Phenolics and Condensed Tannins of Forage Plants from Botswana and their Biological Significance

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ABSTRACT

Phenolics and Condensed Tannins of Forage Plants from Botswana and their Biological Significance

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The main objectives of this study were to isolate and characterise phenolics and CT from Botswanan forage plants, and to investigate their potential anti-parasitic and immunostimulatory properties. *Viscum rotundifolium, Viscum verrucosum, Tapinanthus oleifolius, Grewia flava and Ipomoea sinensis* forage leaves and small stems were harvested over two summers (February 2009 and 2010), extracted twice with acetone:water (7:3) and subsequently defatted with dichloromethane. Each crude extract (6 g) was loaded onto a Sephadex LH-20 column and eluted with aqueous methanol (1:1) to yield four fractions, and subsequently eluted with acetone:water (7:3) to yield three fractions. Phytochemical screening of the fractions for the presence of CT and phenolics was conducted by a reverse phase high performance liquid chromatography coupled to a photodiode array (RP-HPLC-PDA) detector at 280 nm. The butanol-HCl colorimetric assay revealed that the total CT concentrations from the forage plants ranged from 0.2 to 12.7 (g/100g dry matter). These results indicated that significant amounts of CT were present in *V. verrucosum*, *T. oleifolius* and *G. flava*.

The potential impact of each purified CT fraction was evaluated for anti-parasitic effects using a larval development assay (LDA). Three different species of gastrointestinal nematodes (*Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*) from sheep were tested with the fractions at 100 and 500 μg/mL. CT fractions from *V. rotundifolium* and *I. sinensis* samples, collected in 2009 and 2010, did not inhibit larval development (L1 and L2) to the infective L3 stage. CT isolated from *V. verrucosum* and *T. oleifolius* which were collected in 2009 completely inhibited the development of all parasite species at both concentrations. Also, complete inhibition of larval development of all tested parasite species was obtained in CT fractions from *G. flava* collected in 2009, with the lowest inhibitory activity at 62.5 μg/mL. These findings suggest that CT extracts have anti-parasitic effects *in vitro*, which may be translated into reduction of the effects of parasitism in ruminants *in vivo.*

The potential impact of the extracts on priming of γδ T cells from the peripheral blood of lambs, calves and kids at 5 and 10 μg/mL was also evaluated *in vitro*. Condensed tannins (CT) from *G. flava* significantly primed γδ T cells in kids by up to 64.75% at 10 μg/mL, which was statistically significant relative to the negative control at 22.66% (p=0.004). CT from *T. oleifolius* also induced priming of γδ T cells in kids, while fractions from *V. rotundifolium* and *V. verrucosum* induced minimal priming of γδ T cells. These findings suggest that CT from a selected Botswanan forage plants can stimulate the immune system *in vivo* in selected ruminant species.

The anti-parasitic and immunostimulatory effects could be influenced, among other things, by the chemical structure and concentration of CT in the forage sample. The ^{13}C-NMR and thiolysis results revealed that CT from *V. verrucosum, T. oleifolius* and *G. flava* were procyanidin (PC) and *cis* dominant. Further purification of the thiolysis adducts of CT from these plants led to the isolation of (-)-epicatechin which was found to be the dominant compound in the extension units of the CT polymer. The final stage of the research was the re-chromatography of methanolic fractions containing low molecular weight phenolics. The low molecular weight phenolics from the methanolic fractions were successfully purified and isolated. The characterisation of flavonoids by NMR and LC-ESI-MS/MS showed that quercetin was predominant in the purified fractions with attached sugars such as rhamnose, glucose and apiose.

Publications

Part of this project including the sulla research related to my PhD project has been published in:

1. **Tibe**, **O**., Lesperance, L., Fraser, K., and Harding D.R.K (2011). Condensed tannins and flavonoids from sulla (*Hedysarum coronarium*). *Journal of Agricultural and Food Chemistry*, 59 (17), 9402-9409.

2. **Tibe**, **O**., Sutherland, I., Lesperance, L., Pernthaner, A., and Harding, D.R.K (2012). Condensed tannins from Botswanan forage plants are effective priming agents of gamma delta γδ T cells in ruminants. *Veterinary Immunology and Immunopathology*. 146 (2), 237-244.

3. **Tibe, O.**, Sutherland, I., Lesperance, L., and Harding D.R.K (2012). The effect of condensed tannins from Botswanan forage plants on the free-living stages of gastrointestinal nematode parasites of livestock. *Veterinary Parasitology* Journal. In press.

