OD of 0.637±0.025. This means that the increased OD due to supplementation with 1% FOS was 0.364 and with 1% HCE was 0.194. Clearly MRS is a rich medium which supports the growth of *L. plantarum* and there is limited additional growth arising from supplementation by either FOS or HCE. FOS shows significantly improved growth of *L. plantarum* over HCE on the already rich medium. However in MM the picture is quite different with an OD increase of 0.082 for growth on 1% FOS compared with an OD increase of 0.374 for growth on 1% HCE.

The growth promoting effect of FOS-enriched MRS medium on probiotic species was generally better (Table 3.8) when compared to HCE The p values (as significant difference) obtained for probiotics grown on HCE compared to FOS presented Table 3.10. Six out of nine probiotic species exhibited significantly ($p \leq 0.05$) better growth on FOS than on HCE in MRS medium.

MRS medium is a very rich mixture of different components such as mineral salts, organic additives (meat and yeast extract, peptone, etc.) and it is specially designated for cultivation of lactobacilli and other fastidious strains. In contrast, minimal medium consists of basic nutrients such as minerals and only a small amount of organic additives (beef extract) are present.

The growth promoting effect of HCE-enriched minimal medium was greater in comparison to FOS-enriched minimal medium. This was in spite of the difference in

soluble material (0.16% soluble material in HCE versus 1% soluble material from FOS). Dissolved in minimal medium, HCE enriched the medium with organic compounds (proteins, reducing sugars, polyphenols and minerals) that are characteristic of most raw extracts obtained from plant residue. Therefore the fact that the growth of probiotic strains on minimal medium supplemented with FOS was less than on the minimal medium enriched with HCE may be due to one or more of these additional components. The nature of the carbohydrate may also contribute to this phenomenon. The protein present in the HCE is relatively low (2.82 %; section 3.3.4), therefore reducing sugars (20%; section 3.3.5) may provide some acceleration of growth. Polyphenols (2.06%; section 3.3.6) may also influence growth. Haddadin (Haddadin, 2010) demonstrated that phenolics derived from plant extracts have enhancing effects on probiotic bacteria *L. acidophilus and B. infantis*.

The FOS used in this research is a pure carbohydrate product, free from any protein or other organic residue. The reducing sugar content in FOS product according to the Certificate of Analysis (by Sigma-Aldrich) is 3.3% fructose and 4.9% disaccharides. These values are very similar to those estimated in the research (total percentage reducing sugar content of FOS was 8.86% in wet sample; section 3.3.5). Additionally, it has to be emphasized that less HCE actually dissolved in the medium (0.16% soluble matter; section 3.2.2) while the FOS material (1%) was completely soluble.

4.1.4.3 Determination of prebiotic index scores (PI)

Determination of prebiotic index (PI), known also as prebiotic activity score, based on the method of Huebner (Huebner *et al.*, 2007) was also performed. It must be emphasised here that culture of all probiotic bacteria was carried out in MRS medium to determine the quantitative scores of prebiotic activity of commercial FOS, GOS and inulin as well as HCE in relation to growth of lactobacilli and bifidobacteria as specified in the original method established by Huebner *et al*. (2007). The original formula (Huebner *et al.*, 2008) incorporates the probiotic growth on prebiotics (over 24 hours) relative to their growth on non-prebiotic substrate (glucose) and additionally, considers the growth of enteric strains in the presence of prebiotics (over 24 hours) and relative to growth on glucose. The original formula gives rise to a ratio of growth of probiotic (first part of formula) to enteric organisms (second part of formula). Positive PI scores are characteristic of those probiotic strains whose growth is better supported by prebiotics

than by the same concentration of glucose and/or where the prebiotic is less supportive of the growth of enteric strains. PI scores, using the original formula, may also be negative in some cases. This is attributed to circumstances where growth of probiotics is poorly supported by the candidate prebiotic, in comparison to glucose, or where growth of an enteric strain such as *E. coli* is supported equally or to a greater extent when the medium is enriched with the prebiotic. It is possible for a candidate prebiotic to enhance the growth of enteric strains (hence the second part of formula gives higher product than the first part). Where the probiotic organism grew much better on glucose than on prebiotic, the first part of the formula gives a low number.

$$\text{Prebiotic activity score} = \left[\frac{(\text{probiotic log O.D. on the prebiotic at 24h} - \text{probiotic log O.D. on the prebiotic at 0h})}{(\text{probiotic log O.D. on glucose at 24h} - \text{probiotic log O.D. on glucose at 0h})}\right] \text{ minus } \left[\frac{(\text{enteric log O.D. on the prebiotic at 24h} - \text{enteric log O.D. on the prebiotic at 0h})}{(\text{enteric log O.D. on glucose at 24h} - \text{enteric log O.D. on glucose at 0h})}\right]$$

On examining the PI score according to Huebner a number of observations were made:

- Huebner specified the log of the optical density at 600nm
- The formula did not appear to make allowance for growth of any of the microorganisms on unsupplemented MRS/M9 medium.
- Huebner's formula subtracted growth of the enteric organism from that of the prebiotic

Considering the data available from the Huebner method for prebiotic assessment and the factors involved in determining and comparing the efficacy of candidate prebiotic material, some modification of the Huebner formula were made. Modifications of the formula were made for the following reasons:

- Optical Density is in itself a log value
- Significant growth of the probiotics on MRS alone was observed. Private communication in 2010 with Hutkins (co-author Huebner *et al*., 2007) confirmed that the PI scores published had not taken this growth into account. It was felt that this is a very important factor to consider when monitoring the growth promoting effects of prebiotics, especially where two different bacterial species (probiotic and enteric are being compared)

- A better indicator of the effectiveness of prebiotics on probiotic growth relative to that on the growth of the enteric strain would be to obtain a ratio of growth. This was achieved by dividing the growth of probiotic by the growth of the enteric strain.

The principle behind the development of a PI score is to derive a quantitative indicator of the effectiveness of a potential prebiotic. To do this the value obtained must consider a number of factors:

1) Growth of probiotic strain on any potential prebiotic
2) Growth of probiotic strain on glucose
3) Growth of probiotic strain on unsupplemented medium
4) Growth of enteric strain on any potential prebiotic
5) Growth of enteric strain on glucose
6) Growth of enteric strain on unsupplemented medium

After carrying out the test in full as described in the original publication, a modified version of the formula was developed.

$$\text{Prebiotic activity score} = \left[\frac{(\text{probiotic OD at 24h on prebiotic} - \text{probiotic OD at 0h on prebiotic}) - (\text{probiotic OD at 24h on control MRS} - \text{probiotic OD at 0h on control MRS})}{(\text{probiotic OD at 24h on glucose} - \text{probiotic OD at 0h on glucose}) - (\text{probiotic OD at 24h on control MRS} - \text{probiotic OD at 0h on control MRS})} \right]$$

$$\text{divided by}$$

$$\left[\frac{(\text{enteric OD at 24h on prebiotic} - \text{enteric OD at 0h on prebiotic}) - (\text{enteric OD at 24h on control M9} - \text{enteric OD at 0h on control M9})}{(\text{enteric OD at 24h on glucose} - \text{enteric OD at 0h on glucose}) - (\text{enteric OD at 24h on control M9} - \text{enteric OD at 0h on control M9})} \right]$$

From the values (OD at 600nm) obtained, the value of bacterial growth on pure MRS was subtracted to correct for un-supplemented medium. *E. coli* growth was supported in pure M9 medium, a more specific medium; therefore the second part of the formula showing the ratio of growth of enteric strains on prebiotics was also subtracted from the growth in un-supplemented M9 medium. Another important modification pertaining to the original formula was the calculation of the ratio of growth of probiotic to non-probiotic strains. To determine this, the rate of growth of probiotic in the first part of formula was divided by the rate of growth of enteric strain.

Quantitative prebiotic index (PI) for different probiotics paired with various commercial prebiotics (GOS, FOS and inulin) or aqueous extract of horse chestnut residue (HCE)

varied according to the strains of organisms tested (Table 3.11). All probiotic strains gave rise to positive PI scores if they were paired with HCE suggesting that all lactobacilli and bifidobacteria species metabolized the extract at least as well as glucose and this utilization was selective because the HCE was not metabolized by the enteric strain. The growth of *E. coli* was less in M9 medium supplemented with HCE than when supplemented with glucose (357% better growth on 1% glucose than on 1% HCE). This was an important finding as it indicated that reducing sugar in HCE was less potent than glucose on its own, and stimulated much less growth of *E. coli* than of probiotic bacteria when all other conditions remained similar.

Among all of the probiotics grown in medium supplemented with HCE, the highest PI (4.64±0.45) was given by *B. breve* DSM 20091 and this score was higher than for cells grown in MRS medium supplemented with GOS (PI 1.37±0.23). Analyzing the growth of *B. breve* and *E. coli* in medium with GOS and HCE, it was found that GOS stimulated the growth of *E. coli* to a greater extent (by 13%) than glucose. Values for growth of *B. breve* on GOS or HCE relative to glucose were very similar and were 1.149 and 0.967 (mean), respectively. In contrast, growth of *E. coli* on GOS relative to glucose was 0.848 (mean) whereas the ratio obtained for growth of the same strain on HCE relative to glucose was only 0.209 (mean). These two values have profound significance in the determination of PI scores. In the modified formula the ratio of enteric growth in the presence of prebiotic to that with glucose is placed in the second part of the formula, where a high value yields a low PI score. This was found in GOS-enriched medium of *B. breve* (PI=1.37±0.23).

The lowest PI scores for probiotics grown on HCE were for two *Lactobacillus* strains: *L. acidophilus* ATCC 4356 and *L. casei* ATCC 393. Both strains gave rise to the same PI scores (0.13). In these cases the extract stimulated better growth of the *E. coli* strain than *Lactobacillus* strains (*L. acidophilus* and *L. casei*) relative to growth on glucose, even though the growth of *E. coli* was relatively low. The values obtained in the first part of formula were 0.030 and 0.026, respectively and gave low PI scores.

Calculation of PI scores using the original formula (Huebner *et al.*, 2008) was also carried out in order to provide comparison between the values obtained for PI scores, using the modified version and the original version. PI scores of various probiotics strains paired with commercial prebiotics (GOS, FOS and inulin) and HCE calculated according to the original formula established by Huebner *et al*. (2008) are presented in

Table 3.12. However no statistical analysis was carried out. It is seen from the Table 3.12 that the PI values differ substantially from those obtained by using the modified version (Table 3.11).

In Huebner's initial work (Huebner *et al.*, 2007) a Prebiotic Activity Scores, named also as Prebiotic Index (PI), in which log cfu ml^{-1} was used to measure growth in order to compare the effect of several prebiotics on growth of ten probiotic strains. His results presented in Figure 4.1 shows widely varying scores depending on prebiotic and strains of probiotic used. *Lactobacillus paracasei* ATCC 1195 showed the best scores when grown on inulin (1.20 for Inulin-S; 1.10 for Raftiline HP) but when grown on different forms of FOS and GOS the results were extremely variable (1.45 for NutraFlora P-95, 1.00 for Raftilose P95 and 0.15 for purified GOS) .

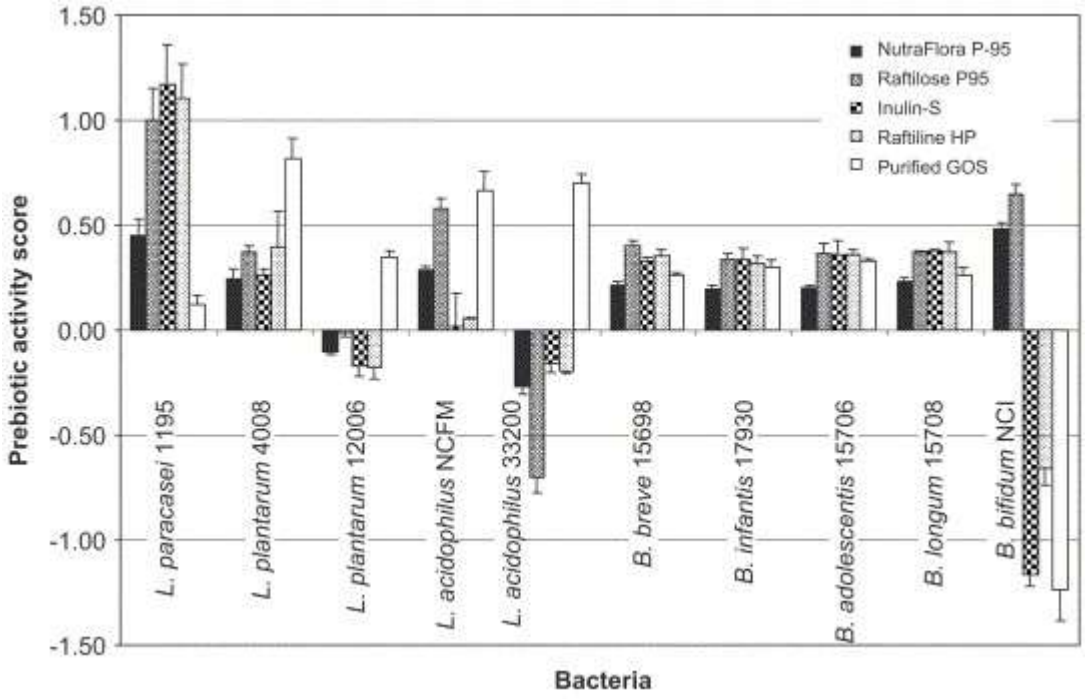


Figure 4.1: Prebiotic activity scores of various bacteria grown on commercial prebiotics.
Taken from: (Huebner *et al.*, 2007).

L. paracasei ATCC 1195 was utilised in further studies (Huebner *et al.*, 2008) where the effect of heat, low pH and Maillard reaction were observed using OD at 600nm to measure growth. Huebner argued that "measurement of OD to determine cell density is advantageous to the plate count method used previously (Huebner *et al.*, 2007) because it is a more simple and rapid technique to determine prebiotic activity". The Prebiotic Activity Score was modified to replace log cfu ml^{-1} by log OD (see section 2.4). The PI scores were dramatically lower when calculated using this formula (Huebner *et al.*,

2008), e.g. Inulin-S gave PI 0.45 compared to a value of 1.20 when log cfu ml^{-1} was used.

It is not possible to directly compare results obtained in this study with Huebner's work even where the formula for calculation of PI scores used was the same. Different prebiotic products and different probiotic strains were used in each case and results from both studies clearly showed that these factors can affect PI scores regardless of which formulae are used.

In this study, calculation of PI scores using the original formula (Huebner *et al.*, 2008) was carried out. From Table 3.12 it can be seen that all probiotic strains exhibited the highest PI score when paired with inulin moreover no strain obtained negative PI values if grown on inulin as happened when the formula modified in this work was employed (section 2.4).

It has to be noted that the growth (expressed as OD at 600nm) of all probiotics (with exception of *B. angulatum*) in medium (MRS) with inulin was almost the same as growth on unsupplemented MRS (see Table 3.8), in other words, inulin did not enhance the growth of probiotic tested. Certainly, high PI values obtained for inulin-grown strains seem not to be correlated with any real increase in cell density when compared with growth on unsupplemented medium. Other PI scores, when calculated by the Huebner formula (Huebner *et al.*, 2008) did not appear to reflect the observed increases in OD of probiotics when paired with different prebiotics or HCE. This may be explained by the fact that Huebner *et al*. did not correct for the OD obtained by growth of probiotic strains on unsupplemented MRS or for growth of *E. coli* on unsupplemented M9 (Hutkins, 2011 personal communication)

In this case, the original formula established by Huebner and co-authors may not be a reliable source of estimation of PI scores. The modified version established in this study considered all controls including growth of probiotic and enteric strains on unsupplemented medium. OD values obtained from spectrophotometer at 0 and 24 hours were directly used in the new formula. Calculation of the ratio of growth of probiotic to enteric strains was expressed in the modified formula. All these modification had significance in calculation of PI scores and has been clearly described in previous section(s).

4.1.4.4 The growth of prebiotic and enteric strains in medium supplemented with different concentrations of HCE (1%-5% w/v)

Further research on prebiotic activity of HCE was carried out using different concentration of horse chestnut extract ranging from 1% to 5%. Residue was added to MRS/M9 medium to obtain the following concentration 1%, 2%, 3%, 4% and 5% (w/v). After extraction and centrifugation, the supernatant was filtered directly to sterile glass universal bottles (as described in section 2.4). It has to be mentioned that the filtration process proved more difficult in the case of more concentrated extract, necessitating regular changes in filter. Bacterial strains *Lactobacillus plantarum* ATCC 8014 and *Escherichia coli* ATCC 35320 were cultivated in MRS/M9 medium (respectively) supplemented with 1% HCE, 2% HCE, 3% HCE, 4% HCE and 5% HCE.

It was observed that both of the strains grew much better in more concentrated HCE (Table 3.13). The increase in cell density (as OD at 600nm) of *L. plantarum* for the highest concentration of HCE (5% w/v) was 1.160 whereas for the lowest concentration of HCE (1% w/v) the same strain exhibited only OD of 0.706. The Terminal Optical Density of *L. plantarum* was 64% better in 5% HCE than in 1% HCE after 24 hours of incubation.

The growth of *E. coli* in M9 medium supplemented with 5% HCE was 0.403 and with 1% HCE was 0.151, yielding 167% better growth. Initial observation of growth suggested that more concentrated HCE in medium had better stimulatory effect on probiotic strains in comparison with the enteric strain, which is a desirable effect in prebiotics. This was reflected in the PI scores.

Calculation of PI scores for all concentration of horse chestnut extracts (1%-5%) and a comparison of PI scores obtained for commercial prebiotics (GOS, FOS and inulin) is presented in Table 3.14.

It is clear from the differences between % OD increase of *L. plantarum* and *E. coli* at each of the respective concentrations that growth of *E. coli* was improved more at concentrations of 3%, 4% and 5% HCE than the growth of *L. plantarum* at these concentrations relative to growth at 1% HCE.

Closer examination of the ODs for *L. plantarum* in comparison with *E. coli* showed the reason for maximal PI score at 2% HCE. Comparison of growth of *L. plantarum* between 1% and the more concentrated HCE preparations showed OD increase of 23%

at 2% HCE, 32% at 3% HCE, 39% at 4% HCE and 64% at 5% HCE. *E. coli* showed greater enhancement of growth relative to 1% HCE at the higher concentrations: 36%, 112%, 139% and 167% respectively for 2%, 3%, 4% and 5% HCE.

However these figures can be viewed differently comparing OD of *E. coli* (showing growth after 24 hours) as a percentage of the OD of *L. plantarum* (after 24 hours of growth). The percentage of growth of *E. coli* at 1% HCE (measured by OD) was 21.4% of growth of *L. plantarum*. This increased slightly up to 23.6% at 2% HCE. At 3%, 4% and 5% HCE the values are 34.4%, 36.8% and 34.7%, respectively. This demonstrates that the growth of *E. coli* as proportion of the growth of *L. plantarum* (measured by OD) was low at an average of 22.5 ± 1.1% at 1% and 2% HCE and increased to an average of 35.3 ± 1.3% for 3%, 4% and 5% HCE. Undoubtedly, there was some stimulation of growth of *E. coli* at the higher HCE concentrations (3%-5%) compared to 1% and 2% HCE, but this remained reasonably consistent, moreover the *L. plantarum* was still outgrowing the *E. coli* substantially in the 24 hour period. This would be of particular benefit *in vivo*.

The highest PI score for *L. plantarum* paired with HCE was obtained for 2% HCE (0.89), whereas for 1%, 3%, 4% and 5% HCE PI were relatively the same (0.59, 0.63, 0.63, and 0.78 respectively).

The data from the graph (Figure 3.30) shows a small divergence at 2% in growth of the two strains which was reflected in the maximal PI score. As this graph was derived from data with low SD (3.0-4.9%), a significant biological effect may exist at 2% HCE. Interestingly, at 5% HCE (PI 0.78) there appeared to be an increase in the rate of *L. plantarum* proliferation compared to *E. coli* with low SD (1.8-4.7%). This also may represent a biological rather than experimental effect.

4.1.4.5 Development and validation of novel 96-well microtitre plate assay for prebiotic efficacy

There were several reasons for examining a novel method for detecting prebiotic effects. Firstly, to develop a method which would allow a high throughput screening of samples and to save time in screening potential prebiotics given the large number of probiotic strains to be tested. Another major consideration was the desire to examine the prebiotic effect of the lyophilised aqueous extract of horse chestnut residue. In the bottle

assays used to date the 1% HCE was equivalent to 0.16% soluble HCE (section 3.2.2). As these results showed significant prebiotic potential, it was felt that using a comparable concentration of soluble HCE to that of the commercial prebiotics (1%) should elicit even better results. As only small quantities of lyophilised HCE (lHCE) could be prepared in the laboratory, it was necessary to scale back the quantity used in assays. The microplate method provided such an option as the total volume used was 200µl compared with 10ml required for the bottle technique.

A validation procedure was carried out to compare growth of *Lactobacillus plantarum* ATCC 8014 using both microtitre plate and bottle techniques. To ensure reproducibility in the microplate assay technique sixteen replicates of each commercial prebiotic or HCE (1%) were used. The results were then compared with values obtained from growth in universal bottles (Table 3.8 and 3.13). A comparison of these values is presented in Table 3.15. It is clear that there is a significant difference in the absolute OD values recorded by both methods. However the difference in the values is very consistent with the OD from microtitre plate being 50.82% of that in the universal bottles, therefore the new method, using microtitre plates appears valid.

Further the path length is a very important factor in measuring absorbance. Absorbance can be expressed as optical density (OD) in the case of bacterial growth. In the formula $A = \varepsilon_\lambda cl$, where A-refers to absorbance, ε_λ- molar absorption coefficient, *c*-concentration (mol $^{-1}$) and *l*- path length (cm) (Srivastava, 2009). In a standard cuvette the path length is 1cm while in 96-well microplate with 200µl volume the path length is reduced to 0.5cm with a corresponding 50% reduction in OD.

The development of a microplate assay presents several challenges and the difference in results may be due to a variety of reasons. Firstly, incubation of universal bottles takes place in a shaking incubator at 50rpm. This encourages greater gas exchange. Shaking reduces clumping, aggregation and biofilm formation hence facilitating bacterial division. Evaporation is likely to be of greater significance when a method uses a maximum volume of 200µl compared to 10ml. While every effort was made to minimize evaporation by incubation of the plate on wet tissue, the extent to which evaporation took place and its consequence remains unknown.

A study carried out by Harinen *et al*. (Harinen *et al.*, 2010) assessed the performance of turbidometric measurements (at OD of 595nm) and liquid evaporation from 96-well microplates over 24 hours. The organism tested was *Salmonella typhimurium.* Interestingly they observed that the OD, when corrected for path length in the

microplates, was more accurate than OD measured in 1cm cuvettes. This was based on observing ODs of sample which was serially diluted 1 in 2 and compared to the theoretical values based on the OD of a 1 in 10 dilution of the culture in logarithmic growth. The study on evaporation showed an overall water loss of 16.6% after 24 hours at 37ºC when the microplate was covered by lid. However they also noted that evaporation occurs primarily from the edge wells. In the work on prebiotics, the 96-well microplate was incubated with a lid and on tray with wet tissue which should further reduce evaporation.

While there are obvious differences between the two methods, the microplate assay did show a high degree of reproducibility in the results obtained and would be useful for comparison of prebiotics. In addition, the two stated objectives were met. Firstly, high throughput potential was established, and secondly the quantity of sample (and reagents) required was reduced by a factor of 50.

4.1.4.6 Heat- and Acid-treated lyophilised horse chestnut aqueous extract

The prebiotic index (PI) was also determined for the lyophilised horse chestnut aqueous extract (lHCE) after exposure to different processing treatments (low pH, low pH with high temperature and after Maillard reaction). Several previous studies have been carried out to evaluate the prebiotic activity of prebiotics subjected to heat and low pH exposure (Huebner *et al.*, 2008), (Böhm *et al.*, 2006). In some cases processing treatments (such as heat) have been reported to improve stimulation of the growth of bifidobacteria in the gut (Böhm *et al.*, 2006). In contrast some fructooligosaccharides can be degraded by high acidity and high temperature (L'homme *et al.*, 2003), (Matusek *et al.*, 2011), and heat treated inulin has been found to be degraded and form new products (Böhm *et al.*, 2005).

This study presents the evaluation of prebiotic-like activity of lHCE after different chemical and physical treatments. For comparison, commercial FOS and GOS were also subjected to the above treatments. In this research powdered and highly soluble lyophilised horse chestnut aqueous extract was used and the concentration of soluble material applied was exactly the same (1% (w/v)) as commercial prebiotics product tested.

Exposure to a low pH (range from 3.0 to 6.0) did not result in a significant change in prebiotic activity of lHCE (Table 3.16). PI values for treated samples at 3.0, 4.0, 5.0 and 6.0 (0.28±0.01, 0.30±0.02, 0.29±0.02 and 0.28±0.01, respectively) did not differ significantly ($p>0.05$) from the control (PI=0.30±0.02).

The horse chestnut extract (lHCE) indicated lower PI scores (0.21±0.01 and 0.24±0.01; $p\leq0.05$) when treated with low pH (3.0 and 4.0, respectively) and heat at 85ºC in comparison to untreated control (PI=0.29±0.01; Table 3.17). Analyzing the values involved in the determination of PI scores, it was found that in the second part of modified formula, showing the ratio of growth of the enteric strain, values increased gradually as pH decreased. This implies that high temperature combined with low pH resulted in some hydrolysis of lHCE which did not stimulate the probiotic strains but stimulated the enteric strain to a greater extent than the untreated sample. Glucose and fructose are thought to be formed during this type of hydrolysis. These sugars may not offer selective stimulation of probiotic bacteria (Huebner *et al.*, 2008). This may have a significant implication in further application of the extract as potential prebiotic supplement in those foods where processing conditions such as high acidity and temperature are needed, especially if the same effect was not observed with existing commercial prebiotics.

Commercial FOS (fructooligosaccharides from chicory supplied by Sigma-Aldrich) treated with low pH (ranged 3.0-6.0) similarly resulted in significantly lower ($p\leq0.05$) PI scores in comparison to the untreated FOS (Table 3.16). Lyophilised HCE does not appear to suffer a different fate on heat-treatment in comparison to FOS. It appeared, however, that the hydrolysis of FOS happened more easily at acidic pH than at neutral pH. Other research on prebiotic showed different outcomes when pretreatment with low pH was employed. The research carried out by Huebner *et al.* (2008) showed that some fructooligosaccharide commercial prebiotics (Raftilose P95, Raftiline HP) retained similar prebiotic activity before and after low pH exposure. On the other hand L'Homme (2003) demonstrated that treatment of fructooligosaccharides with low pH and heat resulted in hydrolysis of the fructooligosaccharides. It would appear that the prebiotic activity in lHCE is influenced in the same manner, in terms of exposure to pH and heat, to commercially available prebiotics.

4.1.4.7 Maillard reaction condition treatments

The stability of lHCE to processing conditions as exemplified by Maillard reaction (Fennema, 1985) was also studied. The Maillard reaction can occur as a result of the interaction between reducing sugars and proteins or amino acids. Maillard reaction products, melanoidins, are higher molecular weight components with significant importance in food industries. Melanoidins can enhance the flavour, aroma and colour of food products (Semenova *et al.*, 2002). These scientists suggested that some melanoidins can selectively enhance the growth of *Bifidobacteria* in the gut, and therefore can be considered to have a prebiotic activity similar to that of dietary fibres (Wang *et al.*, 2011). Other researchers reported that melanoidins support the growth of the gut bacteria (Ames *et al.*, 1999).

The research on lHCE showed that the prebiotic activity of the horse chestnut aqueous extract modified in a Maillard reaction improved significantly in terms of PI score (Table 3.18). After 1, 2, and 3 hours of treatment (as described in section 2.6) the lHCE obtained significantly, ($p \leq 0.05$) higher PI scores (0.21±0.03, 0.24±0.05 and 0.21±0.03, respectively) in comparison to the untreated control (0.15±0.01). In contrast Maillard reaction-based modification of FOS resulted in a significant reduction of prebiotic activity (PI scores with negative values). Therefore the influence of the Maillard reaction with FOS appears to be undesirable. Huebner (2008) also noticed that one of the fructooligosaccharide products-Raftilose P95, obtained significantly lower PI score after 2h of Maillard reaction conditions. It is important to note that in all cases including controls 1% of glycine was added to reaction mixtures. Glycine may have enhancing effect on growth of *E.coli* (Han *et al.*, 2002) This is reflected in a considerably lower PI score for the controls (0.14±0.06 for FOS and 0.15±0.01 for lHCE) when compared with controls from pH/heat treatment studies.

The horse chestnut aqueous extract in lyophilised form (lHCE), a raw extract of plant residue, contained carbohydrates (81%), small amount of protein (2.82%) and reducing sugar which could react to form more products (melanoidins) during the Maillard reaction. These factors may have combined to positively influence the prebiotic-like activity of lHCE. This was supported by further research which showed that a high percentage of browning products were formed during final Maillard reaction of lHCE

(166.05±3.09) compared with FOS (7.72±0.31). These values were calculated in relation to glucose (as control) which at the end of Maillard reaction (3h) yielded 98.15% of browning products (Table 3.25).

4.1.4.8 Comparison of PI values obtained for crude HCE and lyophilised HCE (lHCE)

The maximum PI value obtained for HCE was 0.89 with *Lactobacillus plantarum* ATCC 8014 grown on 2% (w/v) crude extract. This was equivalent to 0.32% soluble HCE. It was felt that this value might be improved by preparing a powder from this solubilised HCE and using it to enhance probiotic growth. The powder was prepared by lyophilisation (lHCE) and 1% lHCE was used to directly compare with 1% FOS and GOS. However the PI value was determined as 0.3 which is low. In this case the microplate assay was used and all prebiotics and lHCE tested were dissolved in citrate-phosphate buffer (pH 7.0) prior to addition to the wells containing double strength of MRS (pH 6.2-6.5). The PI scores obtained for 1% GOS were also lower (at an average 0.67) in these conditions, when compared with PI score of 1.03 when grown in universal bottles. There is obviously an effect on PI scores due to the procedural and format change. As already mentioned (section 4.1.4.5) there are differences between two procedures used such as number of bacteria inoculated, gas exchange, different detector, homogeneity of suspension and path length of light.

It would also appear likely that lyophilisation is not the optimum technique for purification of the water soluble components of HCE. The maximal PI score was obtained when the extract was prepared by directly dissolving horse chestnut residue in MRS medium to 2% concentration (w/v). A study of other purification methods for soluble HCE was beyond the scope of this work but is an area that requires further exploration.

4.1.5 Fatty acid profiles and probiotic growth

The growth of probiotic in MRS supplemented with 1% HCE (w/v) or 1% (w/v) commercial prebiotics (GOS, FOS or inulin) was accompanied by the production of short chain fatty acids.

The short chain fatty acids produced in most abundance by the probiotics tested were lactic acid and acetic acid. The quantity of the SCFA produced differs according to the bacterial strains and carbohydrate substrate tested. The values obtained after 48 hours incubation showed also that production of SCFA by all *Lactobacillus* and *Bifidobacterium* strains in MRS medium directly correlated with the increase in cell biomass (Table 3.20 and Table 3.8).

Acidification of growth medium appeared to depend on organic acid production. The data (Table 3.21) confirmed that low pH in the medium is mainly due to presence of lactic acid. Probiotics grown on glucose (positive control) yielded the highest level of organic acid production and, in turn, the lowest pH was obtained.