One paper in preparation is to be published

1. **Tibe, O.**, Sutherland, I., Lesperance, L., and Harding D.R.K (2012). Condensed tannins and flavonoids from Botswanan forage plants. *Phytochemistry* Journal.

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LIST OF ABBREVIATIONS

aq	Aqueous
APCI	Atmospheric pressure chemical ionisation
AWMT	Adult worm motility test
BCA	Botswana College of Agriculture
Bu	Butanol
C-18	HPLC column coated with a carbon 18 reverse phase
C	Catechin
CCA	R-cyano-4-hydroxycinnamic acid
COSY	Correlated spectroscopy
CSA	5-chlorosalicylic acid
CCC	Countercurrent chromatography
CT	Condensed tannins
d_4-MeOH	Deuterated methanol
d_6-acetone	Deuterated acetone
d	Doublet (spectral)
Da	Daltons
DAMCA	Dimethylamino-cinnamaldehyde
DCM	Dichloromethane
DEPT	Distortionless enhanced by polarisation transfer
DHB	2,5-dihydroxybenzoic acid
DM	Dry matter
EC	Epicatechin
EGC	Epigallocatechin
EHA	Egg hatch assay
ESI-MS	Electrospray Ionization Mass Spectrometry
FTIR	Fourier transform infrared
GC	Gallocatechin or gas chromatography
GIN	Gastrointestinal nematodes
^{1}H NMR	Proton (hydrogen). In reference to NMR spectroscopy
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HSQC	Heteronuclear single quantum correlation

HPLC	High performance liquid chromatography
HT	Hydrolysable tannin
IAA	trans-3-indolacrlylic acid
IR	Infrared spectroscopy
IS	Internal standard
ISA	Immunostimulatory assay
J	Coupling constant (in NMR spectrometry)
LC	Liquid chromatography
LCS	*Lotus corniculatus*
LDA	Larval development assay
LMI	Larval migration inhibition
LMW	Low molecular weight
LP	*Lotus pedunculatus*
MHC	Major histocompatibility complex
MALDI-TOF	Matrix aided laser desorption ionization, time of flight
MeOH	Methanol
mDP	Mean degree of polymerisation
MIP	Molecularly imprinted polymers
MMW	Medium molecular weight
Min	Minute
mL/min	Millilitre per minute
mmol	Millimole
mol	Mole (s)
mol/L	Moles per litre
MS	Mass spectrometry
Mw	Molecular weight
NA	9-nitroanthacene
NDF	Neutral detergent fibre
NIRS	Near infrared reflectance spectroscopy
nm	nanometer
NMR	Nuclear magnetic resonance
NP-HPLC	Normal phase high pressure liquid chromatography
ODS	Octadecyl silica
PA	Proanthocyanindins

PC	Paper chromatography
PCs	Procyanidins
PDs	Prodelphinidins
PDA	Photodiode array detector
PEG	Poly(ethylene glycol)
RP-HPLC	Reverse phase high pressure liquid chromatography
RRF	Relative response factor
s	Singlet (spectral)
SA	Sinapinic acid
SPME	Solid phase microextraction
t	Triplet (spectral)
TCR	T cell receptors
thio-	thiolysis adduct
TLC	Thin-layer chromatography
TOCSY	Total correlated spectroscopy
UV-Vis	Ultra violet-visible

CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Temperate and tropical forage plants are characterised by a large diversity of secondary metabolites and amongst this diversity is an array of compounds with potential biological activity. These secondary metabolites include simple phenols, phenolic acids, coumarins, stilbenes, flavanones, flavones, anthocyanidins, flavonols, hydrolysable tannins (HT), flavan-3-ols, condensed tannins (CT), cyanogenic glycosides, lignans and lignins, and protease inhibitors and lectins (Naczk and Shahidi, 2004). The major secondary metabolites present in forage plants are CT and simple phenolics (Hummer and Schreier, 2008). Therefore, the discussions in the literature review will focus on these plant metabolites and their biological activity.

In chemical ecology, plants produce secondary metabolites such as CT and store them in cell vacuoles (Waghorn and McNabb, 2003). These stored secondary metabolites deter invasion by pathogens (Ayres et al., 1997), predators (Iason, 2005), insects (Barbehenn and Peter Constabel, 2011, Heil, 2002), pests and diseases (Terrill et al., 1992a). Such a defence strategy has been observed in red sorghum varieties, which are more protected from bird predation and pre-harvest molding than white coloured varieties (Watterson and Butler, 1983). Furthermore, these secondary metabolites protect plant tissues against UV radiation (Waterman and Mole, 1994), and are implicated in nutrient cycling and energy conservation (Aerts et al., 1999).