For instance *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4356, *L. casei* ATCC 393 and *L. rhamnosus* ATCC 7469, typical lactic acid producing bacteria (Liong, 2008), produced the highest amount of lactic acid when cultivated on 1% (w/v) glucose in MRS (157.41±4.79, 127.18±9.71, 141.01±0.68 and 144.62±1.72mM, respectively). In contrast the same *Lactobacillus* strains produced very small amount of acetic acid in the same medium (≤7mM). Among all probiotics tested only *L. fermentum* ATCC 9338 produced very low concentrations both lactic acid and acetic acid (38.87±1.13 and 7.09±3.48mM, respectively) when grown in MRS medium supplemented with 1% (w/v) glucose. The pH of the fermented medium of *L. fermentum* (pH=5.64±0.05) was the highest among all probiotic strains grown on glucose.

Growth of probiotics on GOS-supplemented MRS medium resulted in the second (after glucose) highest organic acid production by probiotic strains. *L. plantarum* ATCC 8014 with 102±1.38mM of lactic acid and 102.97±0.48mM of acetic acid was considered to be the best organic acid producer. Low pH (4.56±0.01) also confirmed the presence of high concentration of organic acids in GOS-enriched fermented medium of *L. plantarum*. Considering the high percentage of reducing sugar content (20.6% of glucose and 1.4% galactose) in GOS commercial product, those results were not surprising. Lactose (17.5% of dry matter) is also present in GOS (see Certificate of Analysis- Appendix D). These reducing sugars could accelerate the growth and production of SCFA by *L. plantarum* and other probiotic strains.

Almost all probiotics grown on MRS medium with 1% (w/v) inulin yielded the highest pH with the exception of *B. angulatum* DSM 20098 which indicated the highest increase in cell density as OD at 600 (Table 3.8) and at the same time produced a large amount of acetic acid (102.21±3.35mM) and lactic acid (50.78±0.87mM).

In the case of four probiotic strains (*L. plantarum, L. casei, L. rhamnosus and B. breve*) organic acid levels produced from media supplemented with inulin was less (Table 3.20) than levels produced where supplementation was with any of the other prebiotics tested (including HCE).

Most of the probiotics tested produced organic acids in MRS medium enriched with 1% (w/v) HCE. Only *L. fermentum* ATCC 9338 and *B. longum* DSM 20219 did not produce detectable quantities of lactic acid, while *L. acidophilus* ATCC 4356 did not produce acetic acid when incubated in medium supplemented with HCE. Among all *Lactobacillus* strains grown with HCE, the lowest pH was observed with *L. plantarum* (6.16±0.01) which correlated with the highest production of lactic acid (18.67mM±0.21).

The general phenomenon observed was a large increase in cell biomass associated with a marked decrease in pH and a greater production of organic acid, especially lactic acid.

4.1.6 Anion and Cation exchange chromatography of lyophilised horse chestnut aqueous extract

The compositional analysis of the carbohydrate fraction of lyophilised horse chestnut aqueous extract (lHCE) also included Anion Exchange Chromatography which showed that there may be two major component-types in the lHCE, since the peaks obtained corresponded to monosaccharides (t_R=4.7-7.0) and oligosaccharides (t_R=7.1-13). The proportion of the peak areas corresponding to mono- and oligo-saccharide content were 37% and 63% respectively. However the anion exchange chromatography (Appendix M) did not offer sufficient resolution of the carbohydrate components of lyophilised HCE. Subsequent cation exchange chromatography (Appendix T) provided higher resolution of extract components and allowed identification of the sugar content of lHCE. The qualitative analysis of the lyophilised form of aqueous fraction of horse chestnut residue showed that glucose (t_R=16.01) and fructose (t_R=17.482) are present in the extract. The presence of oligosaccharides was also confirmed by cation exchange chromatography. Three distinct oligosaccharides appeared to exist in lHCE. That with t_R=11.162 corresponds to an oligosaccharide present in inulin and FOS, while the peaks with t_R=11.922 and t_R=12.498 may correspond to oligosaccharides with lower

molecular weight. Sucrose is also present in lHCE and generated a peak at t_R=13.908. The presence of sucrose and glucose in horse chestnut seeds has also been shown by previous researchers (Burlando *et al.*, 2010).

4.1.7 Gel Filtration of lyophilised horse chestnut aqueous extract

Gel filtration chromatography of lyophilised HCE (lHCE) resulted in one major peak (at λ=280nm) which eluted after 20.73ml, and several minor peaks (7.23, 11.61, 17.66, 28.71, 36.71 and 47.40ml; Figure 3.23). In the preliminary study the FPLC fractions were tested for total protein (by Bradford) and total sugar (by Dubois) content. Because of the large number of fractions (91 fractions of 0.5ml) only every second fraction was tested. Carbohydrate and protein analysis showed that the main peak consisted of carbohydrate and that there was very little protein (2.82% total as described in section 3.1.4) in fresh lyophilised HCE. There may also be polyphenolic compounds present. In some cases polyphenols possess functional derivatives such as glycosides which are bound to the phenol component (Mann *et al.*, 1994). In the other words phenolic compounds may be glycosylated. Phenolic compounds have specific absorbance spectra, e.g. for phenolics and phenolic acids the spectra maxima are in the range 250-290nm (Lattanzio *et al.*, 2006). As UV absorbance at 280nm was used to show peak separation in the chromatography process, it is possible that some of the peaks may correspond to polyphenol content.

Following the preliminary study a second fractionation of lHCE was again carried out by gel filtration. In this instance 94 fractions were collected and analysed for sugar content by Dubois assay which was modified in this study (section 2.3.8.4) to facilitate rapid determination of the location of the sugar peaks. A major peak was shown to occur at 20.5ml (Figure 3.29) which confirmed the results obtained in the preliminary study (Figure 3.23)

Estimation of molecular size of the major (mainly carbohydrate) component of lyophilised horse chestnut aqueous extract showed that the average size of carbohydrate in the main fraction was 2735 Da, suggesting an oligosaccharide of approximately 15 monosaccharidic residues. Therefore the degree of polymerization (DP) of sugar

fraction of lHCE was determined to be 15. The molecular mass estimation confirms the previous experiment with qualitative analysis of sugar components in lHCE by anion and cation exchange chromatography: that oligosaccharides are the main sugar components in lyophilised aqueous extract from horse chestnut residue.

4.1.7.1 Modification of the Dubois assay methodology for sugar analysis

The traditional method for total protein and sugar analysis was based on collection of the lHCE fractions into Eppendorfs and then transferring samples into separate set of Eppendorf tubes for analysing of protein (by Bradford) and sugar (by Dubois) content. This method had some disadvantages: A substantial quantity of materials were needed, i.e. Eppendorf tubes - two tubes for each fraction; pipettes, tips, tray, etc. A large number of actions must be performed; insertion of tubes into fraction collector, pipetting into each tube using single pipette, opening and closing of each Eppendorf tube in order to add reagents, mixing by vortex individually and placing the tubes in an appropriate place in tray. An assay which requires a large number of actions has increased potential for human error.

Therefore to detect the location of the major sugar fraction in lHCE, a novel procedure was developed in order to overcome of these disadvantages. The novel method included the following actions:

- Direct collection of all fractions eluted from the column into mega block (96-well with 2ml volume in each). One mega block (96-well) replaces 96 Eppendorf tubes and is easily fitted on the fraction collector.
- All fractions were tested for total sugar content (by Dubois assay) by using a multichannel pipette to transfer 50µl from each fraction to a corresponding position in a new 96-well mega block
- All further steps (addition of reagent, mixing) were carried out in the mega block using a multichannel pipette. Multichannel pipette offers fast replacement of tips and effective mixing at the same time.
- The mega block was then incubated in boiling water for 15 minutes. This was facilitated by covering the block using adhesive aluminium tape. Both mega block and aluminium tape are heat resistant (autoclavable) and acid resistant.

The adhesive tape used allows precise covering of the whole mega block. This prevented spillage and reduced the risk of burning for the handler. After incubation and colour development all fractions were transferred to microplate using multichannel pipette and subjected to spectrophotometric analysis in a microplate reader.

This novel modification of the Dubois assay certainly has many advantages, the most important of which are savings on the costs of materials and especially time.

This modified Dubois assay was used to determine the elution profile of the three dextran standards (Figure 3.26-3.28) as well as lHCE (Figure 3.29) which permitted determination of the molecular mass of sugar component of lHCE.

The procedure provided a convenient, reproducible method for detecting carbohydrate in GFC eluates. Furthermore the absorbances obtained were consistent with the standard assay method and therefore could be used for quantitative purposes.

4.2 Screening of lyophilised horse chestnut aqueous extract for anti-hyaluronidase and anti-elastase activity

4.2.1 Anti-hyaluronidase activity

Hyaluronidase plays a significant role in several medical conditions, for example lung disorders, cardiovascular disorders, atherosclerosis and cancer. cancer (Royce and Steinmann, 2002). Skin ageing may also be caused by the action of hyaluronidase. Destructive effect of hyaluronidase on hyaluronic acid is manifested by the appearance of wrinkles (Sahasrabudhe and Deodhar, 2010). Anti-hyaluronidase compounds can therefore be of significant commercial value in both medical and cosmetic industries.

The lyophilised form of aqueous extract from horse chestnut residue (lHCE) was screened for anti-hyaluronidase activity. The study showed that lHCE inhibits hyaluronidase (HA-ase) in a dose-dependant manner (Table 3.32). Three, four and five milligrams per milliliter of crude lHCE exhibited inhibition effect on hyaluronidase by 5, 28 and 47 percent, respectively. The IC_{50} for HCE was also determined as 4.8mg/ml. Heparin, a well known hyaluronidase inhibitor was also used in this research as the

control inhibitor. This glycosaminoglycan exhibited strong inhibition (IC_{50}= 0.032mg/ml) on HA-ase. Heparin is a non-competitive inhibitor (Mio and Stern, 2002) and is widely used in measurement of inhibitory effect on hyaluronidase enzyme.

The use of lHCE and heparin together had a synergistic effect as mixtures and gave much stronger inhibition on HA-ase than the compounds applied separately. Five milligrams per milliliter of lyophilised HCE mixed with 0.02mg/ml of heparin resulted in an increase of inhibition by 26% in comparison to 5mg/ml of lHCE applied separately (Figure 3.43 D). Heparin, as non-competitive inhibitor, does not bind directly to the active site of the enzyme but reacts with other location on enzyme structure which, in turn leads to changes in structure of the active site of the enzyme (Bosnić *et al.*, 2009). In consequence, substrate cannot bind to the active site and product is not generated.

In this research fructooligosaccharide (FOS) was also used in anti-hyaluronidase assays to determine whether carbohydrate constituents might have an effect on hyaluronidase inhibition. It was demonstrated that FOS did not have any effect on HA-ase (Figure 3.32), which may suggest that components other than carbohydrates are responsible for enzyme inhibition.

4.2.2 Anti-elastase activity

The lyophilised form of the horse chestnut aqueous extract (lHCE) was screened for anti-elastase activity. Similarly to previous research, the inhibitory effect of lHCE observed on elastase activity was dose-dependant. Moreover the percent of elastase inhibition remained constant over a range of different incubation times (30, 90 and 120min. see Figure 3.39). Inhibitory effect of lHCE was observed at quite high concentration (25% of inhibition for 25mg/ml of HCE and 50% of inhibition for 50mg/ml of lHCE). As a model inhibitor *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was used. The *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone is a competitive inhibitor, which binds to the active site of the enzyme preventing substrate binding. This inhibitor irreversibly inhibits elastase (Kore and Shanmugasundaram, 2010).

4.2.3 Study of bioactive components of horse chestnut extract

The competitive inhibition of polyphenol-rich plant extract on hyaluronidase and elastase inhibition has been described by Lee *et al*. (Lee *et al.*, 2001).

The study by Facino *et al.* (Facino *et al.*, 1995) evaluated the inhibitory effect of *Aesculus hippocastanum* on the activity of hyaluronidase. The authors identified escin saponin as the main constituent of *A. hippocastanum* responsible for enzyme inhibition. The IC_{50} of escin saponin obtained from seed tissue was determined as 149.9±2.6μM. Triterpene sapogenin escinol also exhibited weak inhibition on HA-ase (IC_{50} = 1.65±0.03mM).

Components of horse chestnut seeds proanthocyanidin A_2 as well as glucoside coumarin- esculin and their aglycone esculetin (see section 1.1.6) also possess properties similar to escin. These constituents of *Aesculus hippocastanum* may have anti-hyaluronidase and anti-elastase activity (Bombarelli and Morazzoni, 1996). Bioactive components with anti-elastase and anti-hyaluronidase properties obtained from *Aesculus hippocastanum* have a protective effect on degradation of extracellular matrix surrounding tissue and therefore have a potential application in skin cosmetic products (Wilkinson and Brown, 1999).

The polyphenol content in powdered form of horse chestnut aqueous extract (lHCE) was determined as 2% (section 3.3.6), however its composition was not established. Polyphenols and saponins present in horse chestnut seeds may be involved in the anti HA-ase activity.

4.3 Organic extracts of horse chestnut residue and their antibacterial activity

4.3.1 The extraction and chemical profile of the organic extracts

In the next part of the research, the antimicrobial activity of organic extracts of horse chestnut residue was investigated. The extraction of raw material from the residue was

performed using organic solvents that differed in relation to their polarity (methanol, ethanol, dichloromethane, acetone and chloroform). Dichloromethane extracted the highest amount of raw material from the residue (4.56% extracted material), while in contrast, methanol yielded only 2.84% of raw extract from the residue (Figure 3.40).

Preliminary screening of the raw extracts by thin layer chromatography showed that the more effective solvent system for separation of extracts' components was ethyl acetate:methanol:water (EMW) 88:11:8.

The TLC chemical profile of all horse chestnut organic extracts (Figure 3.41) indicated that methanol and ethanol had extracted the highest number of UV active and anisaldehyde/sulphuric acid-visualised compounds (7 and 8, respectively) from horse chestnut residue. In contrast, chloroform had extracted the lowest number of UV active and anisaldehyde/sulphuric acid – visualised compounds (3). Most compounds present in chloroform were non-polar while those in methanol and ethanol extracts were high polarity. This was seen from their effective separation in polar EMW solvent system. Compounds that appeared in the chemical profile of methanol and ethanol were seen to be mostly polar as some of them showed up at the lower part of the chromatogram. EMW as a polar solvent system could effectively separate polar compounds, compounds seated at the lower part of a chromatogram developed with EMW must therefore, be of higher polarity.

In this research, methanol and ethanol extracted the smallest quantity of material from horse chestnut residue (0.851 and 1.196g per 30g residue, respectively) but the highest number of compounds (7 and 8, respectively) showed up in those extracts, therefore methanol and ethanol seem to be useful solvents for selective extraction of material from the residue. HCE been shown have components that are readily soluble in aqueous solvents. Further investigation could examine the extraction efficiency of aqueous solutions of alcoholic solvents over a range of concentrations e.g.90, 80 and 50% alcohol

Inspection of developed TLC plates under UV light also showed that UV-active bands were situated at the top of chromatogram for all organic extracts. These bands, present in all separated extracts, are suggested to correspond to less polar or non-polar compounds which were not retained by silica gel on TLC plate and moved up with the developing solvents.

UV-active spots were also identified in the middle and at start of the separation of methanolic extract (MetHCE) and these were moderately polar and polar (Figure 3.41).

Phenolic compounds are consider to be UV active (Lattanzio *et al.*, 2006) and their presence in chestnuts (Kapusta *et al.*, 2007) may attribute to UV activity of extracts obtained from the residue.

4.3.2 Antibacterial activity of the organic extract from horse chestnut residue

4.3.2.1 Disc diffusion assay

The antibacterial activity of the organic extracts from horse chestnut residue were assayed *in vitro* by a disc diffusion method against Gram-positive strains (*Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228) and Gram-negative (*Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048). The method did not show high inhibitory effects. In the case of methanolic extract (MetHCE) a small degree of inhibition of all pathogenic strains was observed (Figure 3.43). Additionally, some stimulatory effect at a lower concentration was visible. The oligo-dynamic action demonstrated by MetHCE has been described as an occurrence in microbiology (Aneja, 2007).

Organic solvents used for extraction of horse chestnut residue had different polar properties. The most polar, methanol, extracted polar constituents from the residue, in contrast to chloroform, which dissolves non-polar molecules. The diffusion of extracts obtained from non-polar solvents (chloroform, dichloromethane) was non-existent or very poor due to the polar nature of agar. Consequently the disc diffusion method did not appear to be suitable for investigation of these extracts. Diffusion methods have been shown to be a poor choice for testing non-polar or other samples that do not diffuse in the media. The low hydrophilicity of most phenolic compounds present in plant extracts are such that they do not diffuse into water-based medium. This has an important impact on the evaluation of antimicrobial capability (King *et al.*, 2008).

4.3.2.2 Bioautography assay

To assess the major constituents responsible for antimicrobial activity against pathogens, an alternative method, TLC bioautography was performed. All horse chestnut extracts were tested (MetHCE, EtHCE, DiHCE, AcHCE and ChHCE). In contrast to the disc diffusion technique, the bioautography was demonstrated to be very effective in screening the raw extracts for antimicrobial activity. Direct bio-autographic procedures included using soft 0.6% Mueller-Hinton agar (MHA) inoculated with strictly defined number of bacterial cells (10^6 cfu/ml). The other important factor in direct bioautobiography was the use of tetrazolium red for detection of actively growing bacterial cells. The concentration of Muller-Hinton agar (0.6%) probably contributed to the efficacy of this technique. The active components in the extracts appeared to diffuse much more readily into 0.6% agar than into more concentrated medium (such as 1.7% MHA in disc diffusion method).

For all extracts clear inhibition zones were observed for those spots situated at the top of chromatogram with very similar retention factors (Figure 3.44). The bands had an antimicrobial effect on all organisms (*E. coli* ATCC 25922, *E. aerogenes* ATCC 13048 and *S. aureus* ATCC 9144) used in this research and were, most probably, of low polar and non-polar character. Polar constituents of ethanolic and methanolic extracts also showed inhibitory effects on all strains tested. At low concentration (total 4µl of raw MetHCE at concentration of 425.55mg/ml) polar components of raw MetHCE exhibited stimulatory effects on organisms (e.g. *E. coli*, *S. aureus* and *E. aerogenes*). The same effect was observed in the disc diffusion method. Polar components of EtHCE also demonstrated oligodynamic action (stimulation at low concentration, inhibition at higher concentrations) on the following strains: *E. coli* and *E. aerogenes*. Components of AcHCE, DiHCE and ChHCE with higher polarity (Rf=0.13, 0.14 and 0.14 respectively) also indicated antibacterial activity against all strains tested.

4.3.2.3 The checkerboard assay

The methanolic and ethanolic raw extracts were selected for checkerboard assay to determine the minimal inhibitory concentration (MIC). This is a microtitre assay for assessing antimicrobial susceptibility and is based on measurement of inhibition of tested agent/drug in a microtitre plate at varying concentrations. The medium is liquid Muller-Hinton broth and bacterial suspension was added into each well ($1x10^8$-$2x10^9$ cfu/ml; 0.5 McFarland standard). This method permits high throughput screening of

antimicrobial activity of the drug to be tested (Karuppusamy and Rajasekaran, 2009). Detection of viable cells in this research was performed using Alamar blue reagent, a blue dye which is reduced by actively growing cells and at the same time may effectively indicate wells with bacterial inhibition. Alamar blue has important advantages, it allows detection and calculation of the growth of bacterial cells by either spectrophotometric or fluorescent measurement (Habeeb *et al.*, 2007b).

In this research methanolic (MetHCE) and ethanolic (EtHCE) extracts were chosen for high throughput screening of antibacterial activity using gram negative *Escherichia coli* ATCC 25922. Pure methanol and ethanol were also applied as negative controls to determine whether pure solvents exhibited inhibitory effects on the strain tested. The results showed that ethanol had an inhibitory effect on *E. coli* ATCC 25922 at a concentration as low as 12.5 % and therefore screening raw EtHCE dissolved in the same concentration of pure ethanol cannot be reliable (Figure 3.46). The pure methanol also exhibited inhibitory effect on that enteric strain but at much higher concentration (50%), whereas MetHCE containing 25% and 12.5% of pure methanol still exhibited antibacterial activity (Figure 3.47). The MIC value (53mg/ml) for methanolic extract (MetHCE) against *E. coli* ATCC 25922 is a good indication of the efficacy of this residue and shows it to be a good source of bioactive components with antimicrobial potency.

4.3.2.4 Active bio-components of the organic extracts

In the literature the antimycotic activity of ethanolic and aqueous extracts of horse chestnut (*Aesculus hippocastanum*) was demonstrated against different fungal strains (Roy *et al.*, 2011a) however there is little information concerning the antibacterial activity of horse chestnut. The same authors (Roy *et al.*, 2011b) have tested horse chestnut extracts (aqueous and ethanolic) against oral microbes. Both extracts exhibited antibacterial activity on *Streptococcus mutans, Streptococcus sanguis, Streptococcus mitis, Streptococcus salivarius* and *Lactobacillus acidophilus*. Lakshmi and Ravishankar (Lakshmi and Ravishankar, 2011) investigated antibacterial activity of ethanolic seeds extract of *Aesculus hippocastanum* against another endodontic bacterial strain *Enterococcus faecalis*. The findings suggested no antibacterial activity of that extract against the chosen strain. Antimicrobial protein 1 (Ah-AMP-1), isolated from horse chestnuts, plays an important role as plant defensin (Fant *et al.*, 1999), (Broekaert

et al., 1995). Ah-AMP-1 inhibits a broad range of fungi, however, little effect against bacteria has been observed (Osborn *et al.*, 1995).

The total phenolic content in the organic extract of horse chestnut residue was not determined (only aqueous extract of horse chestnut residue was analised for total phenolic content by Folin-Ciocalteau) however a number of flavonoids have been identified in horse chestnut seeds (Kapusta *et al.*, 2007), (Wilkinson and Brown, 1999), (Bombarelli and Morazzoni, 1996). According to Kapusta *et al*. (2007) the powdered seeds contain 0.08% flavonoids in dry matter, whereas their alcoholic extract has 3.46% flavonoid components. Main flavonoid components present in horse chestnut seeds (*Aesculus hippocastanum*) are: quercetin, kaempherol and their derivatives astrogalin, izoquercetin, leucocyanin, rutin, tamatixetin. Coumarins (esculetin, fraxin and scopoletin), epicatechin as well as tannins are also minor flavonoids of horse chestnuts.

Numerous studies have been performed to determine antimicrobial effect of secondary metabolites derived from plant extracts (see Table 1.14/section 1.4.1.7 and Table 1.5/section 1.4.1.12). These classes of natural products possess antifungal, antiviral and antibacterial activity (Cushnie and Lamb, 2005). Quercetin and kaempherol are considered to have antimicrobial effect (Lee and Lee, 2009). Quercetin from plant extract has been shown to be effective in inhibiting the growth of bacterial species including nine different *Escherichia coli* and *Staphylococcus aureus* sp. (Rauha *et al.*, 2000).

Highly polar and polar solvents (water or alcohols) are considered to be good for extraction of phenolics (Koffi *et al.*, 2010). Aqueous and ethanolic plant extracts indicating antibacterial activity have also been demonstrated (Mattana *et al.*, 2010), (Aboaba *et al.*, 2006). Fratianni and colleagues (Fratianni *et al.*, 2011) have examined extract obtained from apple peel for antibacterial potential. Alcoholic extraction (ethanol) was effective in solubilising phenolic compounds. The most abundant phenolic fractions were rutin and epicatechin (27.43% and 24.95%, respectively). The peel extract exhibited antibacterial activity against *E. coli* (serotype O157:H7). Rutin has also been identified as the main phenolic component in aqueous extract from olive leaves (*Olea europaea*). The extract possessed antibacterial activity against *E. coli* and *S. aureus* strains in a concentration-dependent manner (Pereira *et al.*, 2007). Methanolic extracts of some plants are rich sources of coumarins (esculetin and scopoletin) which have also demonstrated antimicrobial potency (Ojala *et al.*, 2000). The influence of tannins on microbial growth has been described by Scalbert (Scalbert, 1991). The

inhibitory effect of plant derived tannins on fungi, bacteria and yeasts depends on the tannins' structure. Antibacterial properties of tannins are widely described by researchers (Hamilton-Miller, 1995), (Akiyama *et al.*, 2001).

In the investigations presented in this thesis, extraction of horse chestnut residue with polar (methanol, ethanol) solvents intermediate (acetone) and non-polar solvents (dichloromethane, chloroform) was employed. The data showed that the highest amount of raw material was obtained from chloroform (ChHCE) and dichloromethanolic (DiHCE) extracts (4.55% and 4.65%, respectively; Figure 3.40). These non-polar organic extracts also exhibited antimicrobial potential using bioautography method. These active spots were mainly situated at the top of the chromatograph as large areas. The volumes of each extract applied on TLC were the same (4µl), but the weight of material varied due to the extraction process (Table 3.22), as observed after TLC development and staining. Non-polar constituents of ChHCE and DiHCE, not retained by silica, moved with developing solvents (EMW). The large spots, which appeared as smears, were a consequence of much greater amount of raw extract. ChHCE and DiHCE as well as AcHCE had a significant oily fraction. As presented in section 3.1.5.1 the crude fat of horse chestnut residue constitutes 1.89% of the dry matter. Those fat fractions could increase the amount of extracted material substantially. Beside phenolic components, coumarins and steroids, tannin and terpenoids can also be extracted from plant material using solvents with low polar and non-polar properties (Murugan *et al.*, 2012). The antibacterial potential of plant extracts in non-polar solvents has been described in literature (Sankar Narayan Sinha *et al.*, 2010), (Murugan *et al.*, 2012). In summary, the horse chestnut extracts appears to contain anti-microbial agents. The identity of these agents was not established, but in combination with the results from prebiotic activity, the consumption of horse chestnuts may well promote growth of probiotic microorganisms while suppressing growth of pathogenic or non-probiotic organisms by several means.

5 Conclusions and Future Work

This thesis presents a comprehensive evaluation of the application of horse chestnut residue to the production of pharmaceutical grade compounds. This project was a challenging collaboration of industrial problem solving and academic research. A number of different analytical methods (protein, fat, total solid determination) were employed to explore the by-product in terms of the quality and quantity of bio-active components. More advanced techniques; HPLC, FPLC, GC, TLC, emission/absorption spectrophotometry were also applied. Microbiological methods such as the prebiotic assay, anti-microbial screening of extracts (disc diffusion, checkerboard and bioautography assays) as well as enzymatic techniques were used in the investigation of the residue. Some research protocols such as the prebiotic assay were modified and transferred to 96-well microplate format, and the Dubois assay was modified for high throughput screening of the FLPC fractions. These novel method formats contributed significantly to the research by allowing the testing of more samples in a short time period, saving on equipment, cost of materials and labour. A further novel outcome of this work was the development and application of a significantly modified formula to the calculation of prebiotic index for assessment of potential prebiotics.

An initial study on the general composition of the residue was carried out. A major component of the horse chestnut residue was carbohydrate; therefore the prebiotic activity of the aqueous extract, known as HCE, was tested. The research also led to the development of an extraction protocol giving rise to a finely powdered prebiotic preparation from horse chestnut residue. The estimation of prebiotic activity of horse chestnut aqueous extract (HCE) led to the conclusion that the extract could be a good candidate as a potential prebiotic product because it met many of the published prebiotic criteria:

- It had a beneficial effect on probiotic strains by supporting their growth in medium.
- It indicated a positive prebiotic index (PI) with all probiotics tested.
- It was selectively fermented by *Lactobacillus* and *Bifidobacterium* species however the capacity to digest depended on the individual strains.

- The fermentation of HCE was manifested by the production of organic acids (mainly lactic acid and acetic acid) and in turn led to a decrease in pH of growth medium.
- HCE was stable and mostly retained the same prebiotic-like properties after different processing treatments.
- Prebiotic-like constituents from HCE were readily soluble in water which may have significance where the destination of the extract is as a food additive.
- Oligosaccharides (with 2735 Da) were determined to be the main sugar fraction of lyophilised form of HCE.

In this study it has been shown that HCE can be used effectively as a prebiotic *in vitro* because of the increase in lactobacilli and bifidobacteria populations with the subsequent increase in lactate and acetate concentration, giving possible added health benefit. The material, stable over a wide pH range and potentiated by Maillard reaction possesses potential commercial value and might be developed as a prebiotic product. The research also provided a preliminary evaluation of the best combinations of probiotic and HCE which could maximize their symbiotic effect in, for example, dairy products and food additives.

Future work should involve the isolation and purification of the sugar component from raw aqueous extract (HCE) which exhibited the greatest prebiotic activity. An upscaling of the extraction and preparation processes would be necessary. Sensory assessment for characteristics such as texture, consistency, colour and the taste of the powdered form of prebiotic-like extract should also be carried out to assess the application in a selected prepared food. In addition toxicity tests should determine any possible toxic effects on human cells.

In the second part of the research the aqueous horse chestnut extract (HCE) was screened for anti-hyaluronidase and anti-elastase properties. However the observed inhibitory effect of the aqueous horse chestnut extract (HCE) on enzymatic activity was weak in comparison to established inhibitors. Some further steps such as purification and isolation of the bioactive components responsible for inhibition of hyaluronidase and elastase may result in a greater specific activity of the inhibitory effect.

In the third part of the research the horse chestnut residue was extracted with different organic solvents. Preliminary research on the organic extracts of horse chestnuts residue showed that this by-product may be considered as a potential source of antimicrobial activity. The organic extracts of the residue demonstrated inhibitory effects on bacteria (*E. coli, E. aerogenes, and S. aureus*) however characterization of active components from horse chestnut organic extracts in terms of their quality and quantity is required. The presence of the flavonoids quercetin and kaempherol and their derivative rutin as well as tannins and coumarins in the seeds of horse chestnut (*Aesculus hippocastanum*) may contribute to antibacterial activity of the chestnuts' residue however the mechanism of biological activity of these compounds in the residue has also to be evaluated in further work. The extraction process may also be modified to optimize large scale extraction: quality and quantity of solvents, weight and ratio of raw material added, temperature and duration of extraction as well as the choice of extraction equipment. It would be necessary to assess the finished organic product in terms of its antimicrobial activities in order to optimize its quality and effectiveness. Further modifications may produce a number of extracts whose activity is effective against different microorganisms - bacteria, yeast, fungi and possibly parasitic protozoans.
This report provides scientific evidence that horse chestnut by-product is a source of antibacterial compounds which have potential application in the pharmaceutical industry.

The study was based on an unusual by-product. The horse chestnut residue is produced locally by a medium-sized industry located in South-West region of Kerry (Ireland). The ability to utilise what is currently a waste material to generate commercially viable products would be of huge benefit to the industry in question. It could also lead to job creation and waste minimisation. Further this would enhance the cooperative links between local industries and academic research, catalyse niche product development, as well as raising the profile of Irish utilisation of natural materials.

6 References

Aboaba, O. O., Smith, S. I. and Olude, F. O. (2006) 'Antibacterial Effect of Edible Plant Extract on *Escherichia coli* 0157:H7', *Pakistan Journal of Nutrition*, 5(4), 325-327.

AbsoluteAstronomy.com (2010a) 'Common Horse-chestnut', [online], available: www.absoluteastronomy.com [accessed 2nd May 2010].

AbsoluteAstronomy.com (2010b) 'Soxhlet extractor', [online], available: www.absoluteastronomy.com [accessed 2nd May 2010].

Adao, C. R., da Silva, B. P. and Parente, J. P. (2011) 'A new steroidal saponin from *Allium ampeloprasum var. porrum* with antiinflammatory and gastroprotective effects', *Phytochemistry Letters*, 4(3), 306-310.