CT are ubiquitous polyphenolic substances in the plant kingdom. These substances are located in the cell walls and vacuoles of plants, especially in angiosperms and gymnosperms. They are biologically synthesised through the shikimate and acetate pathway, and synthesised in the cell cytoplasm from phenylalanine and acetate precursors (Scalbert et al., 1989). Since they reside in the cell vacuoles of some forage legumes, fodder trees and browses, the vacuoles are disrupted during chewing, drying and grinding, and CT form reversible and irreversible complexes with other macromolecules such as polysaccharides and proteins (McMahon et al., 2000, Wolfe et al., 2008). CT can bind to salivary and plant protein by pH-reversible hydrogen bonding. CT have been used in the leather industry from time immemorial when animal hides were converted into leather. Tannins, due to their polymeric structure, can cross-link with fibrous proteins, especially collagen (gelatine) in animal skins. The interaction

of CT with the proteins involves reversible and pH dependent hydrophobic and hydrogen bonding (Hagerman and Butler, 1980).

CT (2-6% DM) can exhibit a variety of beneficial effects in animal nutrition and health (Waghorn et al., 1998). CT can form stable complexes with proteins in the rumen at neutral pH (pH 6.0-7.5). However, the CT-protein complex dissociates in acidic conditions (pH<3.5) in the abomasum and in basic conditions (pH>8.0) in the small intestine, allowing the proteins to be fully digested and absorbed in the small intestine (Terrill et al., 1992a). Therefore, the CT in low to moderate concentrations can protect the proteins from microbial degradation in the rumen and increase the supply of amino acids in the small intestine. Consequently, potential multiple beneficial effects such as increase in milk yield and composition (high protein, low fat), and liveweight gain, and wool growth, and ovulation rate, and reduction of methane emissions in cattle, sheep and goats are achieved (Waghorn, 2008, Waghorn and Clark, 2006). For example, in New Zealand lambs fed sulla containing CT had significant dry matter and protein intake, a more favourable feed conversion ratio, and increase in live-weight gain (Bonanno et al., 2010). Similarly, dagginess and flystrike in cattle and sheep is reduced, and reduced worm burdens in sheep and goats (Robertson et al., 1995), and stimulation of the ruminant immune system (Schreurs et al., 2010). In addition, CT can prevent the growth of *Streptococcus bovis*, a rumen bacterium that produces dextran slime, and destabilise the proteinaceous foam in the rumen that causes bloat. The beneficial effects in ruminants are attributed to the concentration (Min and Hart, 2003, Tanner et al., 1995), chemical structure (Molan et al., 2000b), different ruminant species (Tanner et al., 1995), physiological state of the animal (Adu et al., 1998), and composition of the diet and other secondary metabolites (Barry and McNabb, 1999).

On the other hand, negative effects in CT (>6% DM) in forage and browse plants include inhibition of protein and fibre digestion (Waghorn et al., 1999), complexing with digestive enzymes, depression of voluntary feed intake and digestibility (Bonanno et al., 2011), and inhibition of amino acid absorption (Aerts et al., 1999), and depression of mineral bioavailability (Muir, 2011). For these reasons, tropical forages are known to be of low quality because of high CT and low protein concentrations in the dry matter.

Forages containing only traces of CT (<2% DM) such as lucerne and clover based pastures can have adverse effects in ruminants. For example, rapid fermentation in the rumen can result in protein loss and nitrogen excretion in urine to the

environment (D'Mello, 1992). Furthermore, ingestion of these legumes may cause bloat, which can be lethal to the animal (Reid et al., 1974).

CT are also present in some non-forage food plants including cocoa (Adamson et al., 1999, Hammerstone et al., 1999), grapes (Souquet et al., 2000a), fruits and vegetables (Scalbert and Williamson, 2000). They are also present in beverages (fruit juices, wine, beer, cider and tea) (Santos-Buelga and Scalbert, 2000). CT are responsible for the flavour, bitterness, astringency, colour and odour attributes of some plant-derived food substances. Complexing of CT with proteins makes them astringent, particularly in tea and wine, which is consistent with plant defense against pathogens, fungi, insects and herbivores. CT are responsible for diverse biological activities such as antioxidant (Molan et al., 2009), superoxide radical scavenging (De Bruyne et al., 1999), chelating and antimicrobial properties (Heim et al., 2002) in the non-forage plants.