Ahn, B.-Z., Yoon, Y.-D., Lee, Y. H., Kim, B.-H. and Sok, D.-E. (1998) 'Inhibitory Effect of Bupleuri Radix Saponins on Adhesion of Some Solid Tumor Cells and Relation to Hemolytic Action: Screening of 232 Herbal Drugs for Anti-Cell Adhesion', *Planta Medica*, 64(03), 220,224.

Aider, M. and Halleux, D. d. (2007) 'Isomerization of lactose and lactulose production: review', *Trends in Food Science & Technology*, 18(7), 356-364.

Ajayi, A. O. and Akintola, T. A. (2010) 'Evaluation of antibacterial activity of some medicinal plants on common enteric food-borne pathogens', *African Journal of Microbiology Research*, 4(4), 314-316.

Akaza, H., Miyanaga, N., Takashima, N., Naito, S., Hirao, Y., Tsukamoto, T., Fujioka, T., Mori, M., Kim, W.-J., Song, J. M. and Pantuck, A. J. (2004) 'Comparisons of Percent Equol Producers between Prostate Cancer Patients and Controls: Case-controlled Studies of Isoflavones in Japanese, Korean and American Residents', *Japanese Journal of Clinical Oncology*, 34(2), 86-89.

Akiyama, H., Fujii, K., Yamasaki, O., Oono, T. and Iwatsuki, K. (2001) 'Antibacterial action of several tannins against *Staphylococcus aureus*', *Journal of Antimicrobial Chemotherapy*, 48(4), 487-491.

Alviano, W. S., Alviano, D. S., Diniz, C. u. G., Antoniolli, A. R., Alviano, C. S., Farias, L. M., Carvalho, M. A. R., Souza, M. M. G. and Bolognese, A. M. (2008) 'In vitro antioxidant potential of medicinal plant extracts and their activities against oral bacteria based on Brazilian folk medicine', *Archives of Oral Biology*, 53(6), 545-552.

Ames, J. M., Wynne, A., Hofmann, A., Plos, S. and Gibson, G. R. (1999) 'The effect of a model melanoidin mixture on faecal bacterial populations *in vitro*', *British Journal of Nutrition*, 82, 489-495.

AMRESCO (1998) 'M9 / M9CA Broth (J863, J864)', *Technical Biulletins* [online], available: http://www.amresco-inc.com/media.acux?path=/media/products/dfu/dfu-J864.pdf [accessed 15th May 2010].

AnalChem (2000) 'Visualizing agents for TLC and PC: Preparation of chromatography spray reagents', *Lab Tech resources reagents and Solutions* [online], available: http://delloyd.50megs.com/spray_reagents.html [accessed 20 May 2011].

Aneja, K. R. (2007) *Experiments in microbiology, plant pathology and biotechnology* 4th ed., New Age International Publishers.

Annoni, F., Mauri, A., Marincola, F. and Resele, L. F. (1979) 'Venotonic activity of escin on the human saphenous vein', *Drug Research,* 29(4), 672-5.

Antunes, P. M., de Varennes, A., Rajcan, I. and Goss, M. J. (2006) 'Accumulation of specific flavonoids in soybean (*Glycine max (L.) Merr*.) as a function of the early tripartite symbiosis with arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* (Kirchner) Jordan', *Soil Biology and Biochemistry,* 38(6), 1234-1242.

Apers, S., Baronikova, S., Sindambiwe, J.-B., Witvrouw, M., Clercq, E. D., Berghe, D. V., Marck, E. V., Vlietinck, A. and Pieters, L. (2001) 'Antiviral, Haemolytic and Molluscicidal Activities of Triterpenoid Saponins from *Maesa lanceolata*: Establishment of Structure-Activity Relationships', *Planta Medica,* 67(06), 528,532.

Arabshahi-D, S., Vishalakshi Devi, D. and Urooj, A. (2007) 'Evaluation of antioxidant activity of some plant extracts and their heat, pH and storage stability', *Food Chemistry,* 100(3), 1100-1105.

Araceli, A.-C., Elda, C.-M., Edmundo, L.-G. and Ernesto, G.-P. (2007) 'Capsidiol production in pepper fruits (*Capsicum annuum L*.) induced by arachidonic acid is dependent of an oxidative burst', *Physiological and Molecular Plant Pathology,* 70(1-3), 69-76.

Arora, R. (2010) *Medicinal plant biotechnology,* London, UK: CABI.

Aryaeian, N., Shahram, F., Djalali, M., Eshragian, M. R., Djazayeri, A., Sarrafnejad, A., Salimzadeh, A., Naderi, N. and Maryam, C. (2009) 'Effect of conjugated linoleic acids, vitamin E and their combination on the clinical outcome of Iranian adults with active rheumatoid arthritis', *International Journal of Rheumatic Diseases,* 12(1), 20-8.

Avci, G., Küçükkurt, I., Küpeli Akkol, E. and Yeşilada, E. (2010) 'Effects of escin mixture from the seeds of *Aesculus hippocastanum* on obesity in mice fed a high fat diet', *Pharmaceutical Biology (Formerly International Journal of Pharmacognosy),* 48, 247-252.

Avtzis, N. D., Avtzis, D. N., Vergos, S. G. and Diamandis, S. (2007) 'A contribution to the natural distribution of *Aesculus hippocastanum* (*Hippocastanaceae*) in Greece', *Phytologia Balcanica,* 13(2), 183-187.

Baker, J. W. (1930) *Natural Terpenes,* Methuen & co., ltd.

Ballard, J. L. and Bergan, J. J. (2000) *Chronic Venous Insufficiency; Diagnosis and Treatment*, London: Springer.

Baraldi, C., Bodecchi, L. M., Cocchi, M., Durante, C., Ferrari, G., Foca, G., Grandi, M., Marchetti, A., Tassi, L. and Ulrici, A. (2007) 'Chemical composition and characterisation of seeds from two varieties (pure and hybrid) of *Aesculus hippocastanum*', *Food Chemistry*, 104(1), 229-236.

Barbosa, P., Krischik, V. A. and Jones, C. G. (1991) *Microbial mediation of plant-herbivore interactions*, Canada: John Wiley and Sons, Inc.

Bauer, A. W., Kirby, W. M., Sherris, J. C. and Turck, M. (1966) 'Antibiotic susceptibility testing by a standardized single disk method', *American Journal of Clinical Pathology*, 45(4), 493-6.

Berhow, M. A., Wagner, E. D., Vaughn, S. F. and Plewa, M. J. (2000) 'Characterization and antimutagenic activity of soybean saponins', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 448(1), 11-22.

Bernet, M. F., Brassart, D., Neeser, J. R. and Servin, A. L. (1993) 'Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions', *Applied and Environmental Microbiology,* 59(12), 4121-4128.

Bielecka, M., Biedrzycka, E. and Majkowska, A. (2002) 'Selection of probiotics and prebiotics for synbiotics and confirmation of their in vivo effectiveness', *Food Research International*, 35(2-3), 125-131.

Birosová, L., Mikulásová, M. and Vaverková, S. (2005) 'Antimutagenic effect of phenolic acids', *Biomedical Papers of the Medical Faculty of the University Palacky, Olomouc, Czech Republic*, 149(2), 489-91.

Bleacher, J. C., Adolph, V. R., Dillon, P. W. and Krummel, T. M. (1993) 'Fetal tissue repair and wound healing', *Dermatologic Clinics*, 11(4), 677-683.

Blumwald, E., Aharon, G. S. and C-H. Lam, B. (1998) 'Early signal transduction pathways in plants pathogen interactions', *Trends in Plant Science,* 3(9), 342-346.

BMRD (2012) 'Biological Magnetic Resonance Data Bank', [online], available: http://www.bmrb.wisc.edu [accessed 25th January 2012].

Böhm, A., Kaiser, I., Trebstein, A. and Henle, T. (2005) 'Heat-induced degradation of inulin', *European Food Research and Technology,* 220(5), 466-471.

Böhm, A., Kleessen, B. and Henle, T. (2006) 'Effect of dry heated inulin on selected intestinal bacteria', *European Food Research and Technology,* 222(5), 737-740.

Bombarelli, E. and Morazzoni, P. (1996) '*Aesculus hippocastanum* L.', *Fitoterapia,* 67, 483-511.

Bonev, B., Hooper, J. and Parisot, J. l. (2008) 'Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method', *Journal of Antimicrobial Chemotherapy,* 61(6), 1295-1301.

Bonner Jr, W. M. and Cantey, E. Y. (1966) 'Colorimetric method for determination of serum hyaluronidase activity', *Clinica Chimica Acta,* 13(6), 746-752.

Borg-Karlson, A.-K. and Groth, I. (1986) 'Volatiles from the flowers of four species in the sections arachnitiformes and araneiferae of the genus *Ophrys* as insect mimetic attractants', *Phytochemistry*, 25(6), 1297-1299.

Bosnić, O. M., Gopčević, K. R., Vrvić, M. M. and Karadžić, I. M. (2009) 'Inhibition of trypsin by heparin and dalteparin, a low molecular weight heparin', *Journal of the Serbian Chemical Society,* 74(4), 379-388.

Bothiraja, C., Joshi, P. P., Dama, G. Y. and Pawar, A. P. (2011) 'Rapid method for isolation of plumbagin, an alternative medicine from roots of *Plumbago zeylanica*', *European Journal of Integrative Medicine,* 3(1), 39-42.

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry,* 72(1-2), 248-254.

Breitmaie, E. (2006) *Terpenes: Flavors, Fragrances, Pharmaca, Pheromones,* Weinheim: Wiley-VCH.

Britton, G., Liaaen-Jensen, S. and Pfander, H. (2009) *Carotenoids:Nutrition and Health,* Basel, Switzerland: Birkhauser Verlag.

Brockman, H. E., Stack, H. F. and Waters, M. D. (1992) 'Antimutagenicity profiles of some natural substances', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 267(2), 157-172.

Broekaert, W. F., Terras, F. R. G., Cammue, B. P. A. and Osborn, R. W. (1995) 'Plant Defensins: Novel Antimicrobial Peptides as Components of the Host Defense System', *Plant Physiology,* 108, 1353-1358.

Broekhoven, A. V., Shapiro, F. and Anné, J. (2001) *Novel frontiers in the production of compounds for biomedical use,* Dordrecht: Kluwer Academic Publishers.

Brown, A. (2009) 'Tree Species of the *Aesculus* Genus', [online], available: www.treenames.net [accessed 20th March 2010].

Brown, D. (2003) *Herbal Prescriptions for Health and Healing: Your Everyday Guide to Using Herbs Safely and Effectively*, Twin Lakes, Wr USA: Lotus Press.

Bucke, C. (1999) *Carbohydrate biotechnology protocols, Methods in Biotechnology*, New Jersey: Humana Press Inc.

Burlando, B., Verotta, L., Comara, L. and Bottini-Massa, E. (2010) *Herbal Principles in Cosmetics: Properties and Mechanisms of Action*, Boca Raton: CRC Press, Taylor & Francis Group.

Busan (2006) 'Distillation Unit', [online], available: www.cusana.go.kr [accessed 23rd May 2010].

Butz, D. E., Li, G., Huebner, S. M. and Cook, M. E. (2007) 'A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis', *American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology*, 6, R669-R676.

Carmiel-Haggai, M., Cederbaum, A. I. and Nieto, N. (2005) 'A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats', *FASEB Journal*, 19(1), 136-138.

Cassileth, B. R. and Lucarelli, C. D. (2003) *Herb-Drug Interactions in Oncology*, Hamilton, Ontario: BC Decker.

Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E. and Hermosín-Gutiérrez, I. (2010) 'Flavonol profiles of *Vitis vinifera* white grape cultivars', *Journal of Food Composition and Analysis*, 23(7), 699-705.

Cfm (2012) [online], available: http://www.cfmot.de [accessed 20th January 2012].

Chang, F.-Y., Lee, S.-D., Yeh, G.-H. and Wang, P. (1996) 'Influence of blood glucose levels on rat liquid gastric emptying', *Digestive Diseases and Sciences*, 41(3), 528-532.

Chaudiere, J. and Ferrari-Iliou, R. (1999) 'Intracellular Antioxidants: from Chemical to Biochemical Mechanisms', *Food and Chemical Toxicology*, 37(9-10), 949-962.

Chen, I.-S., Chang, C.-T., Sheen, W.-S., Teng, C.-M., Tsai, I.-L., Duh, C.-Y. and Ko, F.-N. (1996) 'Coumarins and antiplatelet aggregation constituents from formosan *Peucedanum japonicum*', *Phytochemistry*, 41(2), 525-530.

Cho, S., Prosky, L. and L.Dreher, M. (1999) *Complex carbohydrates in foods* New York: Marcel Dekker Inc.

Cho, S. S. and Finocchiaro, E. T. (2010) *Handbook of Prebiotics and Probiotics Ingredients: Health Benefits and Food Applications*, Boca Raton: CRC Press.

Chow, J. (2002) 'Probiotics and prebiotics: A brief overview', *Journal of Renal Nutrition*, 12(2), 76-86.

Chun, S.-S., Vattem, D. A., Lin, Y.-T. and Shetty, K. (2005) 'Phenolic antioxidants from clonal oregano (*Origanum vulgare*) with antimicrobial activity against *Helicobacter pylori*', *Process Biochemistry*, 40(2), 809-816.

Clausen, M. R., Jørgensen, J. and Mortensen, P. B. (1998) 'Comparison of diarrhea induced by ingestion of fructooligosaccharide Idolax and disaccharide lactulose: role of osmolarity versus fermentation of malabsorbed carbohydrate', *Digestive Diseases and Sciences*, 43(12), 2696-2707.

CLSI (2006) *Performance standards for antimicrobial disk susceptibility tests, Approved standard—9th ed.*, Wayne, PA: Clinical Laboratory Standards Institute.

Coakley, M., Ross, R. P., Nordgren, M., Fitzgerald, G., Devery, R. and Stanton, C. (2003) 'Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species', *Journal Applied Microbiology*, 94(1), 138-45.

Cole-Parmer (2010) 'Kjeldahl Method for Determining Nitrogen', [online], available: www.coleparmer.com [accessed 20th September 2009].

Cook, N. C. and Samman, S. (1996) 'Flavonoids; Chemistry, metabolism, cardioprotective effects and dietary sources', *The Journal of Nutritional Biochemistry*, 7(2), 66-76.

Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C. and Gahan, C. G. M. (2007) 'Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius UCC118* ', *Proceedings of the National Academy of Sciences of the USA*, 104, 7617-7621.

Crittenden, R. G. and Playne, M. J. (1996) 'Production, properties and applications of food-grade oligosaccharides', *Trends in Food Science & Technology*, 7(11), 353-361.

Cropper, R. F. and Heywood, A. (1953) 'Analytical separation of the methyl esters of the C12-C22 fatty acids by vapor-phase chromatography', *Nature*, 172, 1101-1102.

Crozier, A., Jaganath, I. B. and Clifford, M. N. (2007) *Phenols, Polyphenols and Tannins: An Overview, Plant Secondary Metabolites*, Chapter 1, Blackwell Publishing Ltd.

Cueva, C., Moreno-Arribas, M. V., Martin-Ãlvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., Rivas, C., Requena, T., Rodriguez, J. M. and Bartolome, B. (2010) 'Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria', *Research in Microbiology*, 161(5), 372-382.

Cushnie, T. P. T. and Lamb, A. J. (2005) 'Antimicrobial activity of flavonoids', *International Journal of Antimicrobial Agents*, 26(5), 343-356.

Dall'Agnol, R., Ferraz, A., Bernardi, A. P., Albring, D., Sarmento, L., Lamb, L., Hass, M., von Poser, G. and Schapoval, E. E. S. (2003) 'Antimicrobial activity of some *Hypericum* species', *Phytomedicine*, 10(6-7), 511-516.

Danone 'Probiotics News', [online], available: www.probioticsnews.co.uk [accessed 20th April 2010].

Dart, R. K. (1996) *Microbiology for the Analytical Chemist*, Cambridge: The Royal Society Of Chemistry.

Davis, C. D. and Milner, J. A. (2009) 'Gastrointestinal microflora, food components and colon cancer prevention', *The Journal of Nutritional Biochemistry*, 20(10), 743-752.

De Gara, L., de Pinto, M. C. and Tommasi, F. (2003) 'The antioxidant systems vis-Ã - vis reactive oxygen species during plant pathogen interaction', *Plant Physiology and Biochemistry*, 41(10), 863-870.

De Mejía, E. G., Castaño-Tostado, E. and Loarca-Piña, G. (1999) 'Antimutagenic effects of natural phenolic compounds in beans', *Mutation Research*, 441(1), 1-9.

de Menezes, C. R., Silva, Í. S., Pavarina, É. C., Guímaro Dias, E. F., Guímaro Dias, F., Grossman, M. J. and Durrant, L. R. (2009) 'Production of xylooligosaccharides from enzymatic hydrolysis of xylan by the white-rot fungi *Pleurotus*', *International Biodeterioration & Biodegradation*, 63(6), 673-678.

de Vos, P., Faas, M. M., Spasojevic, M. and Sikkema, J. (2010) 'Encapsulation for preservation of functionality and targeted delivery of bioactive food components', *International Dairy Journal*, 20(4), 292-302.

Del Rio, D., Calani, L., Cordero, C., Salvatore, S., Pellegrini, N. and Brighenti, F. (2010) 'Bioavailability and catabolism of green tea flavan-3-ols in humans', *Nutrition*, 26(11-12), 1110-1116.

Del Rio, D., Calani, L., Scazzina, F., Jechiu, L., Cordero, C. and Brighenti, F. (2009) 'Bioavailability of catechins from ready-to-drink tea', *Nutrition*, 26(5), 528-533.

Del Rio, J. A., Fuster, M. D., Gomez, P., Porras, I., García-Lidón, A. and Ortuño, A. (2004) '*Citrus limon*: a source of flavonoids of pharmaceutical interest', *Food Chemistry*, 84(3), 457-461.

Del Rio, J. A., Fuster, M. D., Sabater, F., Porras, I., García-Lidón, A. and Ortuño, A. (1997) 'Selection of citrus varieties highly productive for the neohesperidin dihydrochalcone precursor', *Food Chemistry*, 59(3), 433-437.

Dermaxime (2010) 'Horse chestnut in anti-aging skin care products', [online], available: www.dermaxime.com [accessed 4th May 2010].

Dewick, P. M. (2011) *Medicinal Natural Products: A Biosynthetic Approach*, Third Edition, Southern Gate, Chichester, UK: John Wiley & Sons.

Dinsdale, M. (2000) 'Horse chestnut: Nature's help for varicose veins, hemmorhoids, and more', *Better Nutrition*, 32.

Domínguez, H., Alonso, J. L., Garrote, G., Parajó, J. C. and Vázquez, M. J. (2003) 'Xylooligosaccharides: properties and production', *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 2(1), 230-232.

Doonan, S. (1996) *Protein Puryfication Protocols, Methods in Moleculac Biology*, New Jersey: Human Press, Inc.

Drago, L., De Vecchi, E., Nicola, L. and Gismondo, M. (2007) 'In vitro evaluation of antibiotics' combinations for empirical therapy of suspected methicillin resistant *Staphylococcus aureus* severe respiratory infections', *BMC Infectious Diseases*, 7(1), 111.

DSMZ (2007) 'Medium 11', [online], available: http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium11.pdf [accessed 15th May 2010].

DSMZ (2008) 'Bifidobacterium medium', [online], available: http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium58.pdf [accessed 15th May 2010].

Dubin, P. L. (1988) *Aqueous Size-Exclusion Chromatography, Journal Of Chromatography Library*, Amsterdam: Elsevier Science Publishers B.V.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) 'Colorimetric Method for Determination of Sugars and Related Substances', *Analytical Chemistry*, 28(3), 350-356.

Dumitriu, S. (2005) *Polysaccharides: structural diversity and functional versatility*, Second Edition, New York: Marcel Dekker.

Duyff, R. L. (2006) *American Dietetic Association complete food and nutrition guide* New Jersey: John Wiley & Sons, Inc.

Edwards, J. (2009) 'Are spiders scared of conker chemicals?', *Advancing The Chemical Science* [online], available: http://www.rsc.org [accessed 15th March 2010].

Efferth, T., Li, P. C. H., Konkimalla, V. S. B. and Kaina, B. (2007) 'From traditional Chinese medicine to rational cancer therapy', *Trends in Molecular Medicine*, 13(8), 353-361.

El-Abyad, M. S., Morsi, N. M., Zaki, D. A. and Shaaban, M. T. (1990) 'Preliminary screening of some Egyptian weeds for antimicrobial activity', *Microbios,* 62, 47-57.

El-Safory, N. S., Fazary, A. E. and Lee, C.-K. (2010) 'Hyaluronidases, a group of glycosidases: Current and future perspectives', *Carbohydrate Polymers,* 81(2), 165-181.

ElSohly, H. N., Danner, S., Li, X. C., Nimrod, A. C. and Clark, A. M. (1999) 'New Antimycobacterial Saponin from *Colubrina retusa*', *Journal of Natural Products,* 62(9), 1341-1342.

EMEA (2009) 'Committee on Herbal Medicinal Products (HMPC),Community Herbal Monograph on *Aesculus Hippocastanum L.*,semen', *European Medicines Agency; Evaluation of Medicines for Human Use* [online], available: http://www.emea.europa.eu [accessed 4th May 2010].

ESCOP (2003) *Monographs: The Scientific Foundation for Herbal Medicinal Products,* United Kingdom: ESCOP-European Scientific Cooperative on Phytotherapy.

Estrada, A., Katselis, G. S., Laarveld, B. and Barl, B. (2000) 'Isolation and evaluation of immunological adjuvant activities of saponins from *Polygala senega L.*', *Comparative Immunology, Microbiology and Infectious Diseases,* 23(1), 27-43.

Extrasynthese (2011) 'Nature's contribution to chemicals, chemicals to serve the consumers ', [online], available: http://www.extrasynthese.com [accessed 10th January 2012].

Facino, R. M., Carini, M., Stefani, R., Aldini, G. and Saibene, L. (1995) 'Anti-Elastase and Anti-Hyaluronidase Activities of Saponins and Sapogenins from *Hedera helix*, *Aesculus hippocastanum,* and *Ruscus aculeatus*: Factors Contributing to their efficacy in the Treatment of Venous Insufficiency', *Archiv der Pharmazie,* 328(10), 720-724.

Fant, F., Vranken, W. F. and Borremans, F. A. M. (1999) 'The three-dimensional solution structure of *Aesculus hippocastanum* antimicrobial protein 1 determined by 1H nuclear magnetic resonance', *Proteins: Structure, Function, and Bioinformatics,* 37(3), 388-403.

FAO (2007) *FAO Technicial Meeting on Prebiotics,* Food Quality and Standards Service (AGNS) and Food and Agriculture Organization of the United Nations (FAO).

Farnworth, E. R. (2008) *Handbook of Fermented Functional Foods* Boca Raton: CRC Press.

Fennema, O. R. (1985) *Food chemistry,* M. Dekker.

Folkerts, G., Walzl, G. and Openshaw, P. J. M. (2000) 'Do common childhood infections 'teach' the immune system not to be allergic?', *Immunology Today*, 21(3), 118-120.

Fooks, L. J., Fuller, R. and Gibson, G. R. (1999) 'Prebiotics, probiotics and human gut microbiology', *International Dairy Journal*, 9(1), 53-61.

Fralish, J. S. and Franklin, S. B. (2002) *Taxonomy and Ecology of Woody Plants in North American Forests*, Texas: Tarleton State University Texas

Franck, A. (2002) 'Technological functionality of inulin and oligofructose', *British Journal of Nutrition*, 87, 287-291.

Frankenfeld, C. L., Atkinson, C., Thomas, W. K., Gonzalez, A., Jokela, T., Wähälä, K., Schwartz, S. M., Li, S. S. and Lampe, J. W. (2005) 'High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart', *British Journal of Nutrition*, 94(06), 873-876.

Fratianni, F., Coppola, R. and Nazzaro, F. (2011) 'Phenolic Composition and Antimicrobial and Antiquorum Sensing Activity of an Ethanolic Extract of Peels from the Apple Cultivar Annurca', *Journal of Medicinal Food*, 14(9), 957-963.

Freeman, B. C. and Beattie, G. A. (2008) 'An Overview of Plant Defenses against Pathogens and Herbivores', *The Plant Health Instructor*, available: http://www.apsnet.org [accessed 12th November 2011].

Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A. and Fernández-Gutiérrez, A. (2010) 'Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples', *Molecules*, 15(12), 8813-8826.

Garg, H. G. and Hales, C. A. (2004) *Chemistry and biology of hyaluronan*, Elsevier.

Gibson, G. R. (2004) 'Fibre and effects on probiotics (the prebiotic concept)', *Clinical Nutrition Supplements*, 1(2), 25-31.

Gibson, G. R. and Fuller, R. (2000) 'Aspects of In Vitro and In Vivo Research Approaches Directed Toward Identifying Probiotics and Prebiotics for Human Use', *Journal Nutrition*, 130(2), 391-.

Gibson, G. R. and Roberfroid, M. B. (1995) 'Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics', *Journal Nutrition*, 125(6), 1401-1412.

Gladine, C., Morand, C., Rock, E., Bauchart, D. and Durand, D. (2007) 'Plant extracts rich in polyphenols (PERP) are efficient antioxidants to prevent lipoperoxidation in plasma lipids from animals fed $n-3$ PUFA supplemented diets', *Animal Feed Science and Technology*, 136(3-4), 281-296.

Goni, I., Martin, N. and Saura-Calixto, F. (2005) 'In vitro digestibility and intestinal fermentation of grape seed and peel', *Food Chemistry*, 90(1-2), 281-286.

Gonzalez, A. G., Jimenez, I. A., Ravelo, A. G., Coll, J., Gonzalez, J. A. and Lloria, J. (1997) 'Antifeedant activity of sesquiterpenes from *Celastraceae*', *Biochemical Systematics and Ecology,* 25(6), 513-519.

Goodman (2012) 'Taxol', [online], available: http://www-jmg.ch.cam.ac.uk [accessed 20th January 2012].

Gordaliza, M., García, P. A., Miguel del Corral, J. M., Castro, M. A. and Gómez-Zurita, M. A. (2004) 'Podophyllotoxin: distribution, sources, applications and new cytotoxic derivatives', *Toxicon,* 44(4), 441-459.

Gosse, B., Gnabre, J., Bates, R. B., Dicus, C. W., Nakkiew, P. and Huang, R. C. C. (2002) 'Antiviral Saponins from *Tieghemella heckelii*', *Journal of Natural Products,* 65(12), 1942-1944.

Gray, R. and Bonner, J. (1948) 'Structure Determination and Synthesis of a Plant Growth Inhibitor, 3-Acetyl-6-methoxybenzaldehyde, Found in the Leaves of *Encelia Farinosa*', *Journal of the American Chemical Society,* 70(3), 1249-1253.

Greuter, W., Burdet, M. H. and Long, G. (1986) *Med-checklist: Dicotyledones* Berlin: Botanic Garden & Botanic Museum.

Grieve, M. (2009) 'A Modern Herbal: Horse Chestnut', [online], available: www.botanical.com [accessed 10th March 2010].

Guarrera, P. M. (1999) 'Traditional antihelmintic, antiparasitic and repellent uses of plants in Central Italy', *Journal of Ethnopharmacology,* 68(1-3), 183-192.

Guo, W., Xu, B., Yang, X. W., Liu, Q. and Cui, J. R. (2003) 'The anticancer effect of beta-escin sodium', *Chinese Pharmacological Bulletin,* 19(3), 351-352.

Gupta, V. K., Taneja, S. C. and Gupta, B. D. (2010) *Comprehensive Bioactive Natural Products, Volume 8 : Quality Control and Standardization,* Texas, USA: Global Media.

Habeeb, F., Shakir, E., Bradbury, F., Cameron, P., Taravati, M. R., Drummond, A. J., Gray, A. and Ferro , V. A. (2007a) 'Screening methods used to determine the anti-microbial properties of Aloe vera inner gel.', *Methods,* 42(4), 315-320.

Habeeb, F., Shakir, E., Bradbury, F., Cameron, P., Taravati, M. R., Drummond, A. J., Gray, A. L. and Ferro, V. A. (2007b) 'Screening methods used to determine the anti-microbial properties of *Aloe vera* inner gel', *Methods,* 42(4), 315-320.

HACH (1990) *Procedures Manual DR/2000 Spectrophotometer,* USA.

Haddadin, M. S. Y. (2010) 'Effect of Olive Leaf Extracts on the Growth and Metabolism of Two Probiotic Bacteria of Intestinal Origin', *Pakistan Journal of Nutrition,* 9(8), 787-793.

Hamid, R., Rotshteyn , Y., Rabadi, L., Parikh, R. and Bullock, P. (2004) 'Comparison of alamar blue and MTT assays for high through-put screening.', *Toxicology In Vitro*, 18(5), 703-710.

Hamilton-Miller, J. M. (1995) 'Antimicrobial properties of tea (*Camellia sinensis* L.)', *Antimicrobial Agents and Chemotherapy*, 39(11), 2375-7.

Han, L., Doverskog, M., Enfors, S.-O. and Häggström, L. (2002) 'Effect of glycine on the cell yield and growth rate of *Escherichia coli*: evidence for cell-density-dependent glycine degradation as determined by 13C NMR spectroscopy', *Journal of Biotechnology*, 92(3), 237-249.

Hanson, J. R. (2003) *Natural products: the secondary metabolites*, Cambridge, UK: The Royal Society of Chemistry.

Harinen, R.-R., Lampinen, J., Raitio, M. and Kytöniemi, V. (2010) 'Turbidometric Growth Curve Measurements and Liquid Evaporation Studies with a Microplate Photometer', [online], available: www.thermo.com/eThermo/CMA/PDFs/Various/File_54274.pdf [accessed 16th February 2012].

Harish, K. and Varghese, T. (2006) 'Probiotics in humans – evidence based review', *Calicut Medical Journal*, 4(4), e3.

Harper, J. and Moses, M. A. (2006) 'Molecular regulation of tumor angiogenesis: mechanisms and therapeutic implications', *EXS*, 96, 223-268.

Hartzfeld, P. W., Forkner, R., Hunter, M. D. and Hagerman, A. E. (2002) 'Determination of Hydrolyzable Tannins (Gallotannins and Ellagitannins) after Reaction with Potassium Iodate', *Journal of Agricultural and Food Chemistry*, 50(7), 1785-1790.

Hause, B. and Schaarschmidt, S. (2009) 'The role of jasmonates in mutualistic symbioses between plants and soil-born microorganisms', *Phytochemistry*, 70(13-14), 1589-1599.

Hayakawa, K., Mizutani, J., Wada, K., Masai, T., Yoshihara, I. and Mitsuoka, T. (1990) 'Effects of Soybean Oligosaccharides on Human Faecal Flora', *Microbial Ecology in Health and Disease*, 3(6), 293 - 303.

Hayashi, K., Sagesaka , Y. M., Suzuki, T. and Suzuki, Y. (2000) 'Inactivation of human type A and B influenza viruses by tea-seed saponins', *Bioscience, Biotechnology and Biochemistry*, 64(1), 184-6.

He, G., Dou, D., Wei, L., Alliston, K. R. and Groutas, W. C. (2010) 'Inhibitors of human neutrophil elastase based on a highly functionalized N-amino-4-imidazolidinone scaffold', *European Journal of Medicinal Chemistry*, 45(9), 4280-4287.

Healthcare (2011) 'Gel Filtration, Principles and Methods', available: http://www.gelifesciences.com/aptrix/upp00919.nsf/content/LD_153206006-R350 [accessed 30th October 2011].

Hedden, P. and Phillips, A. L. (2000) 'Gibberellin metabolism: new insights revealed by the genes', *Trends in Plant Science,* 5(12), 523-530.