While the chemistry of CT and phenolics from forage and food plants and their biological activity have been extensively investigated, detailed studies on these substances from Botswanan forage plants are unknown. There is, therefore, need for a thorough screening of pasture plants for bioactivity for the improvement of livestock (cattle, goats and sheep) productivity in Botswana. Our study was designed to address this gap on the chemistry and biological activity of CT from forages *in vitro*, particularly in relation to reduction of gastrointestinal nematodes in sheep and goats. CT can interact directly with internal and external proteins of gastrointestinal nematodes and disrupt their normal physiological functions. Also, CT may indirectly reduce the effects of gastrointestinal nematodes through the stimulation of the immune system, and this area was also addressed in the study.

Botswana keeps a vast quantity of goats and sheep for income generation, food, employment and cultural practices. In 2004, the population of goats and sheep was 1.55 million and 244 000, respectively (from traditional and commercial farming) (CS0, 2004)). However, diseases associated with gastrointestinal nematodes contribute enormous economic loss to the farming industry. Therefore, alternative control strategies are urgently needed to address this problem, and one approach may be to assess forage plants with putative anthelmintic activity. Increasingly there is a realisation that plants containing phenolics and condensed tannins can offer such alternatives. Parasitic plants in Botswana, also known as mistletoes, form an interesting alternative and additional feed resource, which could increase protein and mineral

intake (Madibela et al., 2000). Parasitic plants have higher protein and mineral content than grass. These plants also contain condensed tannins, which can lower faecal egg counts in goats (Madibela and Jansen, 2003). Smallholding farmers in Botswana have reported using plant pods from *Acacia* species and parasitic plants for meeting the nutritional requirements of sheep, cattle and goats, particularly during the dry season (Madibela et al., 2000). Also, African mistletoe (*Tapinanthus dodoneifolius*) in Nigeria is used as a remedy for several human and animal ailments, including stomach ache, diarrhoea, and wounds. Screening assays of the plant showed a wide spectrum of antimicrobial activities against certain multiple drug resistant bacterial and fungal isolates of farm animals (Deeni and Sadiq, 2002).

The crude extracts and pure isolates from forages may not eliminate helminth parasitism but may reduce the effects of parasitism. However, the plants may be domesticated and commercialised in farming operations, and processed into hay or pellets, and fed as a pure plant material or drenched as a concentrated plant extract. CT from these plants may offer opportunities for development into nutriceutical or pharmaceutical products. They may find applications in the leather industry, food industry, animal nutrition industry, industrial processing and renewable energy industry. They may also be a gateway to synthesis of products with unique properties for commercial use as speciality chemicals, such as adhesives, resins, surfactants, vasodilators in drug delivery to cancer tumor cells (Hemingway, 1989). In addition, the active components may be used as immune system stimulants in ruminants. The ensuing literature review will therefore focus on the chemistry, biological activity and structure-activity relationships of phenolics and CT in forage plants.

1.2. Chemistry, extraction and analysis of condensed tannins (CT)

Condensed tannins (CT) are ubiquitous polyphenolic secondary metabolites which are widely distributed in the plant kingdom. Several spectroscopic and colorimetric techniques are used for their identification, characterisation and quantification.

1.2.1. Condensed tannins chemistry

CT have a complex chemistry. They should be distinguished from hydrolysable tannins (HT). Both differ in the structure and chemical reactivity toward hydrolytic agents (Strumeyer and Malin, 1975). HT are molecules with a carbohydrate entity, consisting of gallic acid (in gallotannin) and ellagic derivatives (in ellagitannin) with ester linkages (Haslam, 2002). HT are associated with toxicity in ruminants (Jansman et al., 1993). As their name implies they are easily hydrolysed with enzymes and dilute acids into sugar moieties such as glucose, and a phenolcarboxylic acid such as gallic acid which contributes to their toxicity (Schofield et al., 2001, Strumeyer and Malin, 1975). HT are usually found in low concentrations in plants (Mueller-Harvey, 2006). They are present in the following plants: oak (*Quercus* spp), Acacia, Eucalyptus, and a variety of browse and tree leaves. Conversely, CT are a group of oligomers and polymers linked by flavan-3-ol subunits, with a considerable range of structural variation, and are non-hydrolysable due to strong interflavanic carbon-carbon bonds (Haslam, 1989). The three rings of flavan-3-ol units are usually denoted as A, B and C (**Figure 1-1**).