Hobson, P. N. and Stewart, C. S. (1997) *The Rumen microbial ecosystem* Second Edition, London: Blackie Academic & Professional.

Hoffmann-Ribani, R., Huber, L. s. S. and Rodriguez-Amaya, D. B. (2009) 'Flavonols in fresh and processed Brazilian fruits', *Journal of Food Composition and Analysis,* 22(4), 263-268.

Hoffmann, D. (2003) *Medical herbalism: the science and practice of herbal medicine,* Healing Arts Press.

Holzapfel, W. H. and Schillinger, U. (2002) 'Introduction to pre- and probiotics', *Food Research International,* 35(2-3), 109-116.

Horn, R. C. and Vargas, V. M. F. o. (2003) 'Antimutagenic activity of extracts of natural substances in the Salmonella/microsome assay', *Mutagenesis,* 18(2), 113-118.

Hudzicki, J. (2009) 'Kirby-Bauer Disk Diffusion Susceptibility Test Protocol', available: http://www.microbelibrary.org/component/resource/laboratory-test/3189-kirby-bauer-disk-diffusion-susceptibility-test-protocol [accessed 26th October 2011].

Huebner, J., Wehling, R. L. and Hutkins, R. W. (2007) 'Functional activity of commercial prebiotics', *International Dairy Journal,* 17(7), 770-775.

Huebner, J., Wehling, R. L., Parkhurst, A. and Hutkins, R. W. (2008) 'Effect of processing conditions on the prebiotic activity of commercial prebiotics', *International Dairy Journal,* 18(3), 287-293.

Hui, Y. H. (2006) *Handbook of food science, technology, and engineering,* Boca Raton: CRC Press.

Huong, N. T., Matsumoto, K. and Watanabe, H. (1998) 'The antistress effect of majonoside-R2, a major saponin component of Vietnamese ginseng: neuronal mechanisms of action', *Methods and Findings in Experimental and Clinical Pharmacology,* 20(1), 65-76.

Hur, S., Whitcomb, F., Rhee, S., Park, Y. and Good, D. J. (2009) 'Effects of trans-10,cis-12 conjugated linoleic acid on body composition in genetically obese mice', *Journal of Medicinal Food,* 12(1), 56-63.

Hur, S. J. and Park, Y. (2007) 'Effect of conjugated linoleic acid on bone formation and rheumatoid arthritis', *European Journal of Pharmacology,* 568(1-3), 16-24.

Ifere, G. O., Barr, E., Equan, A., Gordon, K., Singh, U. P., Chaudhary, J., Igietseme, J. U. and Ananaba, G. A. (2009) 'Differential effects of cholesterol and phytosterols on cell proliferation, apoptosis and expression of a prostate specific gene in prostate cancer cell lines', *Cancer Detection and Prevention*, 32(4), 319-328.

Iorizzi, M., Lanzotti, V., Ranalli, G., De Marino, S. and Zollo, F. (2002) 'Antimicrobial Furostanol Saponins from the Seeds of *Capsicum annuum L. Var. acuminatum*', *Journal of Agricultural and Food Chemistry*, 50(15), 4310-4316.

Itabashi, M., Segawa, K., Ikeda, Y., Kondo, S., Naganawa, H., Koyano, T. and Umezawa, K. (1999) 'A new bioactive steroidal saponin, furcreastatin, from the plant *Furcraea foetida*', *Carbohydrate Research*, 323(1-4), 57-62.

James, A. T. and Martin, A. J. P. (1954) 'Gas-liquid chromatography: A Technique for the Analysis and Identification of Volatile Materials', *British Medical Bulletin*, 10(3), 170-176.

James, C. S. (1999) *Analytical Chemistry of Foods*, Gaithersburg, Maryland: An Aspen Publication, Inc.

Janick, J. and Paull, R. E. (2008) *The encyclopedia of fruit & nuts* Wallingford: CAB International.

Jansen, P. J., Lütjohann, D., Abildayeva, K., Vanmierlo, T., Plösch, T., Plat, J., Groen, A. K., Ramaekers, F. C., Kuipers, F. and Mulder, M. (2006) 'Dietary plant sterols accumulate in the brain', *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1761(4), 445-453.

Jay, J. M. (1996) *Modern Food Microbiology*, Fifth Edition, New York: Chapman & Hall.

Jung, H. W., Mahesh, R., Lee, J. G., Lee, S. H., Kim, Y. S. and Park, Y.-K. (2010) 'Pinoresinol from the fruits of *Forsythia koreana* inhibits inflammatory responses in LPS-activated microglia', *Neuroscience Letters*, 480(3), 215-220.

Just, M. J., Recio, M. C., Giner, R. M., Cuéllar, M. J., Máñez, S., Bilia, A. R. and Ríos, J. L. (1998) 'Anti-Inflammatory Activity of Unusual Lupane Saponins from *Bupleurum fruticescens*', *Planta Med*, 64(05), 404,407.

Kalliomäki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P. and Isolauri, E. (2001) 'Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial', *The Lancet*, 357(9262), 1076-1079.

Kanzaki, T., Morisaki, N., Shiina, R. and Saito, Y. (1998) 'Role of transforming growth factor-β pathway in the mechanism of wound healing by saponin from Ginseng Radix rubra', *British Journal of Pharmacology*, 125(2), 255-262.

Kaplan, H. and Hutkins, R. W. (2000) 'Fermentation of Fructooligosaccharides by Lactic Acid Bacteria and Bifidobacteria', *Applied and Environmental Microbiology*, 66(6), 2682–2684.

Kapoor, V. K., Dureja, J. and Chadha, R. (2009) 'Herbals in the control of ageing', *Drug Discovery Today*, 14(19-20), 992-998.

Kapusta, I., Janda, B., Szajwaj, B., Stochmal, A., Piacente, S., Pizza, C., Franceschi, F., Franz, C. and Oleszek, W. (2007) 'Flavonoids in Horse Chestnut (*Aesculus hippocastanum*) Seeds and Powdered Waste Water Byproducts', *Journal of Agricultural and Food Chemistry*, 55(21), 8485-8490.

Kartesz, J. T. (2009) 'Plants profile (*Aesculus hippocastanum* L. -horse chestnut)', [online], available: http://plants.usda.gov [accessed 10th March 2010].

Karthy, E., Ranjitha, P. and Mohankumar, A. (2009) 'Antimicrobial Potential of Plant Seed Extracts against Multidrug Resistant Methicillin Resistant *Staphylococcus aureus* (MDR-MRSA)', *International Journal of Biology* 1(1), 34-40.

Karuppusamy, S. and Rajasekaran, K. M. (2009) 'High Throughput Antibacterial Screening of Plant Extracts by Resazurin Redox with Special Reference to Medicinal Plants of Western Ghats', *Global Journal of Pharmacology*, 3(2), 63-68.

Katan, M. B., Grundy, S. M., Jones, P., Law, M., Miettinen, T. and Paoletti, R. (2003) 'Efficacy and Safety of Plant Stanols and Sterols in the Management of Blood Cholesterol Levels', *Mayo Clinic Proceedings*, 78(8), 965-978.

Kawaguti, H. Y. and Sato, H. H. (2007) 'Influence of the fermentation parameters and optimisation of isomaltulose production from free *Erwinia* sp. D12 cells using response surface methodology', *Process Biochemistry*, 42(3), 472-479.

Kazufumi, Y. (2006) 'ABC transporters involved in the transport of plant secondary metabolites', *FEBS Letters*, 580(4), 1183-1191.

Khan, R. (1993) *Low-calorie foods and food ingredients* Glasgow: Blackie Academic & Professionals.

Kiani, L. (2009) 'Bugs in Our Guts-Not All Bacteria Are Bad: How Probiotics Keep Us Healthy', *CSA Discovery Guides*, available: www.csa.com [accessed 10th April 2010].

Killeen, G. F., Madigan, C. A., Connolly, C. R., Walsh, G. A., Clark, C., Hynes, M. J., Timmins, B. F., James, P., Headon, D. R. and Power, R. F. (1998) 'Antimicrobial Saponins of *Yucca schidigera* and the Implications of Their in Vitro Properties for Their in Vivo Impact', *Journal of Agricultural and Food Chemistry*, 46(8), 3178-3186.

Kim, D. S., Oh, S. R., Lee, I. S., Jung, K. Y., Park, J. D., Kim, S. I. and Lee, H.-K. (1998) 'Anticomplementary activity of ginseng saponins and their degradation products', *Phytochemistry,* 47(3), 397-399.

Kim, Y., Noh, Y. K., ImLee, G., Lee, Y. K. K. K. s. and Min, K. R. (1995) 'Inhibitory Effects of Herbal Medicines on Hyaluronidase Activity', *Korean Journal of Pharmacology,* 26(3), 265-272.

King, T., Dykes, G. and Kristianti, R. (2008) 'Comparative evaluation of methods commonly used to determine antimicrobial susceptibility to plant extracts and phenolic compounds', *The Journal of AOAC INTERNATIONAL,* 91(6), 1423-9.

Kinjo, J., Yokomizo, K., Hirakawa, T., Shii, Y., Nohara, T. and Uyeda, M. (2000) 'Anti-herpes virus activity of *fabaceous* triterpenoidal saponins', *Biological & Pharmaceutical Bulletin,* 23(7), 887-9.

Koch, S., Eugster-Meier, E., Oberson, G., Meile, L. and Lacroix, C. (2008) 'Effects of strains and growth conditions on autolytic activity and survival to freezing and lyophilization of *Lactobacillus delbrueckii ssp. lactis* isolated from cheese', *International Dairy Journal,* 18(2), 187-196.

Koffi, E., Sea, T., Dodehe, Y. and Soro, S. (2010) 'Effect of solvent type on extraction of polyphenols from twenty three Ivorian plants', *Journal of Animal and Plant Sciences,* 5(3), 550-558.

Konishi, M., Hano, Y., Takayama, M., Nomura, T., Hamzah, A. S., Ahmad, R. b. and Jasmani, H. (1998) 'Triterpenoid saponins from *Hedyotis nudicaulis*', *Phytochemistry,* 48(3), 525-528.

Koolman, J. and Rahm, K. H. (2005) *Color atlas of biochemistry,* Thieme.

Kore, A. R. and Shanmugasundaram, M. (2010) 'Design, synthesis and inhibitory effect of pentapeptidyl chloromethyl ketones on proteinase K', *Bioorganic & Medicinal Chemistry,* 18(23), 8383-8387.

Küçükkurt, I., Ince, S., Keleş, H., Akkol, E., Avci, G., Yeşilada, E. and Bacak, E. (2010) 'Beneficial effects of *Aesculus hippocastanum* L. seed extract on the body's own antioxidant defense system on subacute administration', *Journal of Ethnopharmacology,* 129(1), 18-22.

L'homme, C., Arbelot, M., Puigserver, A. and Biagini, A. (2003) 'Kinetics of hydrolysis of fructooligosaccharides in mineral-buffered aqueous solutions: influence of pH and temperature', *Journal of Agricultural and Food Chemistry,* 51(1), 224-8.

Lack, H. W. (2000) 'The Discovery and Rediscovery of the Horse Chestnut', *Curtis's Botanical Magazine,* 17(2), 109-137.

Lakshmi, T. and Ravishankar, P. (2011) 'In-Vitro Antibacterial Activity of Ethanolic Seed Extract of *Aesculus Hippocastanum* Against *E. faecalis*', *International Journal of Pharmaceutical Research,* 3(4), 85-87.

Lambert, J. and Muir, T. A. (1968) *Practical chemistry,* Third Edition, London: Heineman.

Lattanzio, V., Lattanzio, V. M. T. and Cardinali, A. (2006) 'Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects', *Phytochemistry: Advances in Research,* 23-67.

Lee, K. K., Cho, J. J., Park, E. J. and Choi, J. D. (2001) 'Anti-elastase and anti-hyaluronidase of phenolic substance from *Areca catechu* as a new anti-ageing agent', *International Journal of Cosmetic Science,* 23(6), 341-346.

Lee, O.-H. and Lee, B.-Y. (2009) 'Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract', *Bioresource Technology,* 101(10), 3751-3754.

Lee, S.-J., Sung, J.-H., Lee, S.-J., Moon, C.-K. and Lee, B.-H. (1999) 'Antitumor activity of a novel ginseng saponin metabolite in human pulmonary adenocarcinoma cells resistant to cisplatin', *Cancer Letters,* 144(1), 39-43.

Lee, Y.-B., Lee, H. J. and Sohn, H. S. (2005) 'Soy isoflavones and cognitive function', *The Journal of Nutritional Biochemistry,* 16(11), 641-649.

Lesschaeve, I. and Noble, A. C. (2005) 'Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences', *The American Journal of Clinical Nutrition,* 81(1), 330S-335S.

Levri, K. M., Ketvertis, K., Deramo, M., Merenstein, J. H. and D'Amico, F. (2005) 'Do probiotics reduce adult lactose intolerance? A systematic review', *The Journal of Family Practice,* 54(7).

lfbiotec (2010) [online], available: www. fbiotec.com [accessed 25th April 2010].

Li, D. W., Lee, E. B., Kang, S. S., Hyun, J. E. and Whang, W. K. (2002) 'Activity-Guided Isolation of Saponins from *Kalopanax pictus* with Anti-inflammatory Activity', *Chemical & Pharmaceutical Bulletin,* 50(7), 900-903.

Li, J., Liu, X., Dong, F., Xu, J., Li, Y., Shan, W. and Zheng, Y. (2011) 'Potential allelopathic effects of volatile oils from *Descurainia sophia (L.)* Webb ex Prantl on wheat', *Biochemical Systematics and Ecology,* 39(1), 56-63.

Li, M., Kai, Y., Qiang, H. and Dongying, J. (2006) 'Biodegradation of gallotannins and ellagitannins', *Journal of Basic Microbiology,* 46(1), 68-84.

Li, W., Xiang, X., Tang, S., Tang, Y., Sun, Y. and Zeng, X. (2008) 'Enzymatic synthesis of functional oligosaccharides as prebiotics by the use of glycosidases', *Journal of Biotechnology,* 136(Supplement 1), S365-S365.

Li, X.-C., ElSohly, H. N., Nimrod, A. C. and Clark, A. M. (1999) 'Antifungal Jujubogenin Saponins from *Colubrina retusa*', *Journal of Natural Products*, 62(5), 674-677.

Li, Y.-J., Zhao, G.-Y., Du, W. and Zhang, T.-J. (2009) 'Effect of dietary isomaltooligosaccharides on nutrient digestibility and concentration of glucose, insulin, cholesterol and triglycerides in serum of growing pigs', *Animal Feed Science and Technology*, 151(3-4), 312-315.

Liao, B., Newmark, H. and Zhou, R. (2002) 'Neuroprotective Effects of Ginseng Total Saponin and Ginsenosides Rb1 and Rg1 on Spinal Cord Neurons in Vitro', *Experimental Neurology*, 173(2), 224-234.

Lim, H.-K., Kim, H.-S., Choi, H.-S., Oh, S. and Choi, J. (2000) 'Hepatoprotective effects of bergenin, a major constituent of *Mallotus japonicus*, on carbon tetrachloride-intoxicated rats', *Journal of Ethnopharmacology*, 72(3), 469-474.

Lin, Y.-S., Chen, W.-C. and Lee, W.-C. (2008) 'Production of galactooligosaccharides using truncated beta-galactosidase from *Bifidobacterium bifidum*', *Journal of Biotechnology*, 136(Supplement 1), S738-S738.

Lindsey, J. K. (2005) '*Aesculus hippocastanum Linnaeus 1753* ', [online], available: www.commanster.eu [accessed 10th March 2010].

Liong, M.-T. (2008) 'Roles of Probiotics and Prebiotics in Colon Cancer Prevention: Postulated Mechanisms and In-vivo Evidence', *International Journal of Molecular Sciences*, 9(5), 854-863.

Liu, Y. Z., Cao, Y. G., Ye, J. Q., Wang, W. G., Song, K. J., Wang, X. L., Wang, C. H., Li, R. T. and Deng, X. M. (2009) 'Immunomodulatory effects of proanthocyanidin A-1 derived in vitro from *Rhododendron spiciferum*', *Fitoterapia*, 81(2), 108-114.

Loew, D., Schroedter, A., Schwankl, W. and Maerz, R. W. (2000) 'Measurement of the bioavailability of aescin-containing extracts', *Methods & Findings in Experimental & Clinical Pharmacology*, 22(7), 537.

López-Molina, D., Navarro-Martínez, M. D., Rojas-Melgarejo, F., Hiner, A. N. P., Chazarra, S. and Rodríguez-López, J. N. (2005) 'Molecular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.)', *Phytochemistry*, 66(12), 1476-1484.

Lowery, T. H. and Richardson, K. S. (1987) *Mechanism and Theory in Organic Chemistry*, Third Edition, New York: Harper Collins Publishers.

Luthria, D. L., Biswas, R. and Natarajan, S. (2007) 'Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean', *Food Chemistry*, 105(1), 325-333.

Macfarlane, G. T., Steed, H. and Macfarlane, S. (2008) 'Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics', *Journal of Applied Microbiology,* 104(2), 305-344.

Macpherson, A. J. and Harris, N. L. (2004) 'Interactions between commensal intestinal bacteria and the immune system', *Nature Reviews Immunology,* 4(6), 478-485.

Madigan, M. T., Martinko, J. M. and Parker, J. (2000) *Brock: Biology of Microorganisms,* 9th ed., New Jersey: Prentice Hall.

Mann, J., Davidson, S., Hobbs, J., Banthorpe, D. and Harborne, J. (1994) *Natural products: their chemistry and biological significance,* Harlow, UK: Longman Scientific & Technical.

Markham, K. R. (1984) 'The structures of amentoflavone glycosides isolated from *Psilotum nudum*', *Phytochemistry,* 23(9), 2053-2056.

Martens, S. and Mithafer, A. (2005) 'Flavones and flavone synthases', *Phytochemistry,* 66(20), 2399-2407.

Marth, C., Lang, T., Widschwendter, M., Müller-Holzner, E. and Daxenbichler, G. (1995) 'Effects of taxol on choriocarcinoma cells', *American Journal of Obstetrics and Gynecology,* 173(6), 1835-1842.

Marvin, C. M. (1994) *HPLC, a practical user's guide,* New York: Wiley-VCH.

Matsuda, H., Li, Y., Murakami, T., Ninomiya, K., Araki, N., Yoshikawa, M. and Yamahara, J. (1997) 'Antiinflammatory effects of escins Ia, Ib, IIa, and IIb from horse chestnut, the seeds of *Aesculus hippocastanum L.*', *Bioorganic & Medicinal Chemistry Letters,* 7(13), 1611-1616.

Matsuda, H., Li, Y., Murakami, T., Yamahara, J. and Yoshikawa, M. (1999) 'Effects of escins Ia, Ib, IIa, and IIb from horse chestnuts on gastric emptying in mice', *European Journal of Pharmacology,* 368(2-3), 237-243.

Mattana, C. M., Satorres, S. E., Sosa, A., Fusco, M. and Alcaráz, L. E. (2010) 'Antibacterial activity of extracts of *Acacia aroma* against methicillin-resistant and methicillin-sensitive *Staphylococcus*', *Brazilian Journal of Microbiology,* 41(581-587).

Matusek, A., Merész, P., Le, T. and Örsi, F. (2011) 'Fructo-oligosaccharide degradation in apple pulp matrix', *Acta Alimentaria,* 40(2), 182-193.

Maurya, D. K. and Devasagayam, T. P. A. (2010) 'Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids', *Food and chemical toxicology an international journal published for the British Industrial Biological Research Association,* 48(12), 3369-3373.

McIlvaine, T. C. (1921) 'A buffer solution for colorimetric comparition', *Journal of Biological Chemistry,* 49(1), 183-186.

McKenna, D. J., Jones, K. and Hughes, K. (2002) *Botanical medicines: the desk reference for major herbal supplements*, 2nd ed., New York: The Haworth Herbal Press, Inc.

McLellan, M. C. (2000) 'Horse Chestnut (*Aesculus hippocastanum*)', available: http://www.longwoodherbal.org/horsechestnut/horsechestnut.pdf [accessed 28th January 2012].

McRae, J. M. and Kennedy, J. A. (2011) 'Wine and Grape Tannin Interactions with Salivary Proteins and Their Impact on Astringency: A Review of Current Research', *Molecules*, 16(3), 2348-2364.

Mecham, R. P. (2011) *The Extracellular Matrix: An Overview*, Springer.

Meissner, M. H., Eklof, B., Smith, P. C., Dalsing, M. C., DePalma, R. G., Gloviczki, P., Moneta, G., Neglén, P., O' Donnell, T., Partsch, H. and Raju, S. (2007) 'Secondary chronic venous disorders', *Journal of Vascular Surgery*, 46(6, Supplement 1), S68-S83.

Mendilaharsu, M., De Stefani, E., Deneo-Pellegrini, H., Carzoglio, J. and Ronco, A. (1998) 'Phytosterols and risk of lung cancer: A case-control study in Uruguay', *Lung Cancer*, 21(1), 37-45.

Michels, L. (2008) 'Pflanzenbilder', [online], available: http://www.imagines-plantarum.de/ [accessed 15th March 2010].

Mimaki, Y., Kuroda, M., Kameyama, A., Yokosuka, A. and Sashida, Y. (1998a) 'Aculeoside B, a New Bisdesmosidic Spirostanol Saponin from the Underground Parts of *Ruscus aculeatus*', *Journal of Natural Products*, 61(10), 1279-1282.

Mimaki, Y., Kuroda, M., Kameyama, A., Yokosuka, A. and Sashida, Y. (1998b) 'Steroidal saponins from the rhizomes of *Hosta sieboldii* and their cytostatic activity on HL-60 cells', *Phytochemistry*, 48(8), 1361-1369.

Mio, K. and Stern, R. (2002) 'Inhibitors of the hyaluronidases', *Matrix Biology*, 21(1), 31-37.

MN (2011) 'Basic principles of TLC', 268-312, available: www.mn-net.com [accessed 31st October 2011].

Mohammed, M., J, Al-Bayati and Firas, A. (2009) 'Isolation and identification of antibacterial compounds from *Thymus kotschyanus* aerial parts and *Dianthus caryophyllus* flower buds', available: http://www.thefreelibrary.com [accessed 28th October 2011].

Molecular (2012) [online], available: http://www.molecular-networks.com [accessed 20th January 2012].

Morais, M. B. d. and Jacob, C. M. A. (2006) 'The role of probiotics and prebiotics in pediatric practice', *Jornal de Pediatria,* 82, S189-S197.

Morgan, W. T. and Elson, L. A. (1933) ' A colorimetric method for the determination of glucosamine and chondrosamine', *Biochemical Journal,* 27(6), 1824-1828.

Morita, S., Nakatani, S., Koshiba, T., Masumura, T., Ogihara, Y. and Tanaka, K. (2011) 'Differential Expression of Two Cytosolic Ascorbate Peroxidases and Two Superoxide Dismutase Genes in Response to Abiotic Stress in Rice', *Rice Science,* 18(3), 157-166.

Mshvildadze, V., Favel, A., Delmas, F., Elias, R., Faure, R. and Decanosidze G, K. E., Balansard G. (2000) 'Antifungal and antiprotozoal activities of saponins from *Hedera colchica*', *Pharmazie,* 55(4), 325-6.

Mueller, C. and Macpherson, A. J. (2006) 'Layers of mutualism with commensal bacteria protect us from intestinal inflammation', *Gut,* 55(2), 276-284.

Murugan, M., Mohan, V. R. and Thamodharan, V. (2012) 'Phytochemical screening and antibacterial activity of *Gymnema sylvestre* (Retz) R . Br ex. Schultes and *Morinda pubescens* J.E. Smith var. *pubescens*', *Journal of Applied Pharmaceutical Science,* 2(2), 73-76.

Mussatto, S. I. and Mancilha, I. M. (2007) 'Non-digestible oligosaccharides: A review', *Carbohydrate Polymers,* 68(3), 587-597.

Nadarajah, D. (2008) *Purification and characterization of mouse pancreatic elastase,* Stephen F. Austin State University.

Nakakuki, T. (2002) 'Present status and future of functional oligosaccharide development in Japan', *Pure and Appied Chemistry,* 74(7), 1245-1251.

Navarro, M. C., Montilla, M. P., Cabo, M. M., Galisteo, M., Cáceres, A., Morales, C. and Berger, I. (2003) 'Antibacterial, Antiprotozoal and Antioxidant Activity of Five Plants Used in Izabal for Infectious Diseases', *Phythotherapy Research,* 17, 325-329.

Niu, Y.-P., Li, L.-D. and Wu, L.-M. (2008) 'Beta-aescin: A potent natural inhibitor of proliferation and inducer of apoptosis in human chronic myeloid leukemia K562 cells in vitro', *Leukemia & Lymphoma,* 49(7), 1384-1391.

Nocerino, E., Amato, M. and Izzo, A. A. (2000) 'The aphrodisiac and adaptogenic properties of ginseng', *Fitoterapia,* 71(Suppl 1:S-5).

O'Flaherty, S. and Klaenhammer, T. R. (2010) 'The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and gut/host', *International Dairy Journal,* 20(4), 262-268.

O'Neil, M. J., Heckelman, P. E., Koch, C. B. and Roman, K. J. (2006) *The Merck Index: An encyclopedia of chemicals, drugs and biologicals,* 14th ed., USA: Merck & Co., Inc.

O'Toole, P. W. and Claesson, M. J. (2010) 'Gut microbiota: Changes throughout the lifespan from infancy to elderly', *International Dairy Journal,* 20(4), 281-291.

Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T. and Yoshikawa, M. (2000) 'Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants', *The Journal of Biological Chemistry,* 381(1), 67-74.

Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, K. and Vuorela, P. (2000) 'Antimicrobial activity of some coumarin containing herbal plants growing in Finland', *Journal of Ethnopharmacology,* 73(1-2), 299-305.

Oleszek, W. and Marston, A. (2000) *Saponins in food, feedstuffs and medicinal plants* Dordrecht: Kluwer Academic Publishers.

Osborn, R. W., De Samblanx, G. W., Thevissen, K., Goderis, I., Torrekens, S., Van Leuven, F., Attenborough, S., Rees, S. B. and Broekaert, W. F. (1995) 'Isolation and characterisation of plant defensins from seeds of *Asteraceae*, *Fabaceae*, *Hippocastanaceae* and *Saxifragaceae*', *FEBS Letters,* 368(2), 257-262.

Ozturk, M., Aydogmus-Ozturk, F., Duru, M. E. and Topcu, G. (2007) 'Antioxidant activity of stem and root extracts of Rhubarb (*Rheum ribes*): An edible medicinal plant', *Food Chemistry,* 103(2), 623-630.

Papadopoulos, G. and Boskou, D. (1991) 'Antioxidant effect of natural phenols on olive oil', *Journal of the American Oil Chemists' Society,* 68(9), 669-671.

Parab, R. S. and Mengi, S. A. (2002) 'Hypolipidemic activity of *Acorus calamus L.* in rats', *Fitoterapia,* 73(6), 451-5.

Parasa, L. S., Sunita T, Babu Rao, K., Hanumantha Rao, A., Srinivasa Rao, J. and Cyril Arun Kumar, L. (2011) 'Acetone extract of Cashew (*Anacardium occidentale, L.*) nuts shelliquid against Methicillin resistant *Staphylococcus aureus* (MRSA) by minimum inhibitory concentration (MIC)', *Journal of Chemical and Pharmaceutical Research,* 3(5), 736-742.

Park, Y. (2009) 'Conjugated nonadecadienoic acid is more potent than conjugated linoleic acid on body fat reduction', *The Journal of Nutritional Biochemistry*.

Pathak, Y. (2009) *Handbook of Nutraceuticals Ingredients. Formulations and Applications,* Boca Raton: CRC Press.

Patri, G., Silano, V. and Anton, R. (2006) *Plants in Cosmetics: Potentially Harmful Components,* Strasbourg: Council of Europa Publishing.

Pearse, A. G. E. and Stoward, P. J. (1980) *Histochemistry: theoretical and applied,* Forth Edition, Edinburgh: Churchill Livingstone.

Pei-Ren, L., Roch, C., Cheng-Chun, C. and Ya-Hui, T. (2002) 'Antimutagenic activity of several probiotic bifidobacteria against Benzo[a]pyrene', *Journal of Bioscience and Bioengineering*, 94(2), 148-153.

Peña-Méndez, E. M., Hernández-Suárez, M., Díaz-Romero, C. and Rodríguez-Rodríguez, E. (2008) 'Characterization of various chestnut cultivars by means of chemometrics approach', *Food Chemistry*, 107(1), 537-544.

Pereira, A. P., Ferreira, I. C. F. R., Marcelino, F., Valentão, P., B., P., Andrade, Seabra, R., Estevinho, L., Bento, A. and Pereira, o. A. (2007) 'Phenolic Compounds and Antimicrobial Activity of Olive (*Olea europaea* L. Cv. Cobrançosa) Leaves', *Molecules*, 12, 1153-1162.

Perez-Jimenez, J., Arranz, S. and Saura-Calixto, F. (2009) 'Proanthocyanidin content in foods is largely underestimated in the literature data: An approach to quantification of the missing proanthocyanidins', *Food Research International*, 42(10), 1381-1388.

Phenomenex (2011) 'Solvent miscibility table ', [online], available: http://www.phenomenex.com/HPLC2011 [accessed 31 October 2011].

Piechal, A., Blecharz-Klin, K. and Widy-Tyszkiewicz, E. (2005) 'Horse chestnut (*Aesculus hippocastani*) in contemporary therapy', *Przewodnik lekarza*, 4, 74-81.

Piwowarski, J. P., Kiss, A. K. and Kozlowska-Wojciechowska, M. (2011) 'Anti-hyaluronidase and anti-elastase activity screening of tannin-rich plant materials used in traditional Polish medicine for external treatment of diseases with inflammatory background', *Journal of Ethnopharmacology*, 137(1), 937-941.

Pryce, R. J. (1972) 'Metabolism of lunularic acid to a new plant stilbene by *Lunularia cruciata*', *Phytochemistry*, 11(4), 1355-1364.

Pulido, R., Bravo, L. and Saura-Calixto, F. (2000) 'Antioxidant Activity of Dietary Polyphenols As Determined by a Modified Ferric Reducing/Antioxidant Power Assay', *Journal of Agricultural and Food Chemistry*, 48(8), 3396-3402.

Rabiu, B. A., Jay, A. J., Gibson, G. R. and Rastall, R. A. (2001) 'Synthesis and Fermentation Properties of Novel Galacto-Oligosaccharides by {beta}-Galactosidases from *Bifidobacterium* Species', *Applied Environmental Microbiology*, 67(6), 2526-2530.

Rameshwar Singh, R. (2010) 'Mechanism of action of insecticidal secondary metabolites of plant origin', *Crop Protection*, 29(9), 913-920.

Rastall, R. A. and Maitin, V. (2002) 'Prebiotics and synbiotics: towards the next generation', *Current Opinion in Biotechnology*, 13(5), 490-496.

Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kujala, T., Pihlaja, K., Vuorela, H. and Vuorela, P. (2000) 'Antimicrobial effects of Finnish plant extracts

containing flavonoids and other phenolic compounds', *International Journal of Food Microbiology,* 56(1), 3-12.

Rechner, A. R. and Kroner, C. (2005) 'Anthocyanins and colonic metabolites of dietary polyphenols inhibit platelet function', *Thrombosis Research,* 116(4), 327-334.

Resta-Lenert, S. and Barrett, K. E. (2003) 'Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC)', *Gut,* 52(7), 988-997.

Ridoux, O., Di Giorgio, C., Delmas, F., Elias, R., Mshvildadze, V., Dekanosidze, G., Kemertelidze, E., Balansard, G. and Timon-David, P. (2001) 'In vitro antileishmanial activity of three saponins isolated from ivy, α-hederin, β-hederin and hederacolchiside A1, in association with pentamidine and amphotericin B', *Phytotherapy_Research,* 15(4), 298-301.

Roberfroid, M. B. (2005) *Inulin-type fructans: functional food ingredients, CRC Series in Modern Nutrition,* Boca Raton: CRC Press.

Rode, H., Koerbe, S., Besch, A., Methling, K., Loose, J. and Otto, H.-H. (2006) 'Synthesis and in vitro evaluation of pseudosaccharinamine derivatives as potential elastase inhibitors', *Bioorganic & Medicinal Chemistry,* 14(8), 2789-2798.

Ross, R. P., Mills, S., Hill, C., Fitzgerald, G. F. and Stanton, C. (2010) 'Specific metabolite production by gut microbiota as a basis for probiotic function', *International Dairy Journal,* 20(4), 269-276.

Roy, A., Geetha, R. V. and Lakshmi, T. (2011a) 'Evaluation of the Antimycotic Activity of Aqueous and Ethanolic Extracts of *Aesculus hippocastanum* – An In Vitro Study', *International Journal of Drug Development & Research,* 3(3), 335-338.

Roy, A., Geetha, R. V. and Lakshmi, T. (2011b) 'In Vitro Evaluation of Antibacterial Activity Of Aqueous And Ethanolic Extracts Of *Aesculus Hippocastanum* On Oral Microbes', *Asian Journal of Pharmaceutical and Clinical Research,* 4(4), 90-92.

Royce, P. M. and Steinmann, B. U. (2002) *Connective tissue and its heritable disorders: molecular, genetic, and medical aspects,* Second Edition, New York: Wiley-Liss.

Rycroft, C. E., Jones, M. R., Gibson, G. R. and Rastall, R. A. (2001) 'A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides', *Journal of Applied Microbiology,* 91(5), 878-887.

Sahasrabudhe, A. and Deodhar, M. (2010) 'Anti-hyaluronidase, Anti-elastase Activity of *Garcinia indica*', *International Journal of Botany,* 6(3), 299-303.

Salminen, S., Wright, A. and Ouwehand, A. (2004) *Lactic acid bacteria: microbiological and functional aspects* New York: Marcel Dekker, Inc.

Sandie, L. (1992) *High performance liquid chromatography, Analytical Chemistry by Open Learning*, second ed., London: John Wiley & Sons.

Sato, I., Suzuki, T., Kobayashi, H. and Tsuda, S. (2005) 'Antioxidative and antigenotoxic effects of Japanese horse chestnut (*Aesculus turbinata*) seeds', *The Journal of Veterinary Medical Science*, 67(7), 731-4.

Scalbert, A. (1991) 'Antimicrobial properties of tannins', *Phytochemistry*, 30(12), 3875-3883.

Schofield, P., Mbugua, D. M. and Pell, A. N. (2001) 'Analysis of condensed tannins: a review', *Animal Feed Science and Technology*, 91(1-2), 21-40.

Schulzke, J. D., Ploeger, S., Amasheh, M., Fromm, A., Zeissig, S., Troeger, H., Richter, J., Bojarski, C., Schumann, M. and Fromm, M. (2009) 'Epithelial tight junctions in intestinal inflammation', *Annals of the New York Academy of Sciences*, 1165, 294-300.

Sebedio, J. L., Christie, W. W. and Adolf, R. (2003) *Advances in Conjugated Linoleic Acid Research*, AOCS Press.

Seigler, D. S. (1998) *Plant secondary metabolism*, Massachusetts, USA, Kluwer Academic.

Semenova, M. G., Antipova, A. S. and Belyakova, L. E. (2002) 'Food protein interactions in sugar solutions', *Current Opinion in Colloid & Interface Science*, 7(5-6), 438-444.

Sharma, A., Patel, V. K., Rawat, S., Ramteke, P. and Verma, R. (2010) 'Identification of the antibacterial component of some Indian medicinal plants against *Klebsiella pneumoniae*', *International Journal of Pharmacy and Pharmaceutical Sciences*, 2(3).

Sharma, O. P. and Bhat, T. K. (2009) 'DPPH antioxidant assay revisited', *Food Chemistry*, 113(4), 1202-1205.

Shea-Donohue, T., Urban Jr, J. F., Notari, L. and Zhao, A. (2010) 'Immune regulation of epithelial cell function: Implications for GI pathologies', *International Dairy Journal*, 20(4), 248-252.

Shetty, K. (2006) *Food biotechnology*, Second Edition., Boca Raton: CRC Press.

Shimada, T. (2001) 'Nutrient compositions of acorns and horse chestnuts in relation to seed-hoarding', *Ecological Research*, 16(4), 803-808.

Shinguh, Y., Yamazaki, A., Inamura, N., Fujie, K., Okamoto, M., Nakahara, K., Notsu, Y., Okuhara, M. and Ono, T. (1998) 'Biochemical and pharmacological characterization of FR134043, a novel elastase inhibitor', *European Journal of Pharmacology*, 345(3), 299-308.

Shortt, C. and O'Brien, J. (2004) *Handbook of functional dairy products,* Boca Raton: CRC Press.

Sies, H. (1997) 'Oxidative stress: oxidants and antioxidants', *Experimental Physiology,* 82(2), 291-295.

Sies, H., Stahl, W. and Sundquist, A. R. (1992) 'Antioxidant Functions of Vitamins', *Annals of the New York Academy of Sciences,* 669(1), 7-20.

Silanikove, N., Perevolotsky, A. and Provenza, F. D. (2001) 'Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants', *Animal Feed Science and Technology,* 91(1-2), 69-81.

Simões, C. M. O., Amoros, M. and Girre, L. (1999) 'Mechanism of antiviral activity of triterpenoid saponins', *Phytotherapy_Research,* 13(4), 323-328.

Sindambiwe, J. B., Calomme, M., Geerts, S., Pieters, L., Vlietinck, A. J. and Vanden Berghe, D. A. (1998) 'Evaluation of Biological Activities of Triterpenoid Saponins from *Maesa lanceolata*', *Journal of Natural Products,* 61(5), 585-590.

Singh, M., Sharma, R. and Banerjee, U. C. (2002) 'Biotechnological applications of cyclodextrins', *Biotechnology Advances,* 20(5-6), 341-359.

Sinha, N. K., Hui, Y. H., Evranuz, E., Siddiq, M. and Ahmed, J. (2010) *Handbook of Vegetables and Vegetable Processing,* Iowa, USA: John Wiley & Sons.

Sinha, S. N., Saha, G. C. and Biswas, M. (2010) 'Screening of Various Solvent Extracts of *Gymnema sylvestre* R.Br. Leaf for Antibacterial Activity', *Advances in Bioresearch,* 1(2).

Sirtori, C. R. (2001) 'Aescin: pharmacology, pharmacokinetics and therapeutic profile', *Pharmacological Research,* 44(3), 183-193.

Son, K. H., Kwak, W. J., Han, C. K., Chang, H. W., Kim, Y. P. and Kang, S. K. (2003) 'Loniceroside C, a new antiinflammatory triterpene glycoside from the aerial parts of *Lonicera japonica* (5 kg)', *Chemical & Pharmaceutical Bulletin,* 51(3), 333-335.

Sparg, S. G., Light, M. E. and van Staden, J. (2004) 'Biological activities and distribution of plant saponins', *Journal of Ethnopharmacology,* 94(2-3), 219-243.

Sproll, C., Ruge, W., Andlauer, C., Godelmann, R. and Lachenmeier, D. W. (2008) 'HPLC analysis and safety assessment of coumarin in foods', *Food Chemistry,* 109(2), 462-469.

Srivastava, G. (2009) *Fundamentals Of Bioanalytical Techniques And Instrumentation,* Prentice-Hall Of India Pvt. Limited.

Stahl, E. (1983) 'A Quarter Century of Thin-Layer Chromatography—An Interim Report', *Angewandte Chemie International Edition in English*, 22(7), 507-516.

Stalikas, C. D. (2007) 'Extraction, separation, and detection methods for phenolic acids and flavonoids', *Journal of Separation Science*, 30(18), 3268-3295.

Stanic, G., Juristic, B. and Brkic, D. (1999) 'HPLC Analysis of Esculin and Fraxin in Horse Chestnut Bark (*Aesculus hippocastanum L.*)', *Croatica Chemica Acta*, 72(4), 827-834.

Stavros, K. (2000) *Practical Problem Solving in HPLC*, Weinheim: Wiley-Vch.

Tamime, A. Y. (2005) *Probiotic dairy products*, Oxford: Blackwell Publishing Ltd.

Tang, T., Yin, L., Yang, J. and Shan, G. (2007) 'Emodin, an anthraquinone derivative from *Rheum officinale* Baill, enhances cutaneous wound healing in rats', *European Journal of Pharmacology*, 567(3), 177-185.

Tannock, G. W. (2002) *Probiotics and prebiotics: where are we going?*, Norfolk: Caister Academic Press.

Tannock, G. W. (2005) *Probiotics and prebiotics: scientific aspects* Norfolk: Caister Academic Press.

Taylor, K. (1995) 'A modification of the phenol/sulfuric acid assay for total carbohydrates giving more comparable absorbances', *Applied Biochemistry and Biotechnology*, 53(3), 207-214.

Technologies, L. (2011) 'AlamarBlue® - Rapid & Accurate Cell Health Indicator', [online], available: http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/alamarBlue-Rapid-and-Accurate-Cell-Health-Indicator.html [accessed 28 October 2011].

Tomaševič, M., Vukmirovič, Z., Rajšič, S., Tasič, M. and Stevanovič, B. (2008) 'Contribution to biomonitoring of some trace metals by deciduous tree leaves in urban areas', *Environmental Monitoring and Assessment*, 137(1), 393-401.

Topol, E. J. and Califf, R. M. (2007) *Textbook of cardiovascular medicine*, Third Edition, Philadelphia: Lippincott Williams & Wilkins.

Tortuero, F., Fernández, E., Rupérez, P. and Moreno, M. (1997) 'Raffinose and Lactic Acid Bacteria Influence Caecal Fermentation and Serum Cholesterol in Rats', *Nutrition Research*, 17(1), 41-49.

Tran, Q. L., Tezuka, Y., Banskota, A. H., Tran, Q. K., Saiki, I. and Kadota, S. (2001) 'New Spirostanol Steroids and Steroidal Saponins from Roots and Rhizomes of *Dracaena angustifolia* and Their Antiproliferative Activity', *Journal of Natural Products*, 64, 1127-1132.

Traore, F., Faure, R., Ollivier, E., Gasquet, M., Azas, N., Debrauwer, L., Keita, A., Timon-David, P. and Balansard, G. (2000) 'Structure and Antiprotozoal Activity of Triterpenoid Saponins from *Glinus oppositifolius*', *Planta Med*, 66(04), 368,371.

Treyvaud, V., Marston, A., Dyatmiko, W. and Hostettmann, K. (2000) 'Molluscicidal saponins from *Phytolacca icosandra*', *Phytochemistry*, 55(6), 603-609.

Tzounis, X., Vulevic, J., Kuhnle, G. G. C., George , T., Leonczak, J., Gibson, G. R., Kwik-Uribe, C. and Spencer, J. P. E. (2008) 'Flavanol monomer-induced changes to the human faecal microflora', *British Journal of Nutrition*, 99(4), 782-792.

UC (2011) 'TLC - Retention Factor (Rf)', [online], available: http://orgchem.colorado.edu/hndbksupport/TLC/TLCrf.html [accessed

UG (2009) 'Determination of protein content by Bradford method', [online], available: www.ocean.univ.gda.pl [accessed 19th July 2009].

UMMC (2008) 'The Digestive System-An Overview', *Digestive Disorders* [online], available: www.umm.edu [accessed 2nd February 2010].

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J. and Telser, J. (2004) 'Role of oxygen radicals in DNA damage and cancer incidence', *Molecular and Cellular Biochemistry*, 266(1), 37-56.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M. and Telser, J. (2007) 'Free radicals and antioxidants in normal physiological functions and human disease', *The International Journal of Biochemistry & Cell Biology*, 39(1), 44-84.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. and Mazur, M. (2006) 'Free radicals, metals and antioxidants in oxidative stress-induced cancer', *Chemico-Biological Interactions*, 160(1), 1-40.

Van Laere, K. M. J., Hartemink, R., Bosveld, M., Schols, H. A. and Voragen, A. G. J. (2000) 'Fermentation of Plant Cell Wall Derived Polysaccharides and Their Corresponding Oligosaccharides by Intestinal Bacteria', *Journal of Agricultural and Food Chemistry*, 48(5), 1644-1652.

Vázquez, M. J., Alonso, J. L., Domínguez, H. and Parajó, J. C. (2006) 'Enhancing the potential of oligosaccharides from corncob autohydrolysis as prebiotic food ingredients', *Industrial Crops and Products*, 24(2), 152-159.

Vincken, J.-P., Heng, L., de Groot, A. and Gruppen, H. (2007) 'Saponins, classification and occurrence in the plant kingdom', *Phytochemistry*, 68(3), 275-297.

Voragen, A. G. J. (1998) 'Technological aspects of functional food-related carbohydrates', *Trends in Food Science & Technology*, 9(8-9), 328-335.

Voutquenne, L., Guinot, P., Thoison, O., Sevenet, T. and Lavaud, C. (2003) 'Oleanolic glycosides from *Pometia ridleyi*', *Phytochemistry,* 64(3), 781-789.

Vrese, M. d. and Marteau, P. R. (2007) 'Probiotics and prebiotics: effects on diarrhea', *Journal of Nutrition,* 137, 803S-11S.

Wang, H.-Y., Qian, H. and Yao, W.-R. (2011) 'Melanoidins produced by the Maillard reaction: Structure and biological activity', *Food Chemistry,* 128(3), 573-584.

Wang, N. S. (2009) 'Glucose assay by dinitrosalicylic colorimetric method', [online], available: www.eng.umd.edu/~nsw [accessed 20th July 2009].

Wang, X.-H., Xu, B., Liu, J.-T. and Cui, J.-R. (2008) 'Effect of [beta]-escin sodium on endothelial cells proliferation, migration and apoptosis', *Vascular Pharmacology,* 49(4-6), 158-165.

Wang, Y. (2009) 'Prebiotics: Present and future in food science and technology', *Food Research International,* 42(1), 8-12.

Waterhouse, A. L. (2002) *Current Protocols in Food Analytical Chemistry: Determination of Total Phenolics,* New Jersey: John Wiley & Sons, Inc.

Watson, D. G. (1999) *Pharmaceutical Analysis: a textbook for pharmacy students and pharmaceutical chemists,* New York: Churchill Livingstone.

Watts, H. (1871) *A dictionary of chemistry and the allied branches of other sciences,* Longmans.

Weston, A. and Brown, P. R. (1997) *HPLC and CE. Principle and Practice,* San Diego, California: Academic Press.

Wildman, R. E. C. (2007) *Handbook of Nutraceuticals and Functional Foods,* Boca Raton: CRC Press.

Wilkinson, J. A. and Brown, A. M. G. (1999) 'Horse Chestnut-*Aesculus hippocastanum*: Potential Application in Cosmetic Skin-care Products"', *International Journal of Cosmetic Science,* 21, 437-447.

Wink, M. (2003) 'Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective', *Phytochemistry,* 64(1), 3-19.

Woldemichael, G. M. and Wink, M. (2001) 'Identification and Biological Activities of Triterpenoid Saponins from *Chenopodium quinoa*', *Journal of Agricultural and Food Chemistry,* 49(5), 2327-2332.

Wolf, R. A., Glogar, D. and Chaung, L. Y. (1984) 'Heparin inhibits bovine testicular hyaluronidase activity in myocardium of dogs with coronary artery occlusion', *American Journal of Cardiology,* 53(7), 941-944.

Wood, B. J. B. and Holzapfel, W. H. (1995) *The Lactic Acid Bacteria: The genera of lactic acid bacteria* Glasgow: Blackie Academic & Professional.

Xia, D., Wu, X., Shi, J., Yang, Q. and Zhang, Y. (2010) 'Phenolic compounds from the edible seeds extract of Chinese Mei (*Prunus mume Sieb. et Zucc*) and their antimicrobial activity', *LWT - Food Science and Technology*, 44(1), 347-349.

Xiao, K., Yi, Y.-H., Wang, Z.-Z., Tang, H.-F., Li, Y.-Q. and Lin, H.-W. (1999) 'A Cytotoxic Triterpene Saponin from the Root Bark of *Aralia dasyphylla*', *Journal of Natural Products*, 62(7), 1030-1032.

Yang, X.-R., Zhao, B.-Z. and Cui, J.-R. (2006) 'Anti-angiogenesis of sodium aescinate in vivo', *Chinese Journal of New Drugs*, 15(11), 868-870.

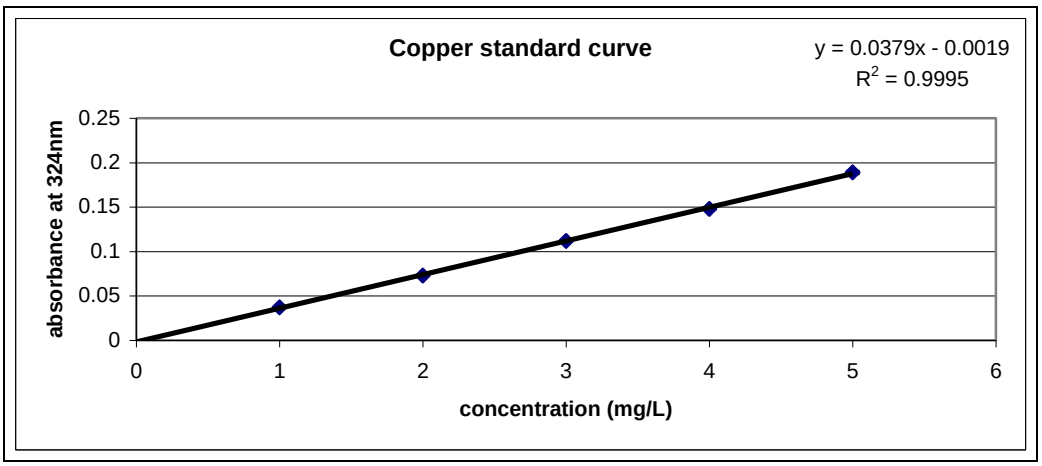
Yang, X.-W., Zhao, J., Cui, Y.-X., Liu, X.-H., Ma, C.-M., Hattori, M. and Zhang, L.-H. (1999) 'Anti-HIV-1 Protease Triterpenoid Saponins from the Seeds of *Aesculus chinensis*', *Journal of Natural Products,* 62(11), 1510-1513.

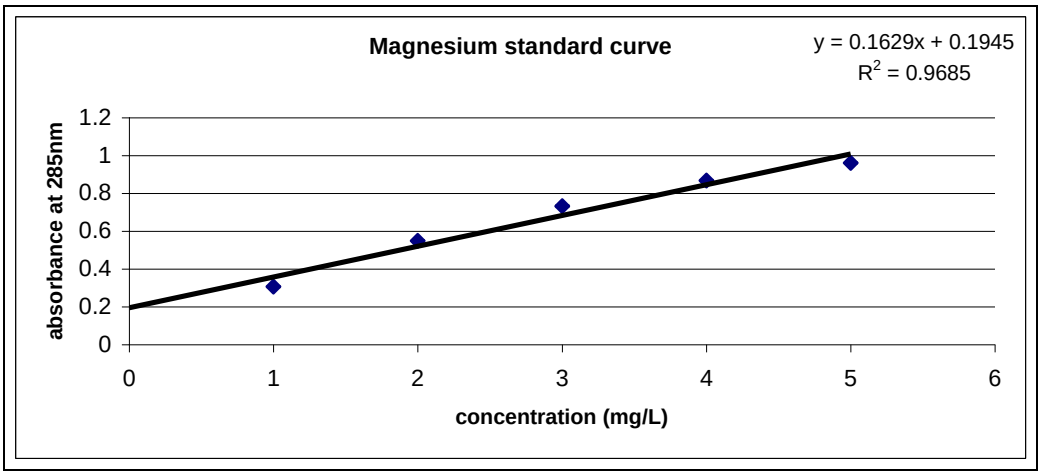
Yoshikawa, M., Morikawa, T., Kashima, Y., Ninomiya, K. and Matsuda, H. (2003) 'Structures of New Dammarane-Type Triterpene Saponins from the Flower Buds of *Panax notoginseng* and Hepatoprotective Effects of Principal Ginseng Saponins1', *Journal of Natural Products*, 66(7), 922-927.

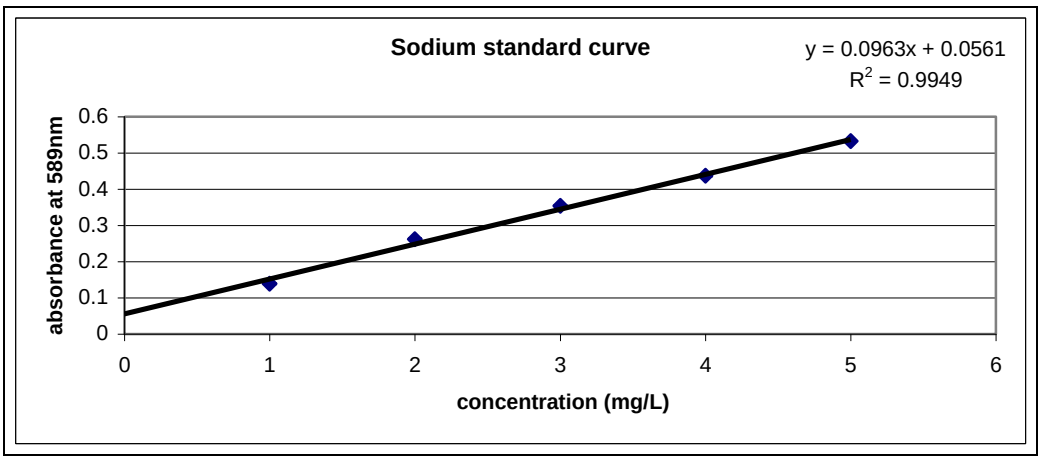
Zamora, A. (2010) [online], available: www.scientificpsychic.com [accessed 30th April 2010].

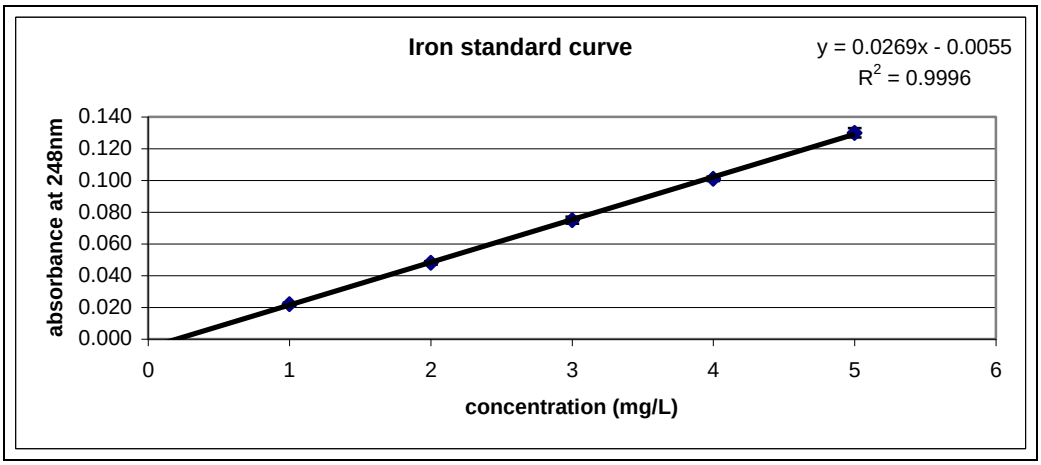
Appendices

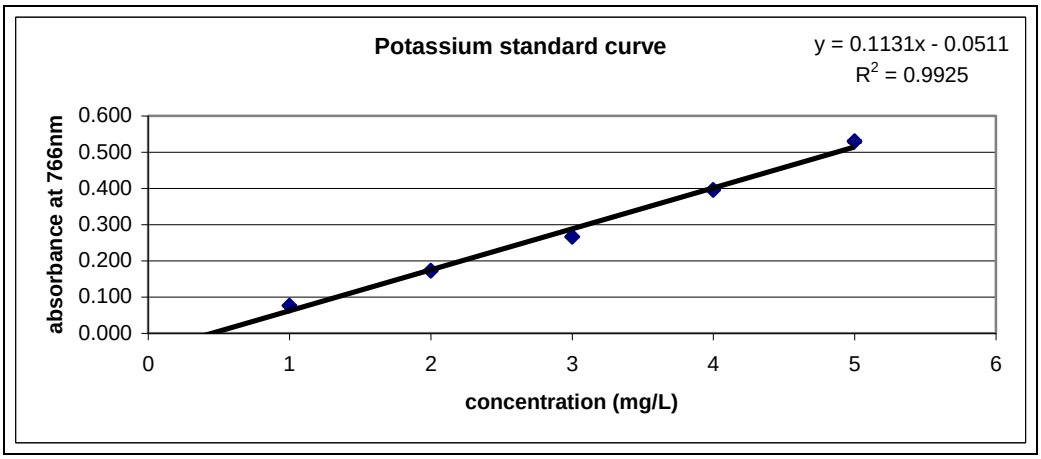
Appendix A: Standard curves of mineral elements

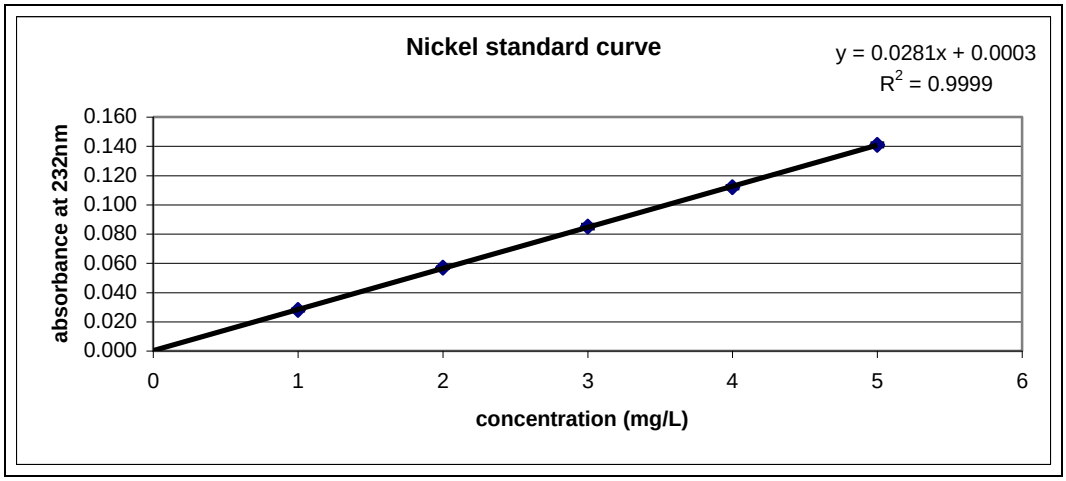


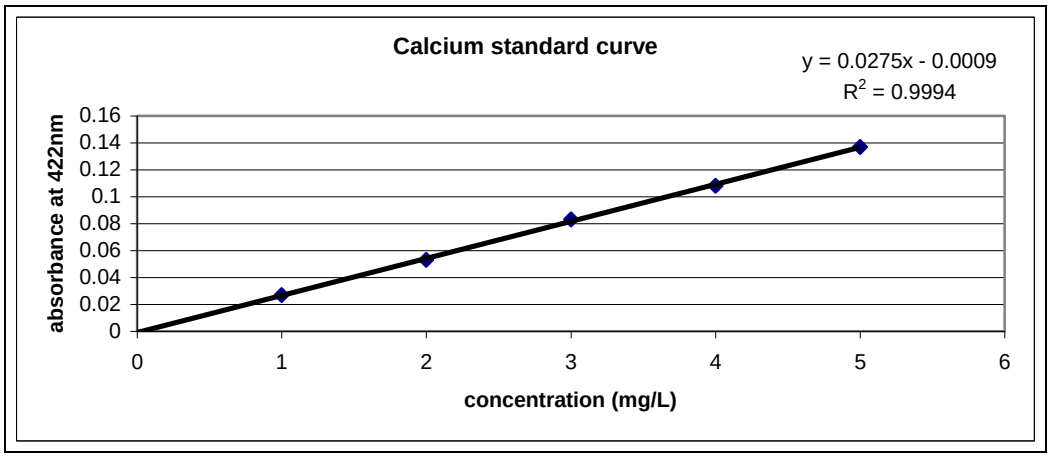




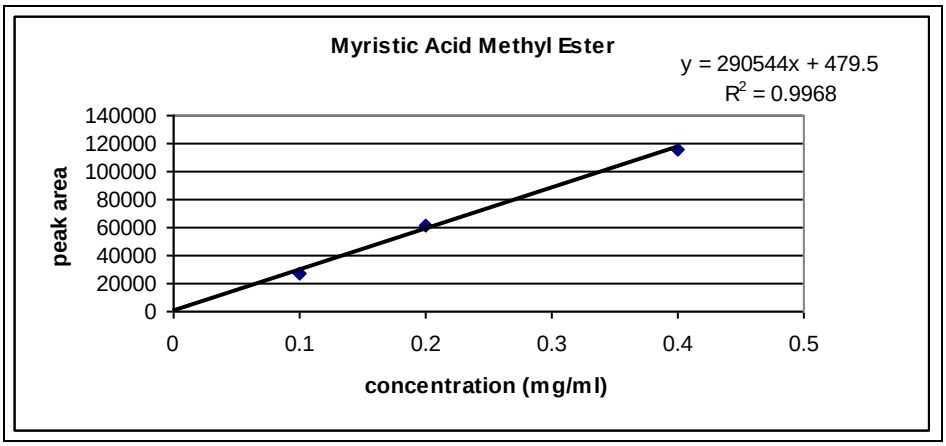

Iron standard curve
y = 0.0269x - 0.0055
R2 = 0.9996
absorbance at 248nm
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0
1
2
3
4
5
6
concentration (mg/L)

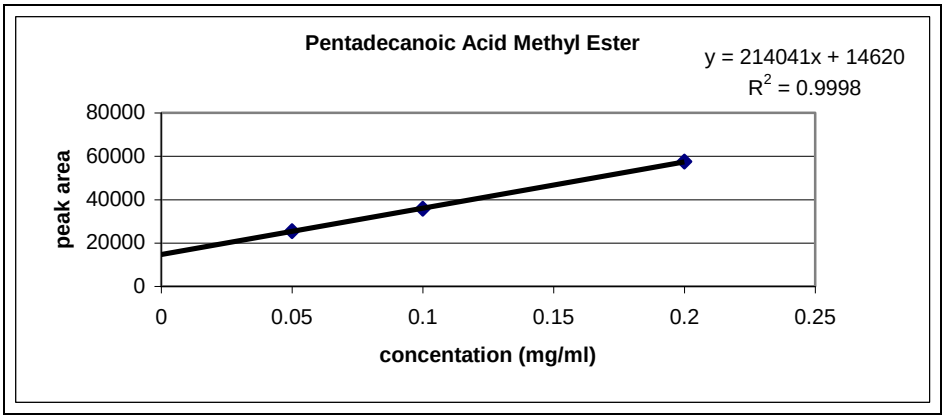

Potassium standard curve
y = 0.1131x - 0.0511
R2 = 0.9925
absorbance at 766nm
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
1
2
3
4
5
6
concentration (mg/L)

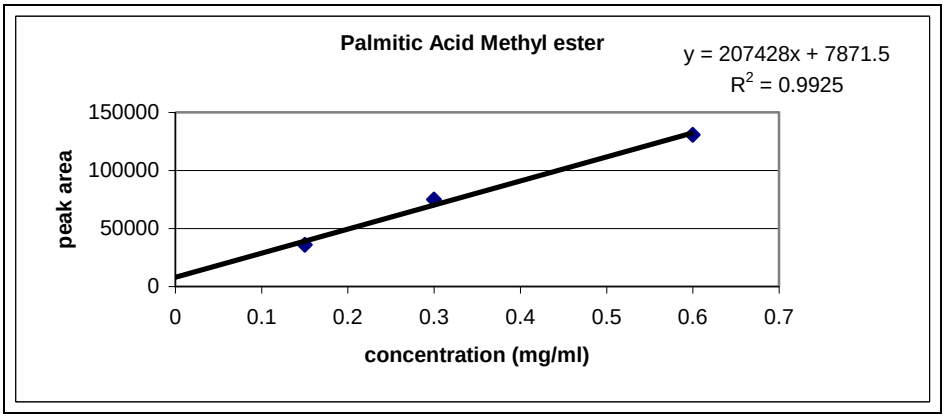

Nickel standard curve
y = 0.0281x + 0.0003
R2 = 0.9999
absorbance at 232nm
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0
1
2
3
4
5
6
concentration (mg/L)

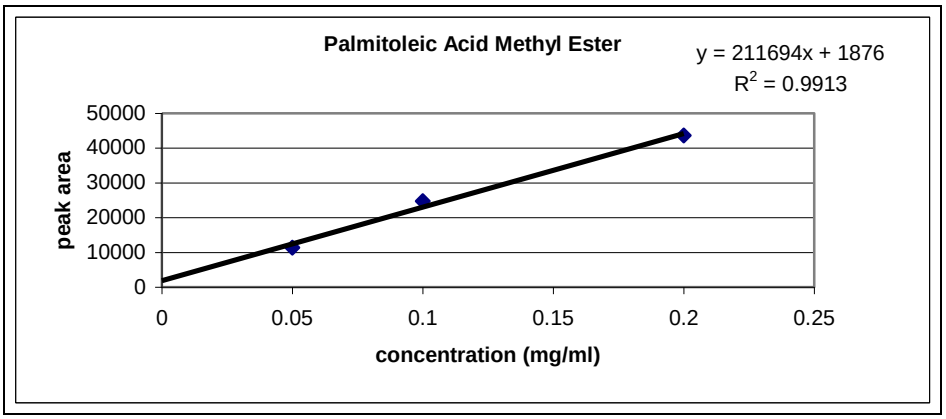

Calcium standard curve
y = 0.0275x - 0.0009
R2 = 0.9994
absorbance at 422nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1
2
3
4
5
6
concentration (mg/L)

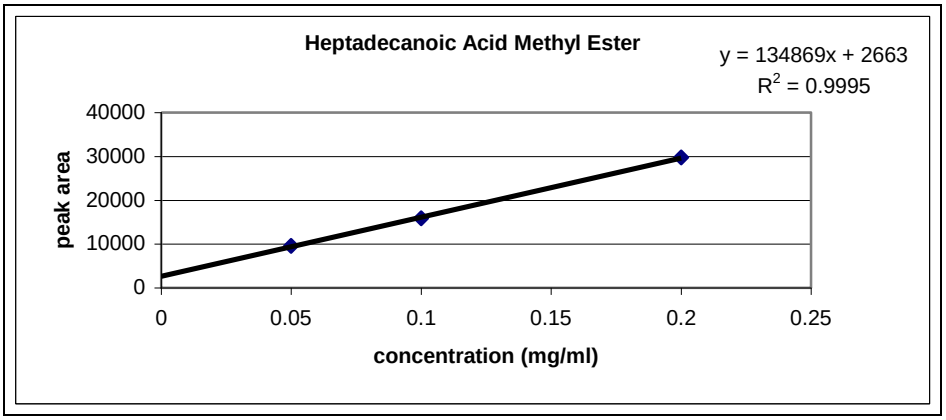
Appendix B: Standard curves of methyl ester of fatty acids

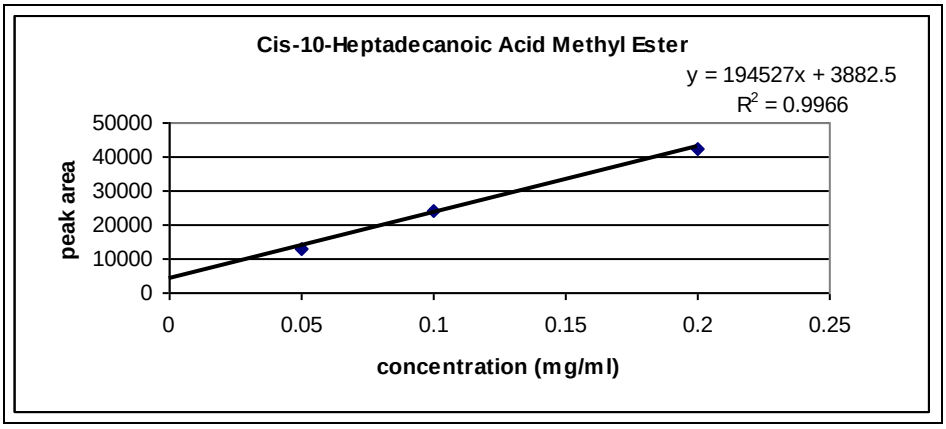


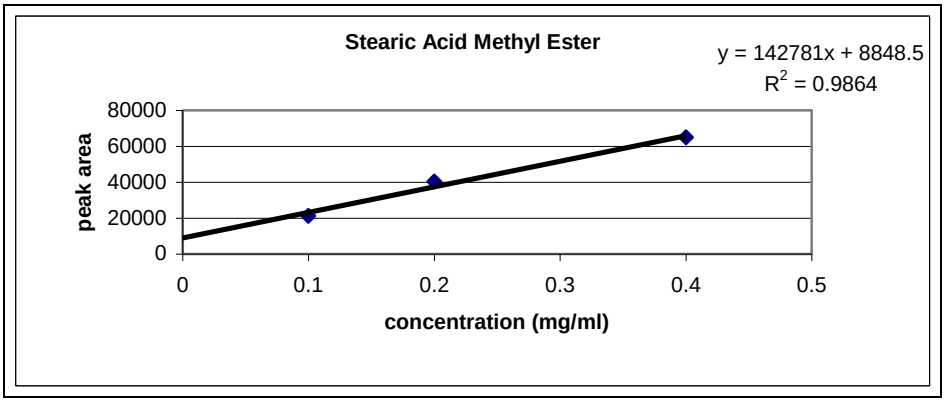


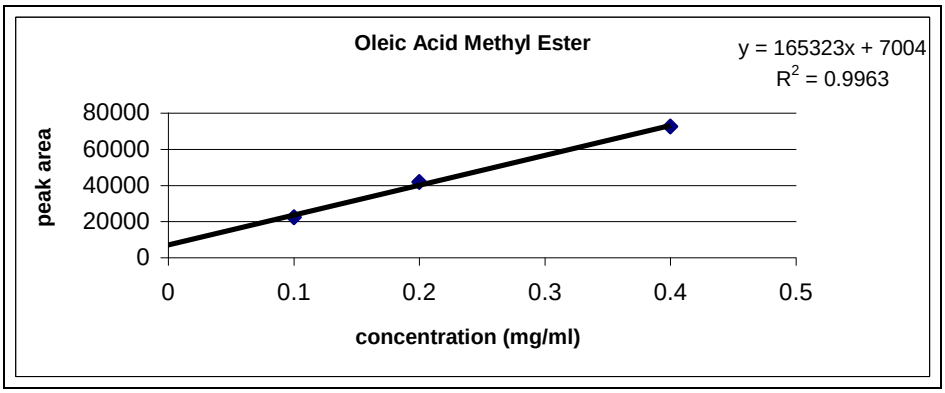


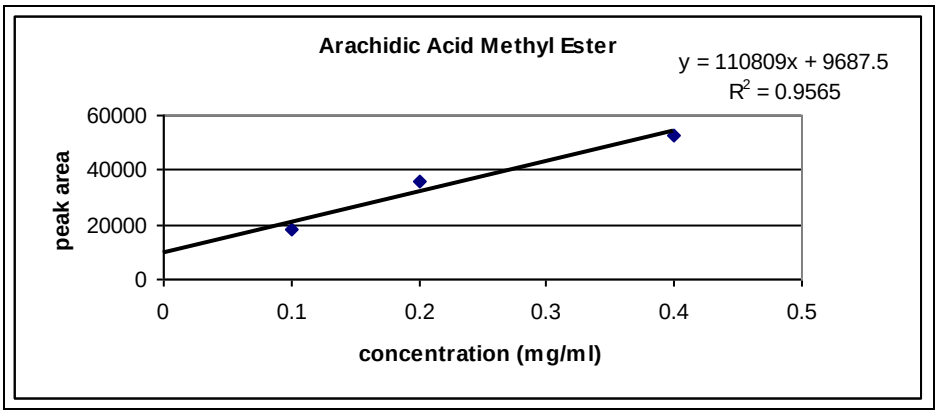

Palmitoleic Acid Methyl Ester
y = 211694x + 1876
R2 = 0.9913
peak area
50000
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25
concentration (mg/ml)

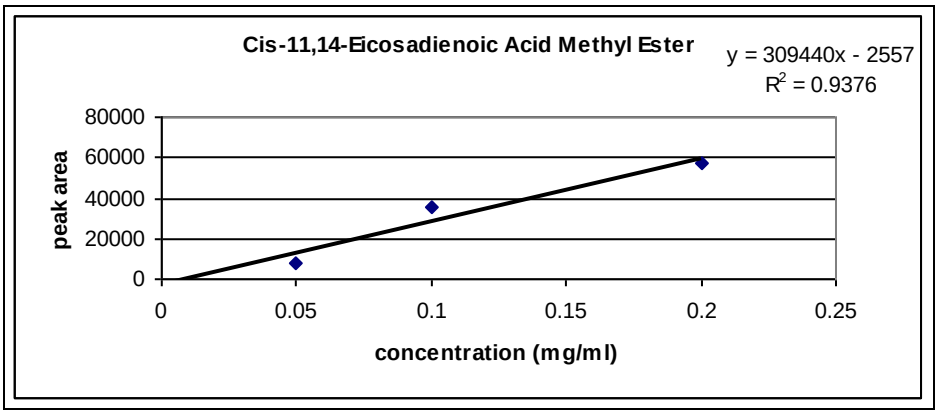

Heptadecanoic Acid Methyl Ester
y = 134869x + 2663
R2 = 0.9995
peak area
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25
concentration (mg/ml)

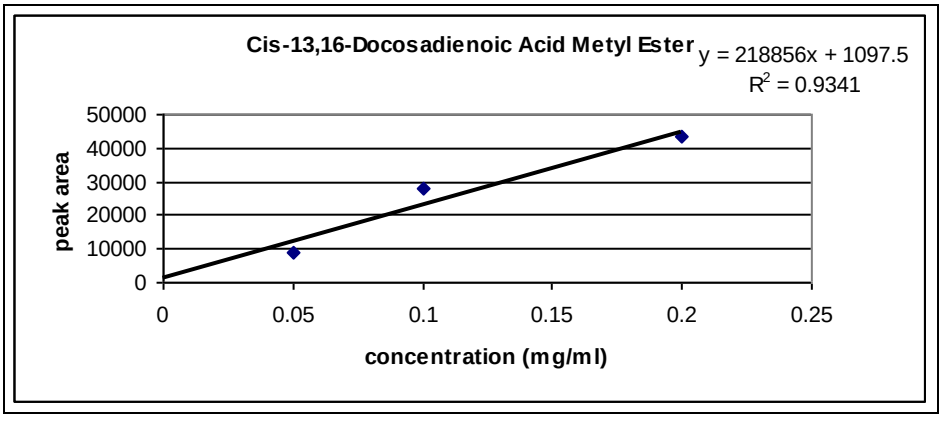

Cis-10-Heptadecanoic Acid Methyl Ester
y = 194527x + 3882.5
R2 = 0.9966
peak area
50000
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25
concentration (mg/ml)

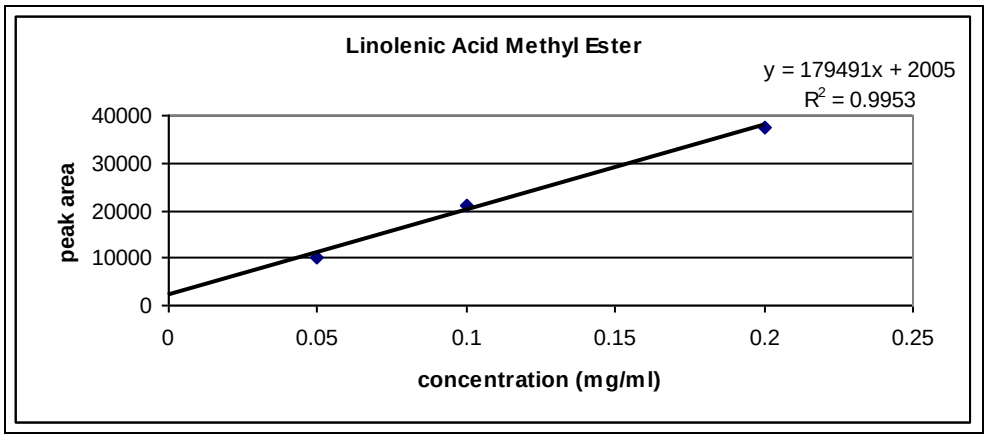

Stearic Acid Methyl Ester
y = 142781x + 8848.5
R2 = 0.9864
peak area
80000
60000
40000
20000
0
0
0.1
0.2
0.3
0.4
0.5
concentration (mg/ml)


Oleic Acid Methyl Ester
y = 165323x + 7004
R2 = 0.9963
peak area
80000
60000
40000
20000
0
0
0.1
0.2
0.3
0.4
0.5
concentration (mg/ml)


Arachidic Acid Methyl Ester
y = 110809x + 9687.5
R2 = 0.9565
peak area
60000
40000
20000
0
0
0.1
0.2
0.3
0.4
0.5
concentration (mg/ml)


Cis-11,14-Eicosadienoic Acid Methyl Ester
y = 309440x - 2557
R2 = 0.9376
peak area
80000
60000
40000
20000
0
0
0.05
0.1
0.15
0.2
0.25
concentration (mg/ml)


Cis-13,16-Docosadienoic Acid Metyl Ester
y = 218856x + 1097.5
R2 = 0.9341
peak area
50000
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25
concentration (mg/ml)


Linolenic Acid Methyl Ester
y = 179491x + 2005
R2 = 0.9953
40000
30000
20000
10000
0
peak area
0
0.05
0.1
0.15
0.2
0.25
concentration (mg/ml)

Appendix C: Product Specification Sheet of DOMO Vivinal® GOS

DOMO®

VIVINAL®
GOS

Product Specification Sheet

Description	:	galacto-oligosaccharide syrup.
Typical analysis	:	dry matter 75% of which galacto-oligosaccharides 59%, lactose 21%, glucose 19% and galactose 1%
Sensorial	:	clear syrup, slightly sweet taste.

Product specification :

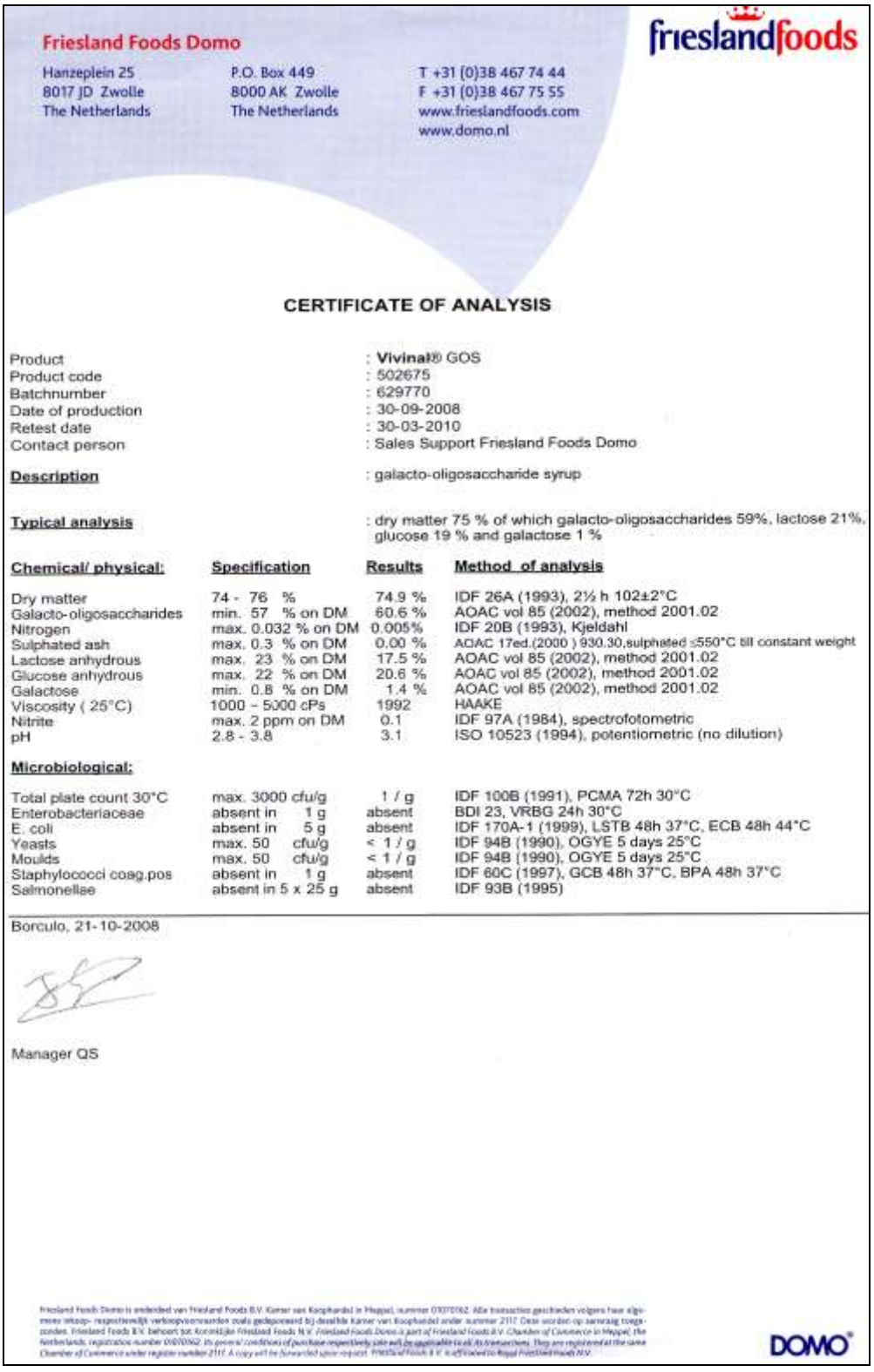
	Specification	**Method of analysis**
Chemical/ physical:		
Dry matter	74-76%	IDF 26A (1993), 2½ h 102±2°C
Galacto-oligosaccharides	min. 57% on DM	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.032% on DM	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3% on DM	AOAC 17ed.(2000) 930.30, sulphated ≤550°C till constant weight
Lactose anhydrous	max. 23% on DM	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22% on DM	AOAC vol 85 (2002), method 2001.02
Galactose	min. 0.8% on DM	AOAC vol 85 (2002), method 2001.02
Viscosity	1000-5000 cPs	HAAKE
Nitrite	max. 2 ppm on DM	IDF 97A (1984), spectrophotometric
pH	2.8 - 3.8	ISO 10523 (1994), potentiometric (no dilution)
Microbiological:		
Total plate count 30°C	max. 3000 cfu/g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 1 g	BDI 23, VRBG 24h 30°C
E. coli	absent in 5 g	IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50 cfu/g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	IDF 94B (1990), OGYE 5 days 25°C
Staphylococci coagulase-positive	absent in 1 g	IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in 5x25 g	IDF 93B (1995)

Packaging	:	container, 650 or 1200 kg.
Storage	:	keep in clean, dry and dark conditions, keep away from strongly odorous materials.
Shelf life	:	18 months after production date.

June 4th, 2008 (version 15)

Friesland Foods Domo
Hanzeplein 25, 8017 JD Zwolle, P.O. Box 449 8000 AK Zwolle, The Netherlands,
Telephone: +31 38 46 77 444, Fax: +31 38 46 77 555, www.domo.nl, e-mail: info.domo@frieslandfoods.com

Appendix D: Certificate of Analysis of DOMO Vivinal® GOS



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8017 JD Zwolle
The Netherlands

P.O. Box 449
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www.domo.nl

frieslandfoods

CERTIFICATE OF ANALYSIS

Product	: **Vivinal®** GOS
Product code	: 502675
Batchnumber	: 629770
Date of production	: 30-09-2008
Retest date	: 30-03-2010
Contact person	: Sales Support Friesland Foods Domo
Description	: galacto-oligosaccharide syrup
Typical analysis	: dry matter 75 % of which galacto-oligosaccharides 59%, lactose 21%, glucose 19 % and galactose 1 %

Chemical/ physical:	**Specification**	**Results**	**Method of analysis**
Dry matter	74 - 76 %	74.9 %	IDF 26A (1993), 2½ h 102±2°C
Galacto-oligosaccharides	min. 57 % on DM	60.6 %	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.032 % on DM	0.005%	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3 % on DM	0.00 %	AOAC 17ed.(2000) 930.30,sulphated ≤550°C till constant weight
Lactose anhydrous	max. 23 % on DM	17.5 %	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22 % on DM	20.6 %	AOAC vol 85 (2002), method 2001.02
Galactose	min. 0.8 % on DM	1.4 %	AOAC vol 85 (2002), method 2001.02
Viscosity (25°C)	1000 – 5000 cPs	1992	HAAKE
Nitrite	max. 2 ppm on DM	0.1	IDF 97A (1984), spectrofotometric
pH	2.8 - 3.8	3.1	ISO 10523 (1994), potentiometric (no dilution)

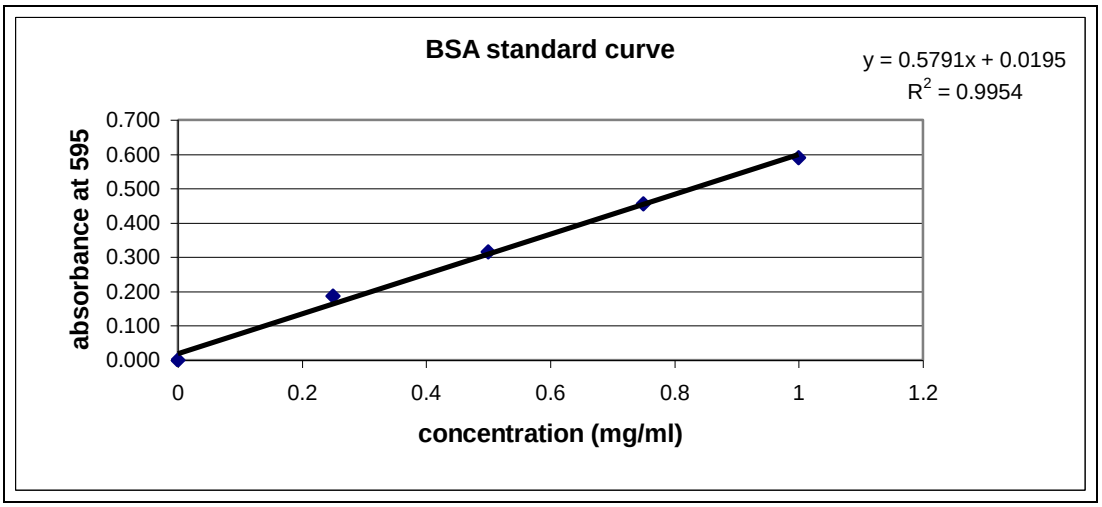
Microbiological:			
Total plate count 30°C	max. 3000 cfu/g	1 / g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 1 g	absent	BDI 23, VRBG 24h 30°C
E. coli	absent in 5 g	absent	IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50 cfu/g	< 1 / g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	< 1 / g	IDF 94B (1990), OGYE 5 days 25°C
Staphylococci coag.pos	absent in 1 g	absent	IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in 5 x 25 g	absent	IDF 93B (1995)

Borculo, 21-10-2008

Manager QS

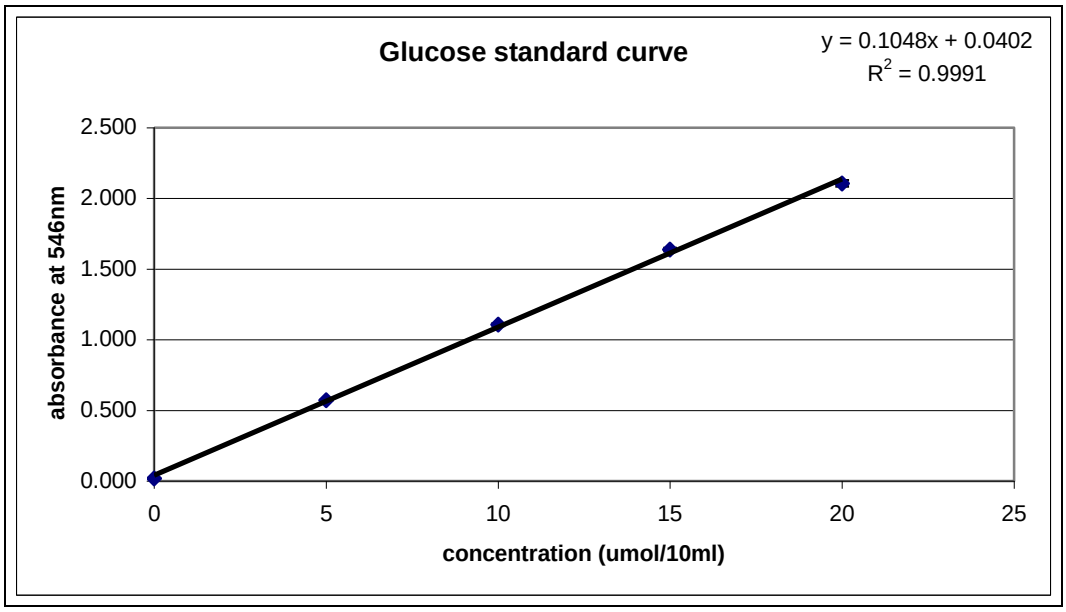
DOMO®

Appendix E: Standard curve of BSA standard



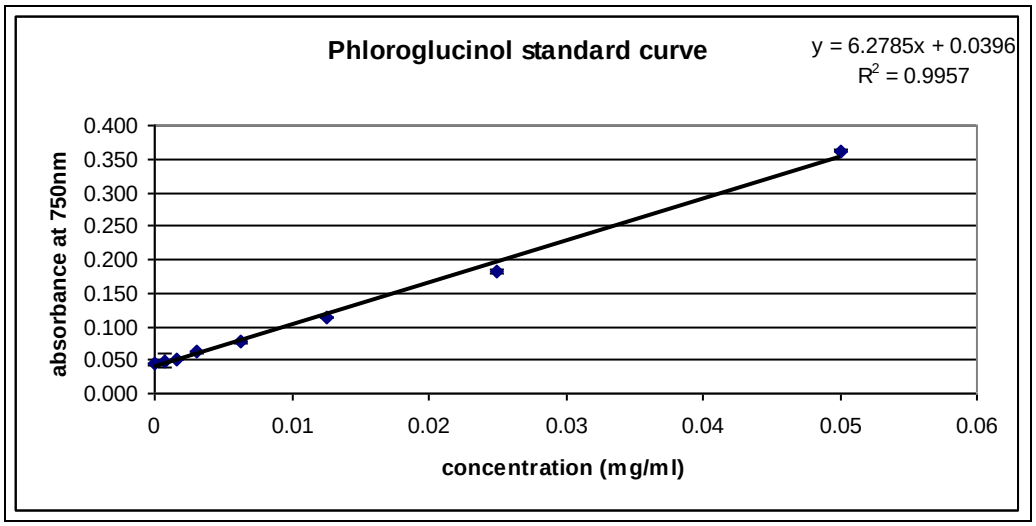
Values for each point of measure reported as mean ± standard deviation (n=3)

Appendix F: Standard curve of glucose standard



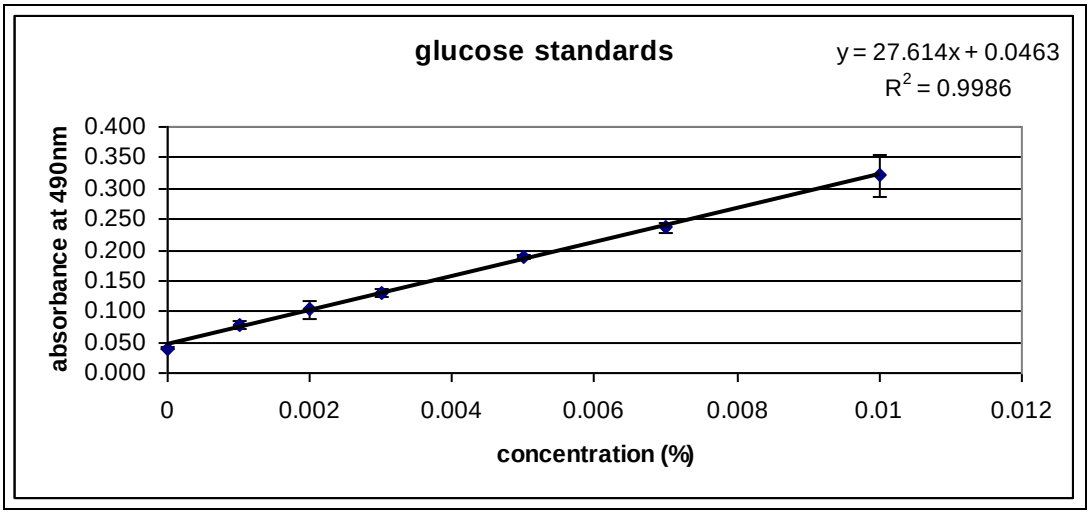
Values for each point of measure reported as mean ± standard deviation (n=3)

Appendix G: Standard curve of phlorolucinol standard



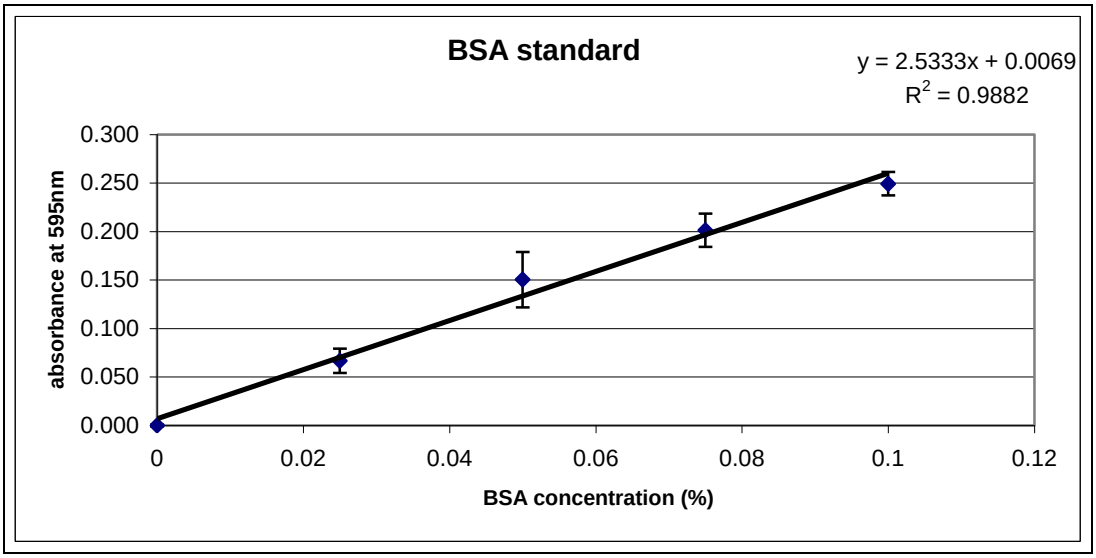
Values for each point of measure reported as mean ± standard deviation (n=3)

Appendix H: Calibration curve for glucose using Dubois assay



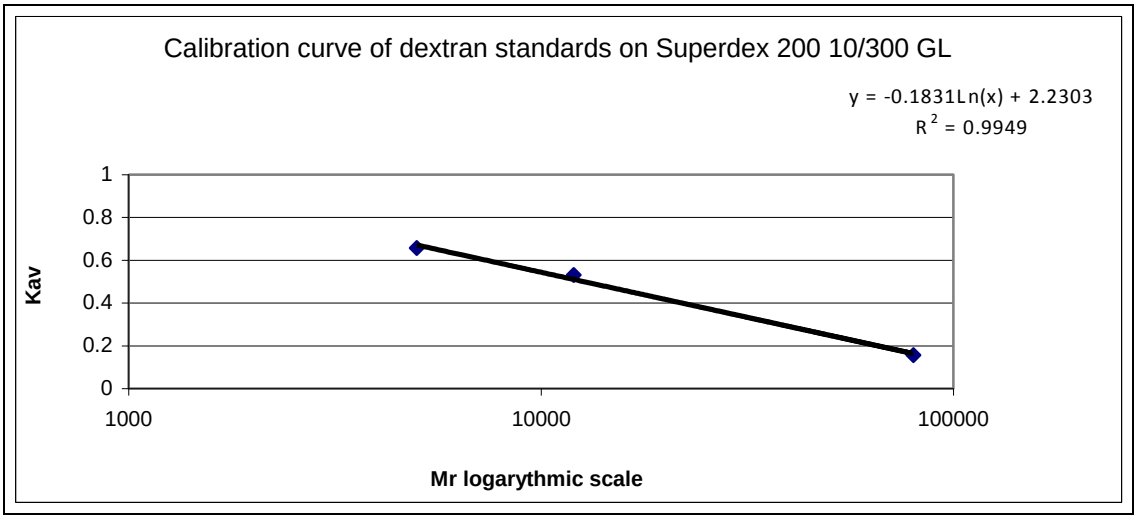
Each value for glucose absorbance represents mean ± standard deviation (n=3)

Appendix I: Standard curve of BSA standard by Bradford assay



Each value for BSA absorbance represents mean ± standard deviation (n=3)

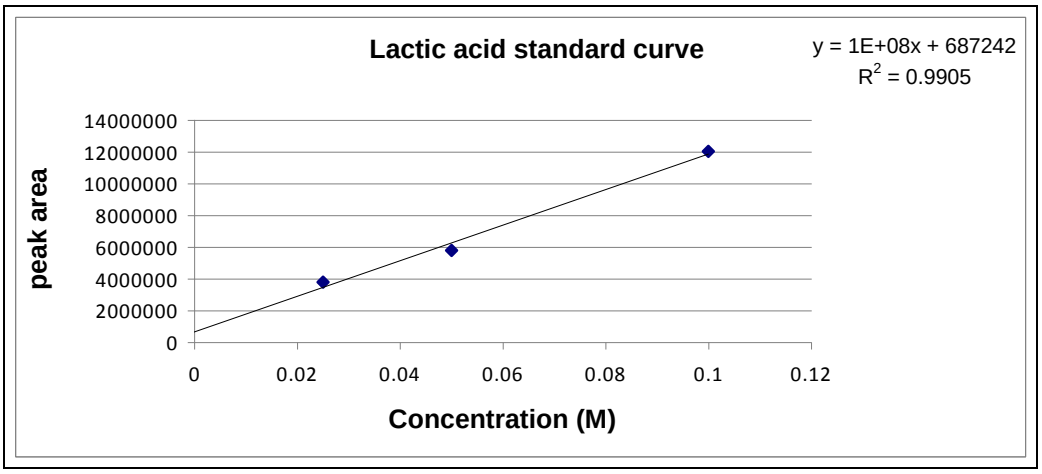
Appendix J: Calibration curve of dextran standards fractionated on Superdex 200 10/300GL



Appendix K: Retention time and peak area for lactic acid and its standard curve

Retention times and peaks area obtained for different concentration of lactic acid eluted on Supelcogel C-610H column

Retention time				Peak area	
Lactic acid concentration (M)	**t_R**	**MEAN**	**SD**	**Lactic acid concentration (M)**	**peak area (n=3)**
0.1	13.837	13.839	0.003	0.1	12036207
0.05	13.844			0.05	5807669
0.025	13.837			0.025	3801511

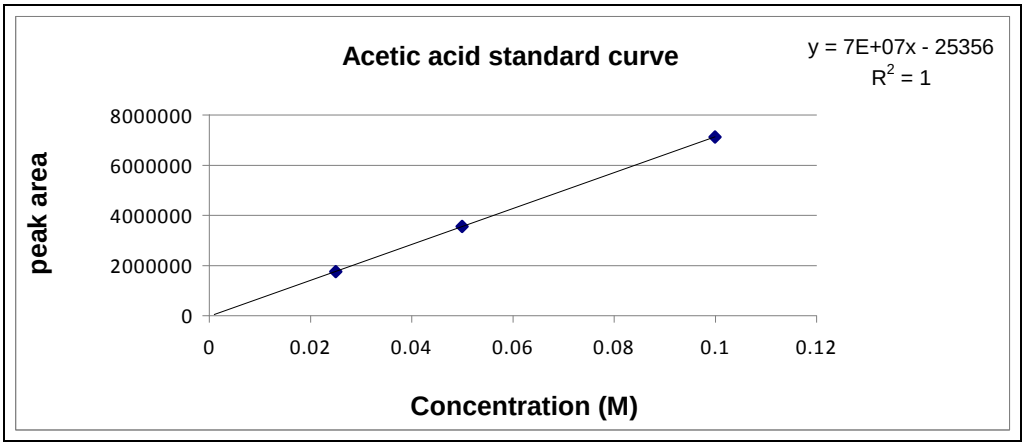


Concentration of lactic acid standard vs. peak area

Appendix L: Retention time and peak area for acetic acid and its standard curve

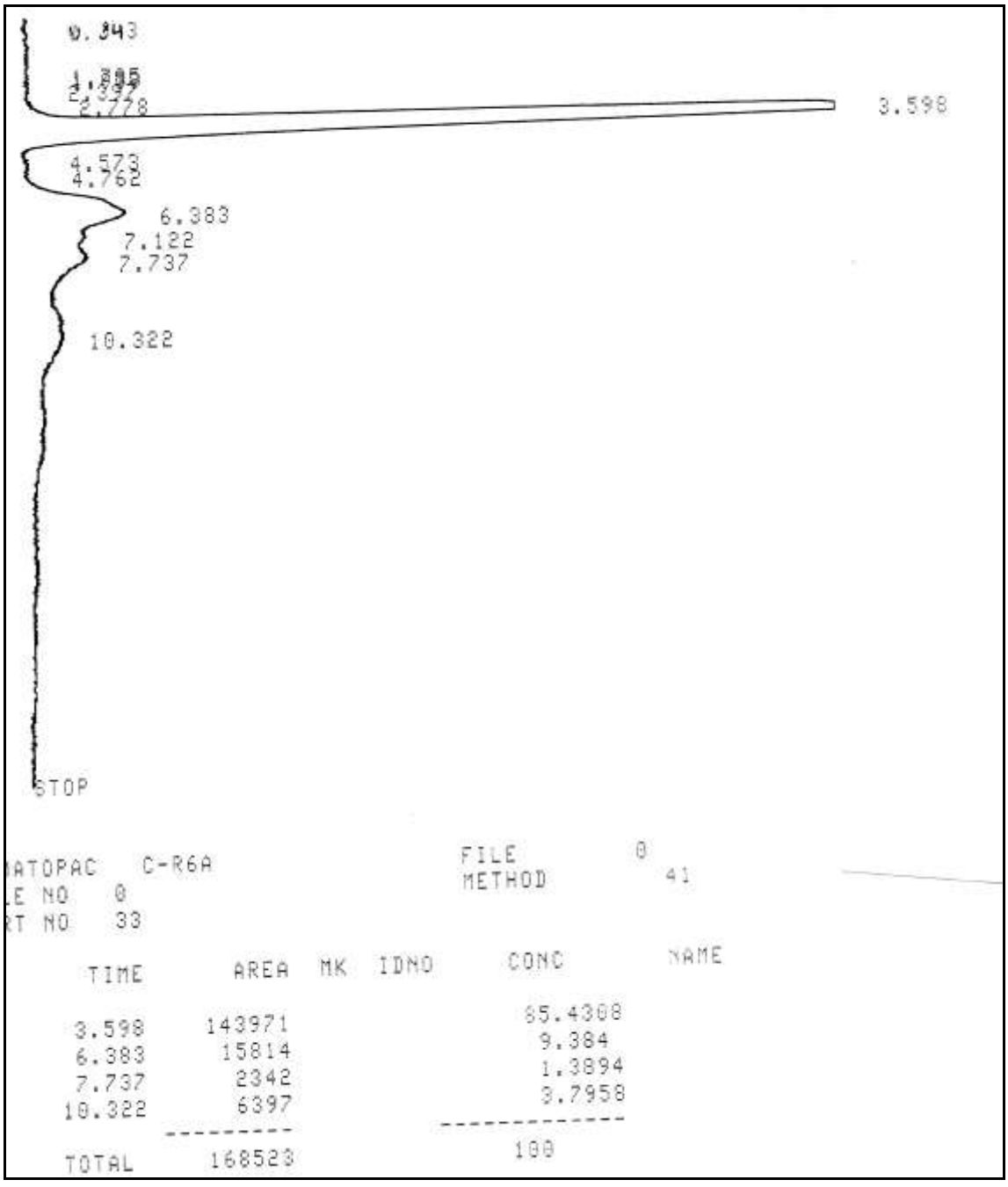
Retention times and peaks area obtained for different concentration of acetic acid eluted on Supelcogel C-610H column

Retention time				Peak area	
Acetic acid concentration (M)	**t_R**	**MEAN**	**SD**	**Acetic acid concentration (M)**	**Peak area (n=3)**
0.1	16.578	16.590	0.010	0.1	7122137
0.05	16.591			0.05	3557605
0.025	16.602			0.025	1756910



Concentration of acetic acid standard vs. peak area

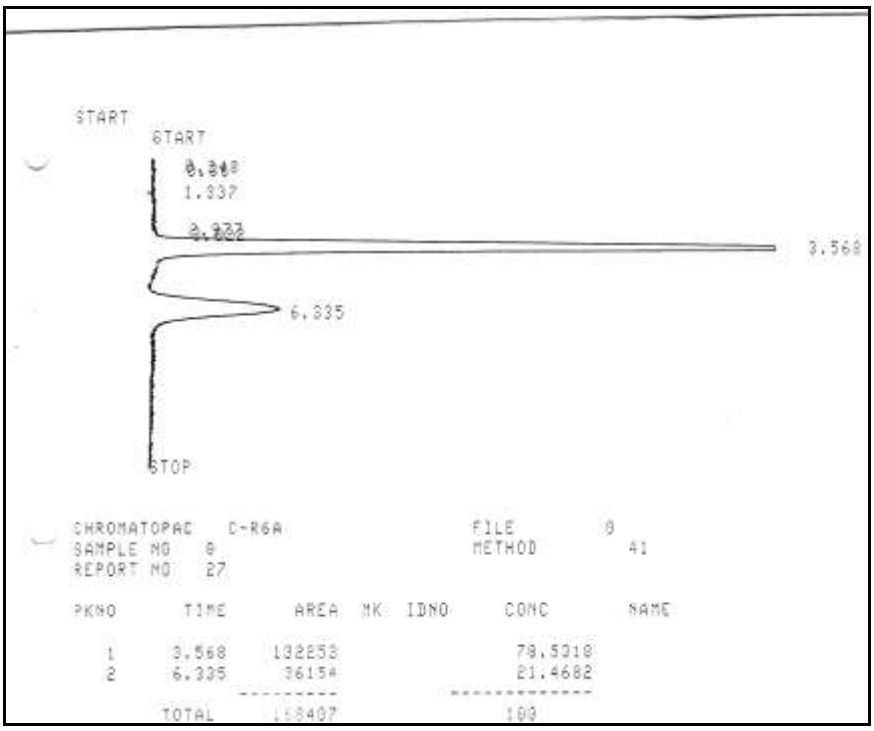
Appendix M: Anion exchange chromatography of 5% (w/v) lyophilised horse chestnut aqueous extract using Supelcosil-LC-NH2 column



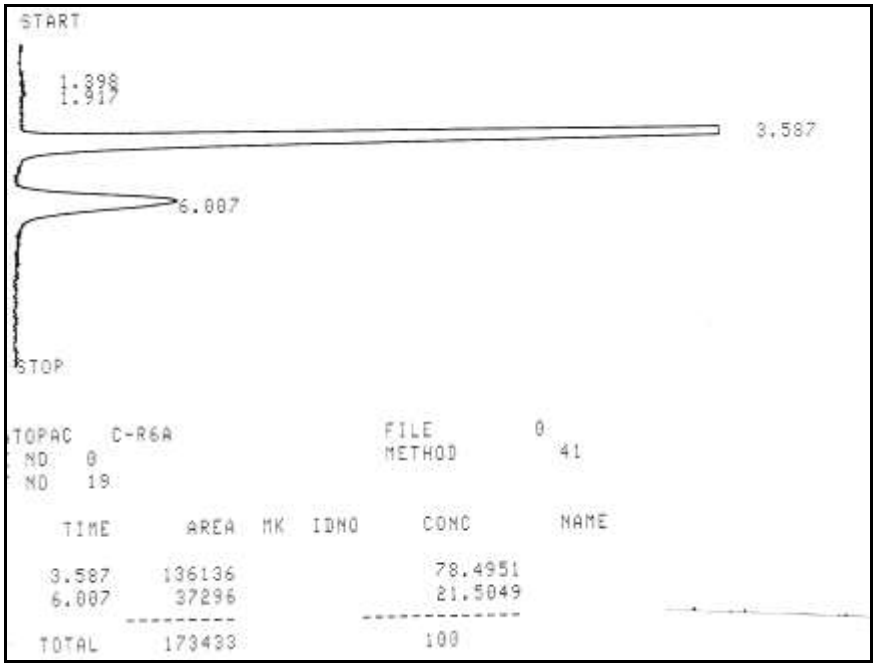
Retention time (t_R) is in minutes

Appendix N: Anion exchange chromatography of 1% (w/v) monosaccharides: glucose, fructose, galactose and 1% (w/v) disaccharide maltose on Supelcosil-LC-NH2 column

Glucose

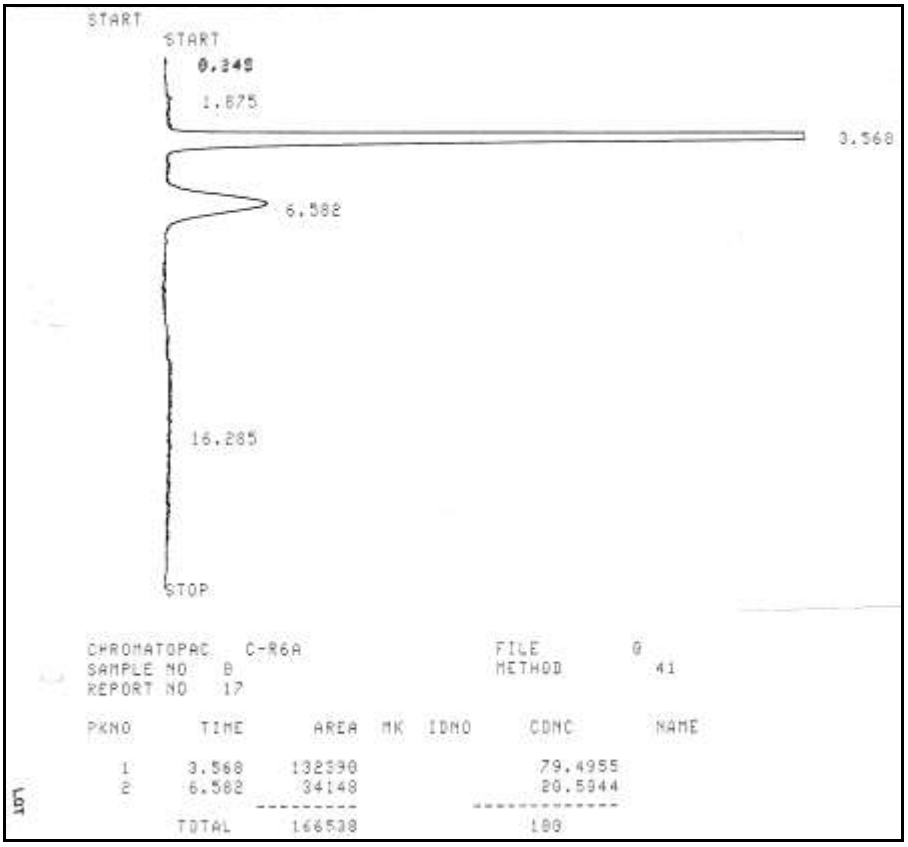


Fructose

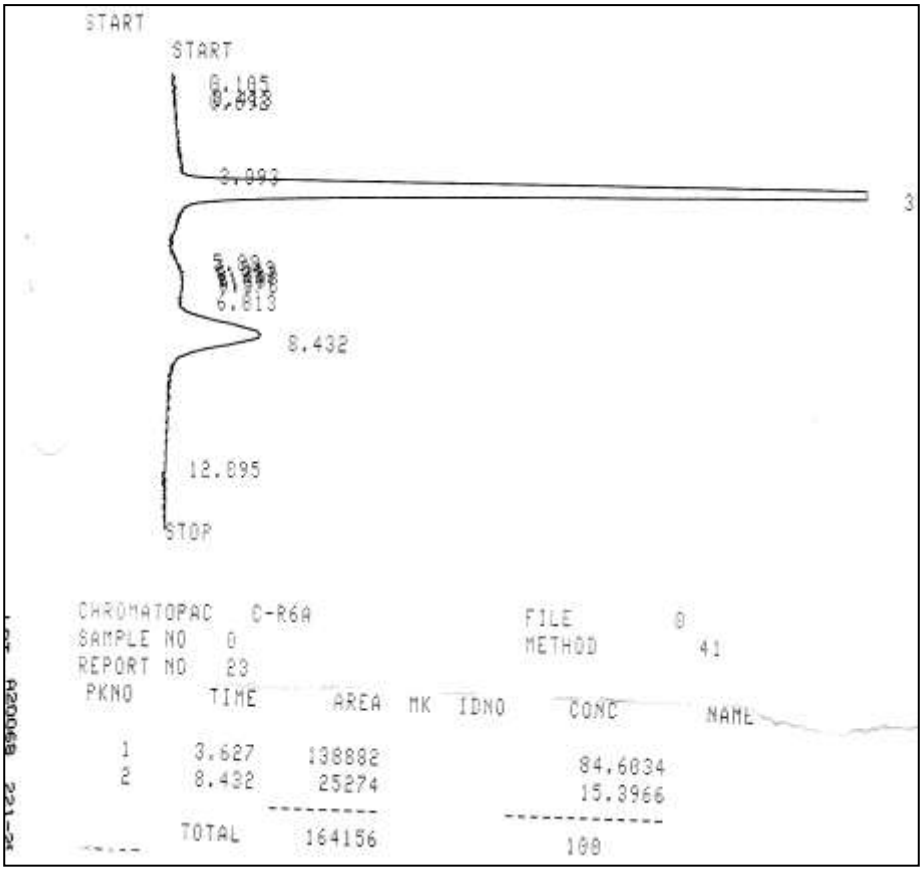


Retention time (t_R) is in minutes

Galactose

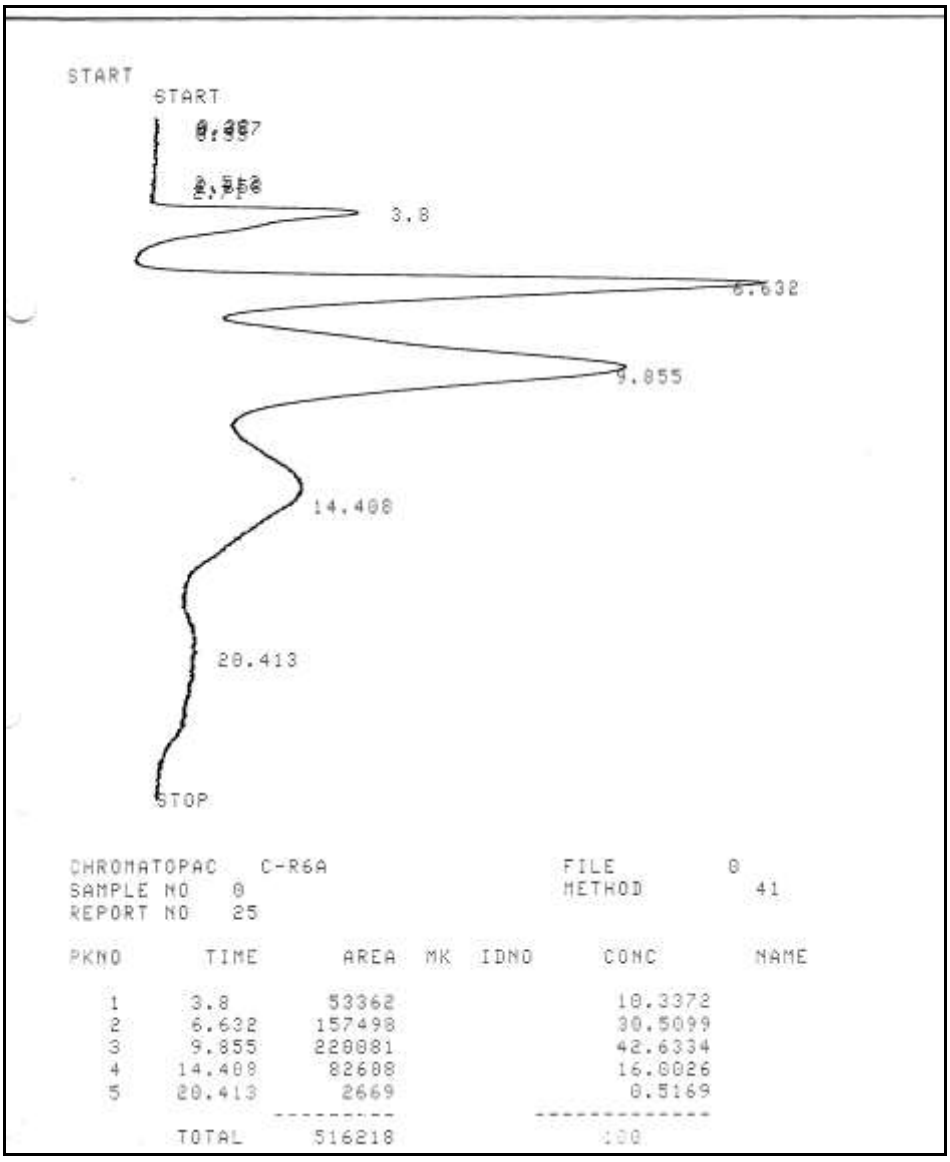


Maltose



Appendix O: Anion exchange chromatography of 1% (w/v) GOS and 1% (w/v) FOS on Supelcosil-LC-NH2 column

GOS

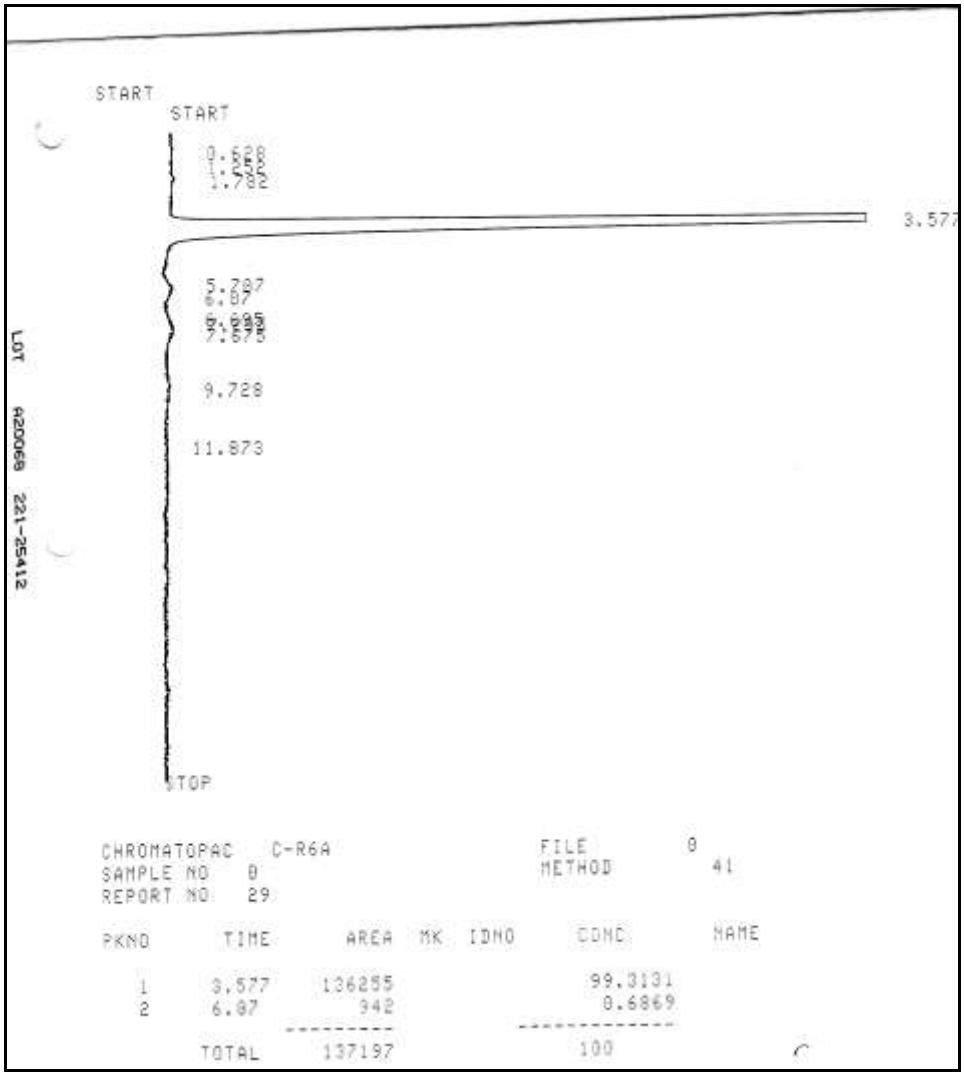


CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 25

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.8	53362			10.3372	
2	6.632	157498			30.5099	
3	9.855	220081			42.6334	
4	14.408	82608			16.0026	
5	20.413	2669			0.5169	
	TOTAL	516218			100	

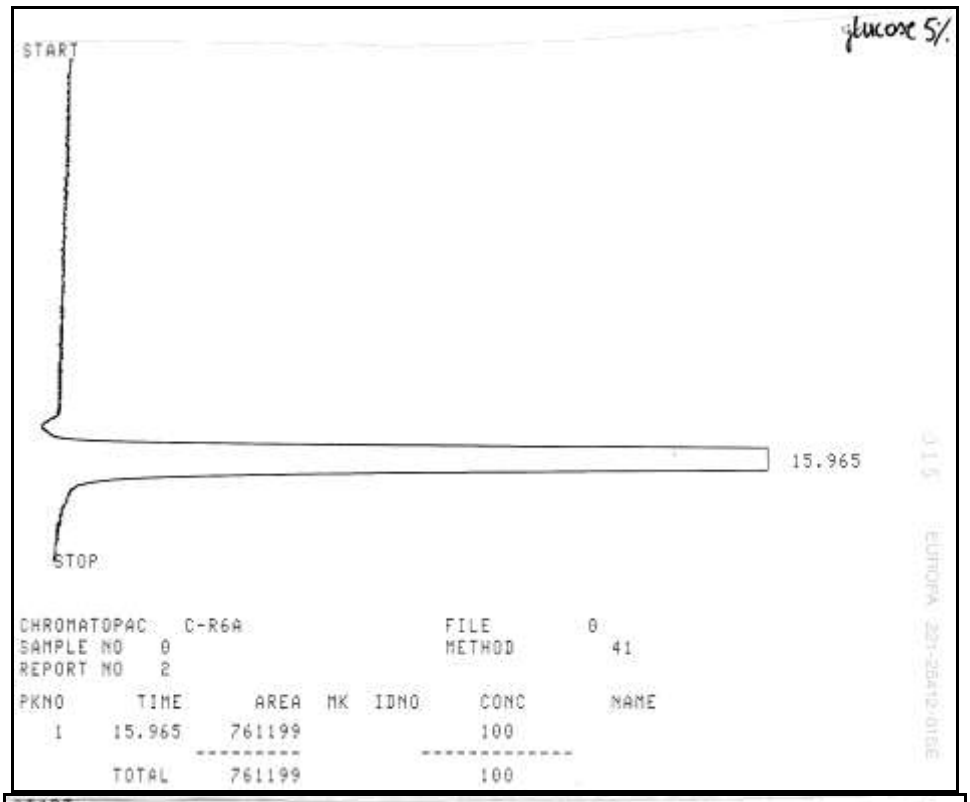
Retention time (t_R) is in minutes

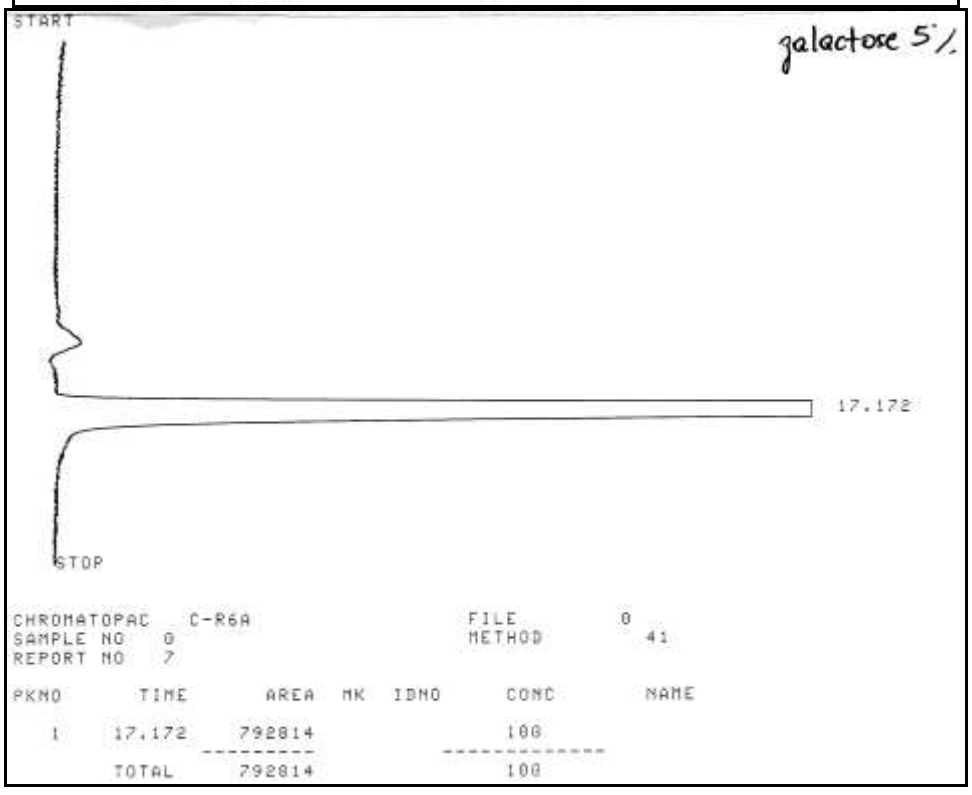
FOS

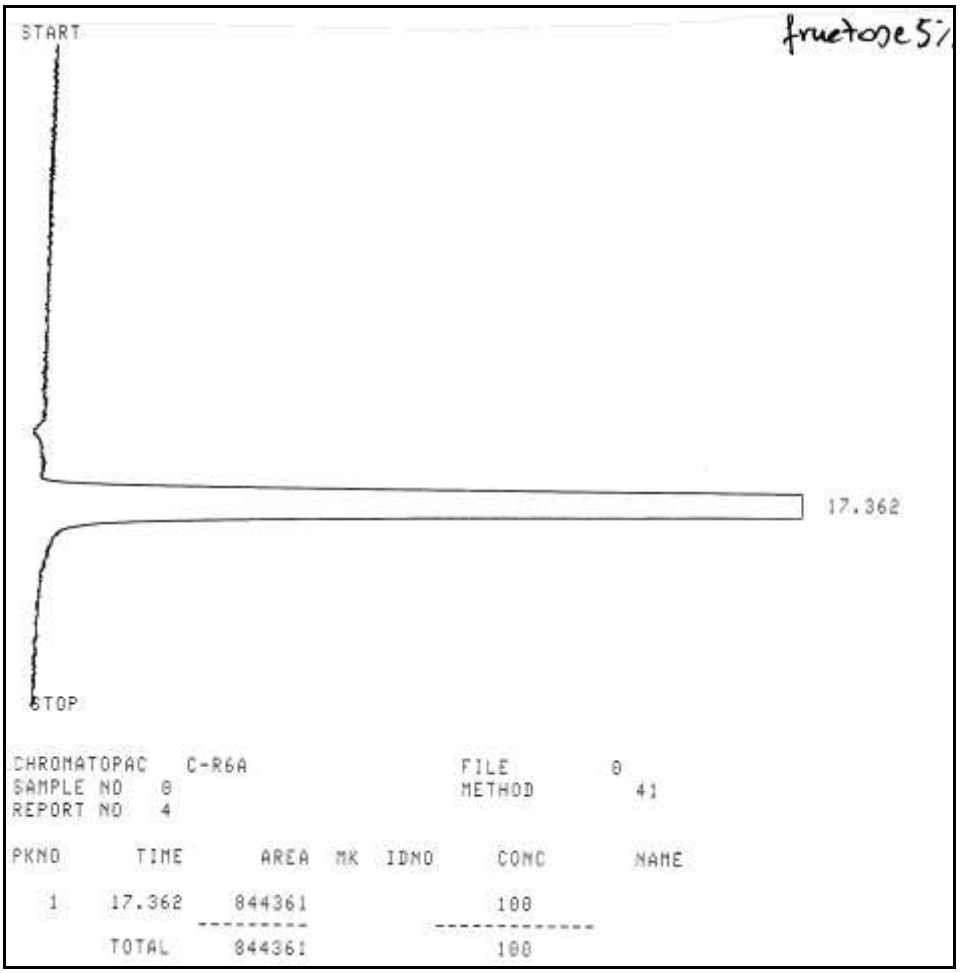


Retention time (t_R) is in minutes

Appendix P: Cation exchange chromatography of 5% (w/v) monosaccharides: glucose, galactose and fructose on Supelcogel C-610H column

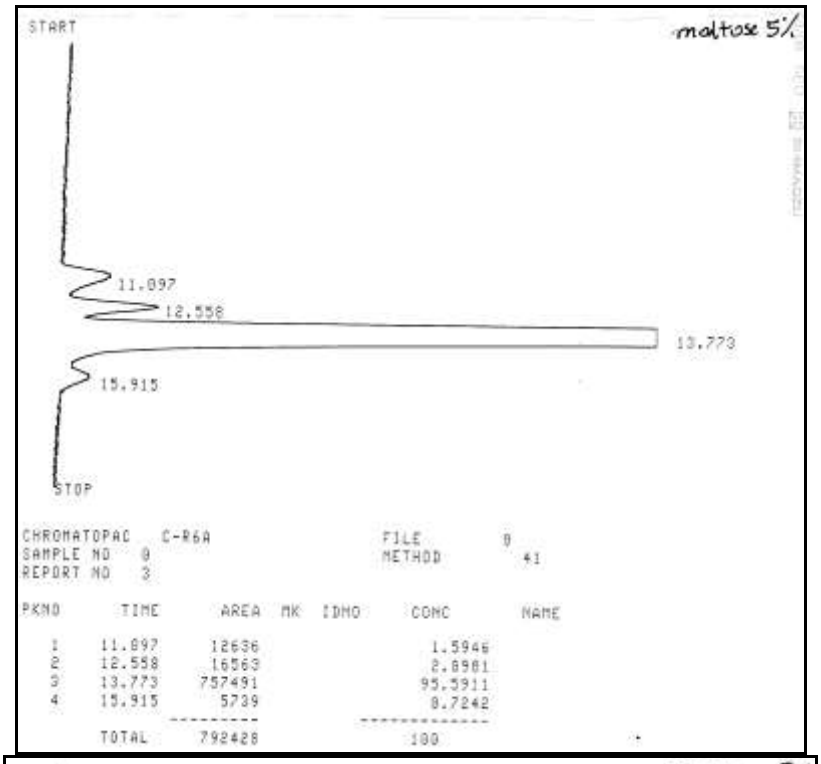






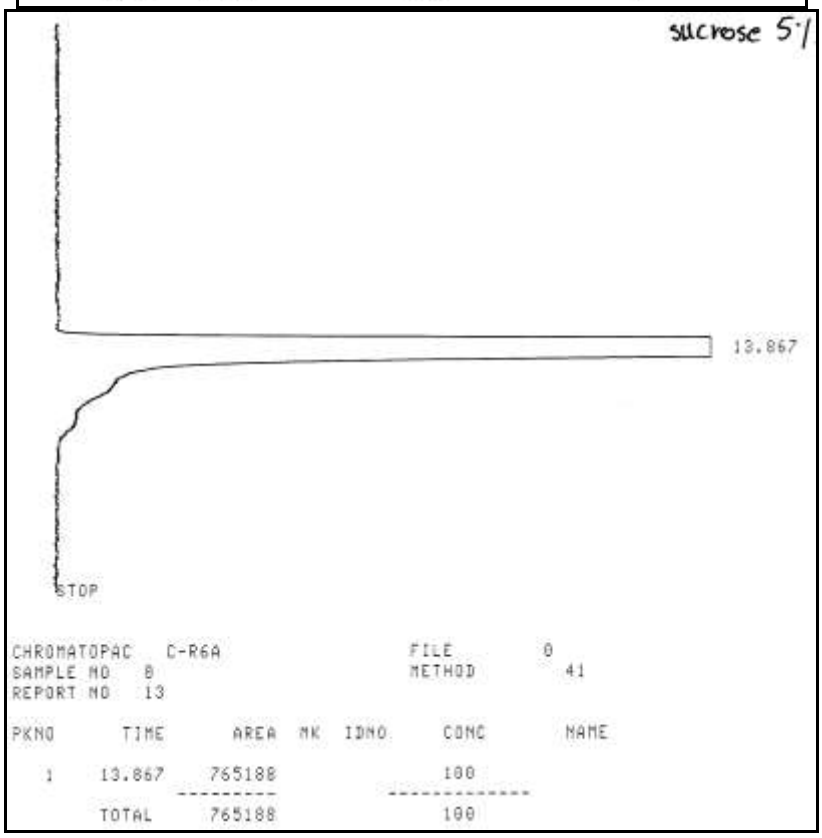
Retention time (t_R) is in minutes

Appendix Q: Cation exchange chromatography of 5% (w/v) disaccharides: maltose, sucrose and lactose on Supelcogel C-610H column



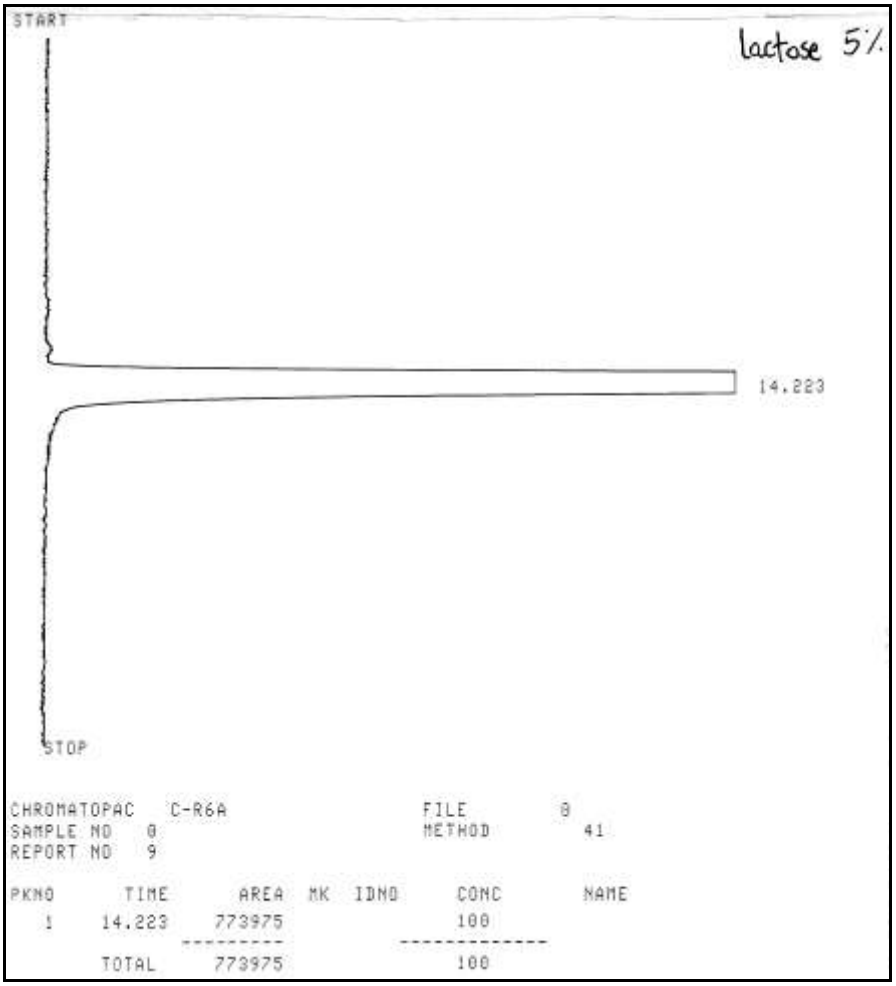
CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 3

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	11.097	12636			1.5946	
2	12.558	16563			2.0981	
3	13.773	757491			95.5911	
4	15.915	5739			0.7242	
	TOTAL	792428			100	



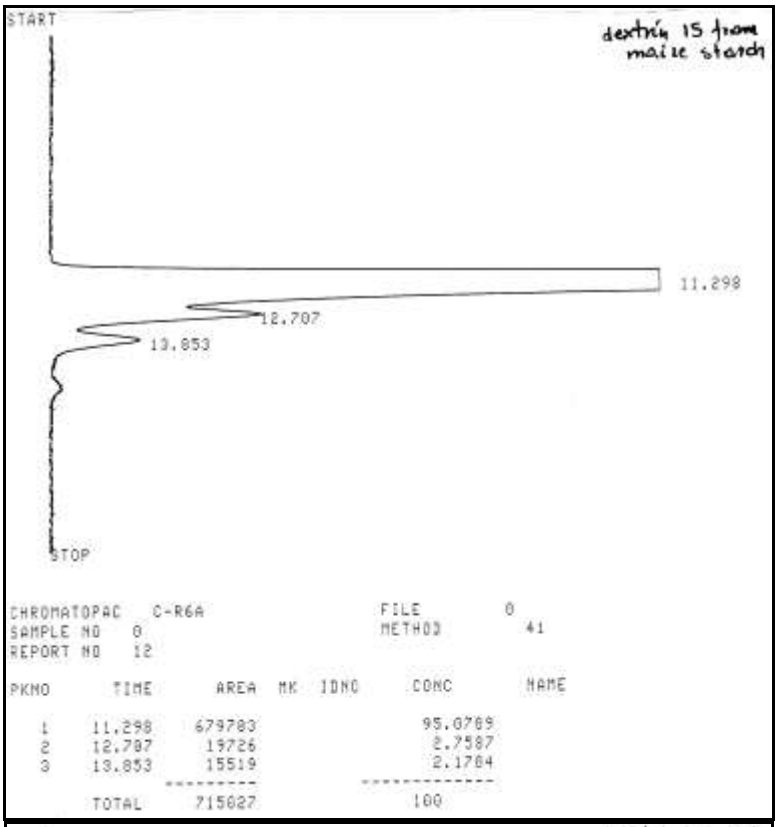
CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 13

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	13.867	765188			100	
	TOTAL	765188			100	



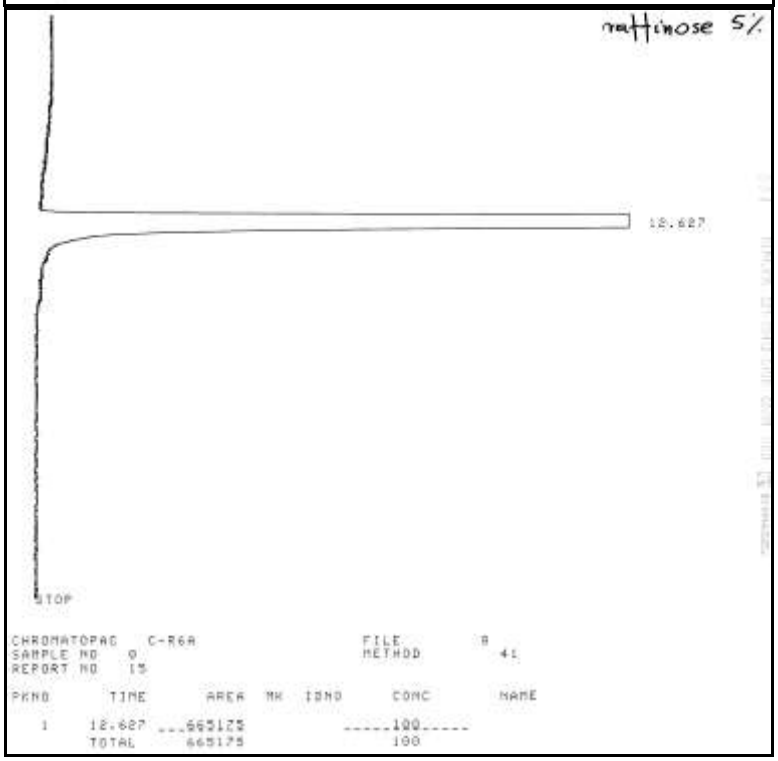
Retention time (t_R) is in minutes

Appendix R: Cation exchange chromatography of 5% (w/v) oligosaccharides: dextrin 15 and raffinose on Supelcogel C-610H column



CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 12

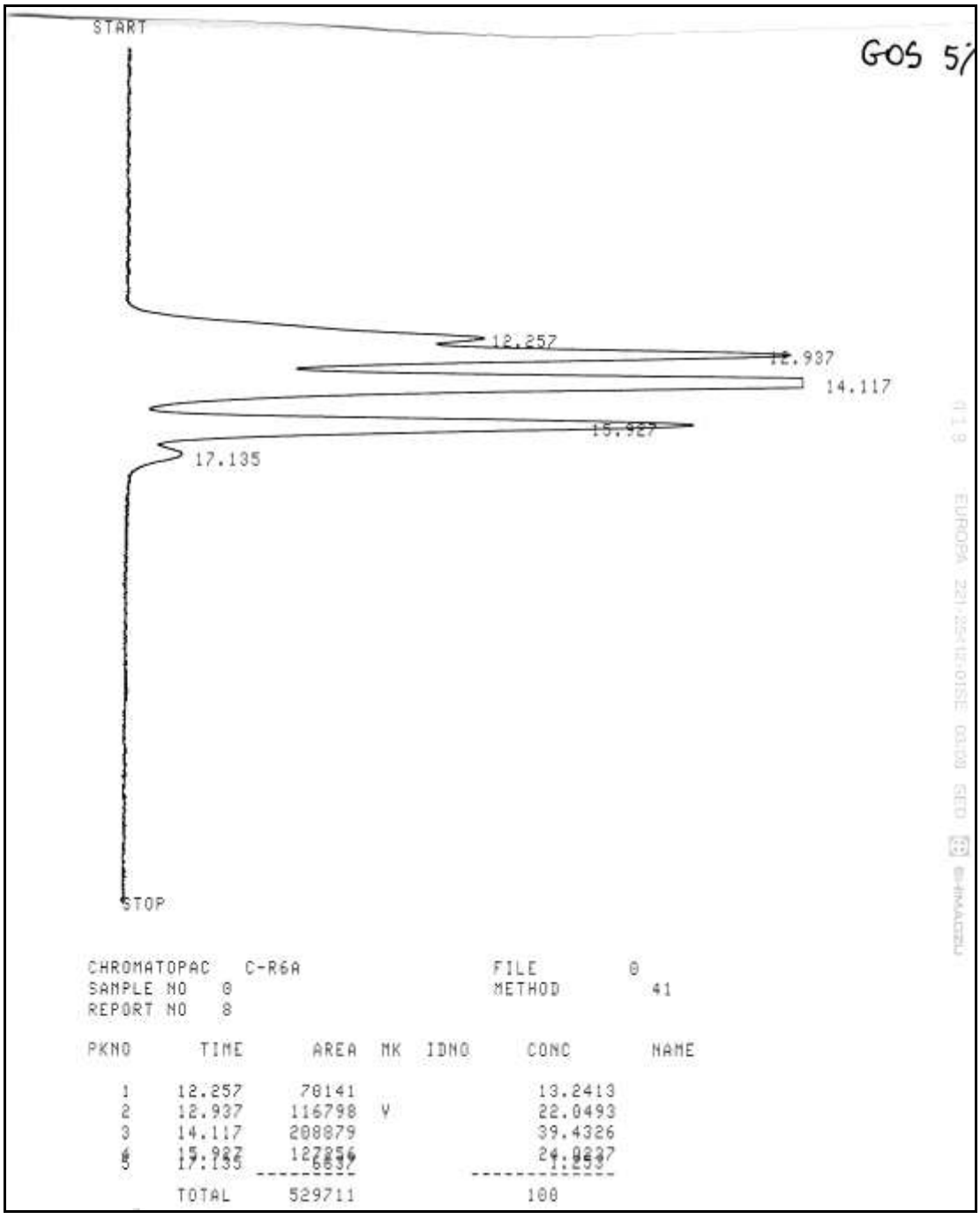
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	11.298	679703			95.0709	
2	12.707	19726			2.7587	
3	13.853	15519			2.1704	
	TOTAL	715027			100	



CHROMATOPAC C-R6A FILE 8
SAMPLE NO 0 METHOD 41
REPORT NO 15

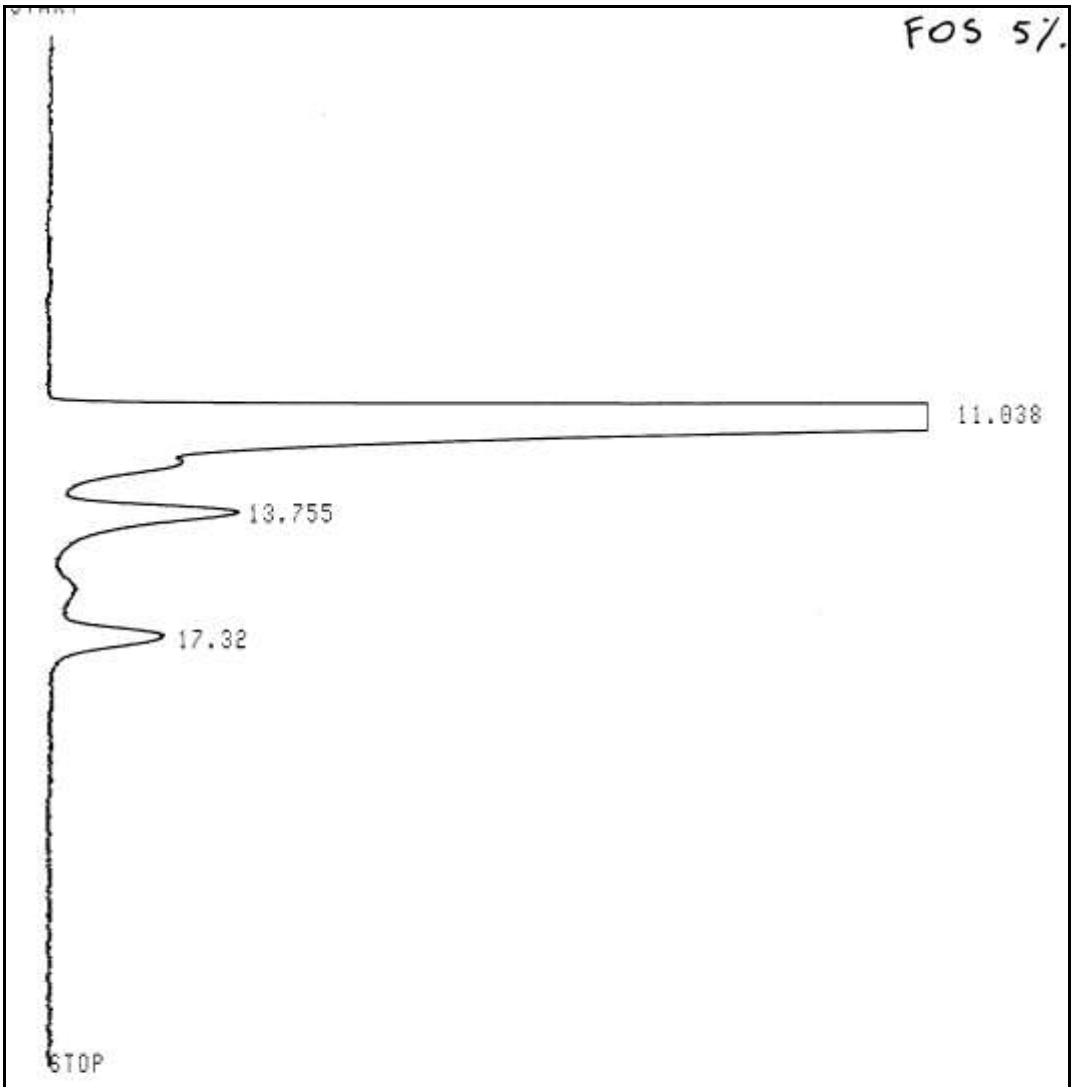
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	12.627	665175			100	
	TOTAL	665175			100	

Appendix S: Cation exchange chromatography of 5% (w/v) prebiotics: GOS, FOS and 2.5% (w/v) inulin on Supelcogel C-610H column



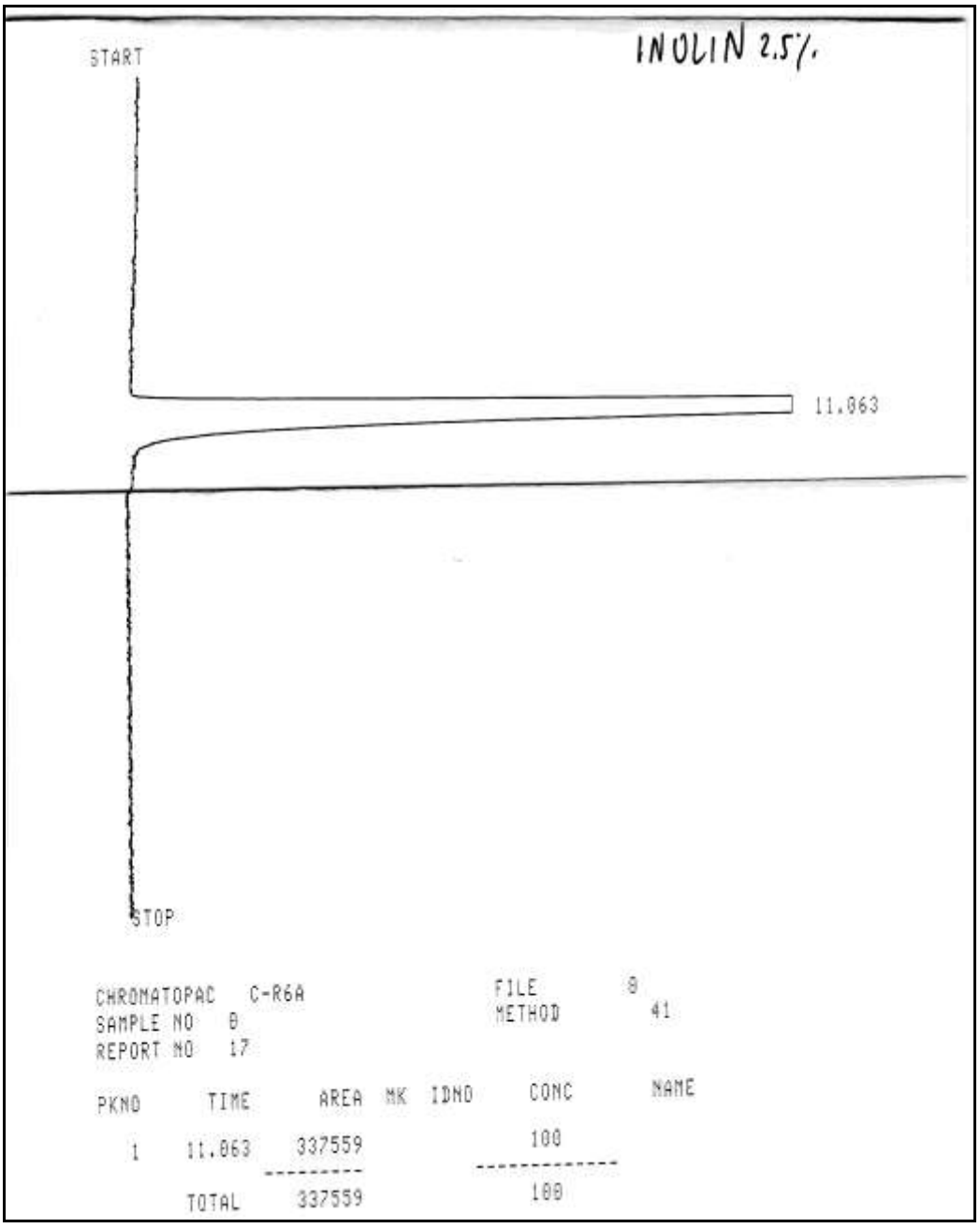
CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 8

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	12.257	70141			13.2413	
2	12.937	116798	V		22.0493	
3	14.117	208879			39.4326	
4	15.927	127256			24.0337	
5	17.135	6637			1.2531	
	TOTAL	529711			100	



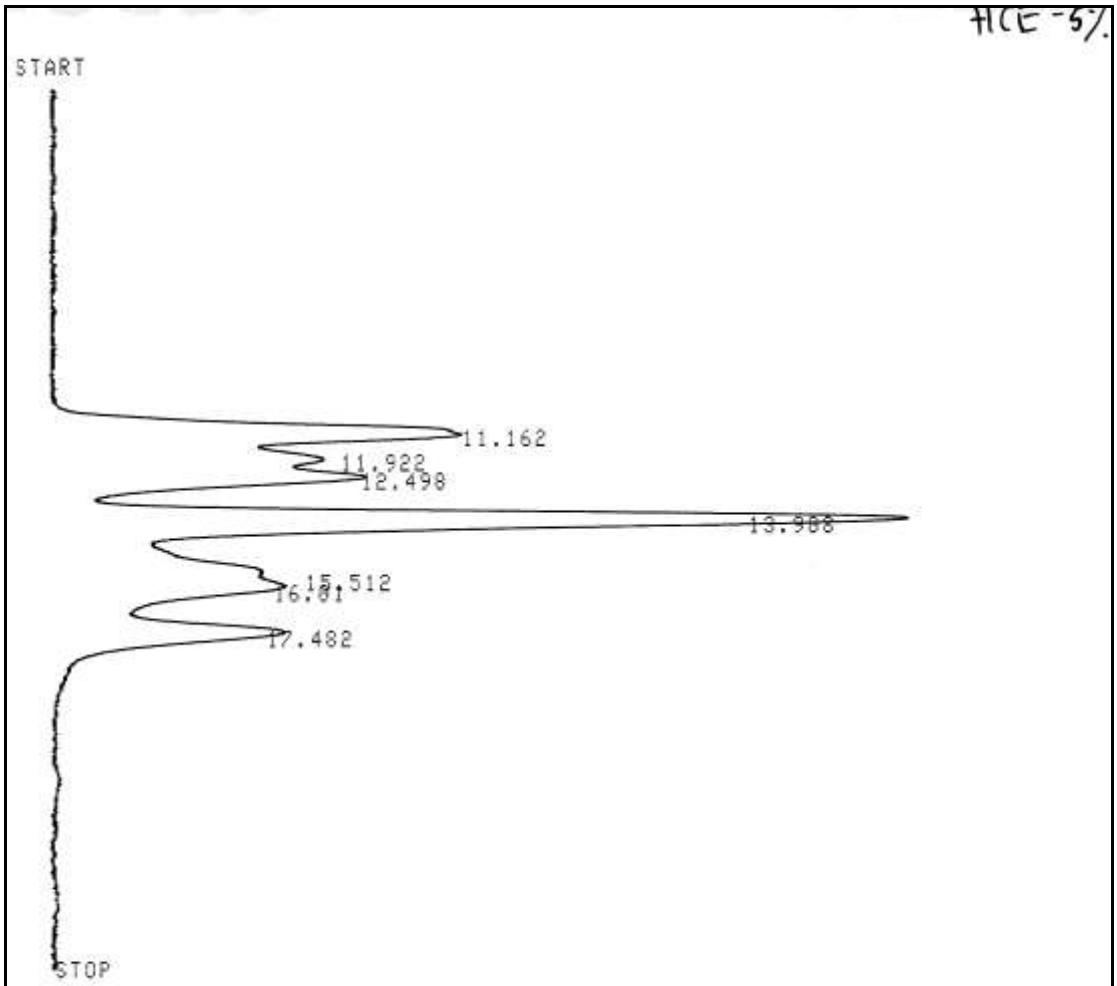
CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 10

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	11.038	624779			92.5565	
2	13.755	32206			4.7711	
3	17.32	18039			2.6724	
	TOTAL	675024			100	



Retention time (t_R) is in minutes

Appendix T: Cation exchange chromatography of 5% (w/v) lyophilised horse chestnut aqueous extract (lHCE) on Supelcogel C-610H column



CHROMATOPAC C-R6A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 11

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	11.162	47953			19.5981	
2	11.922	4249			1.7367	
3	12.498	17561			7.1771	
4	13.908	133675			54.6325	
5	16.01	8049			3.2898	
6	17.482	33193			13.5658	
	TOTAL	244680			100	

Retention time (t_R) is in minutes