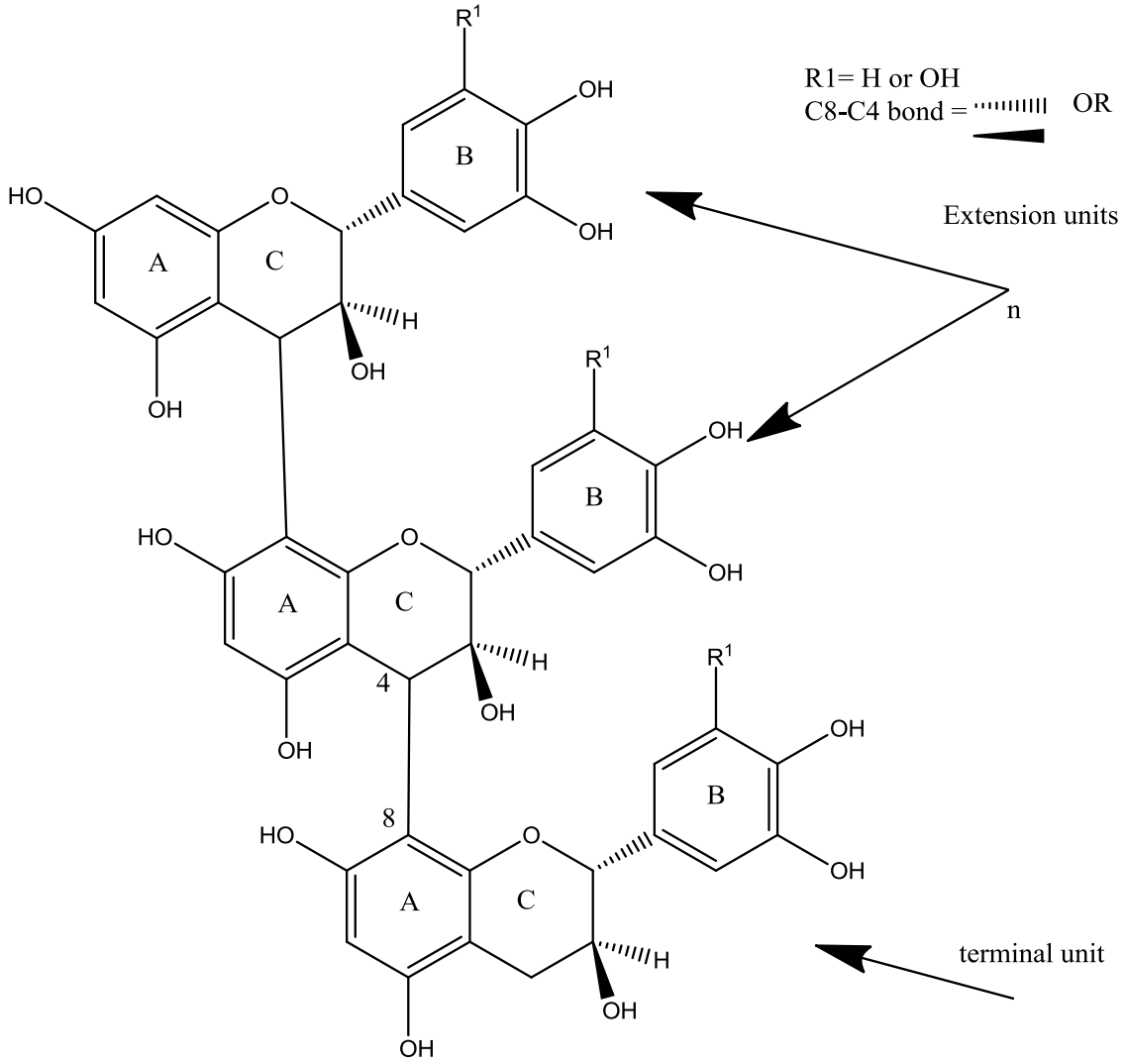
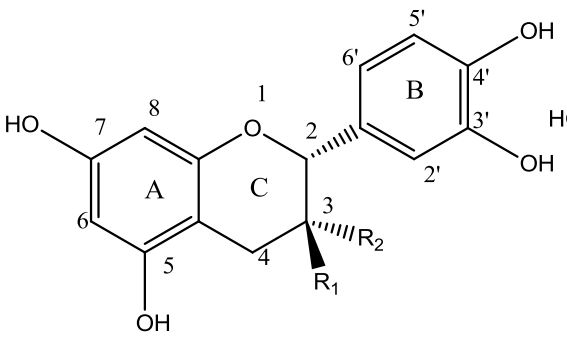
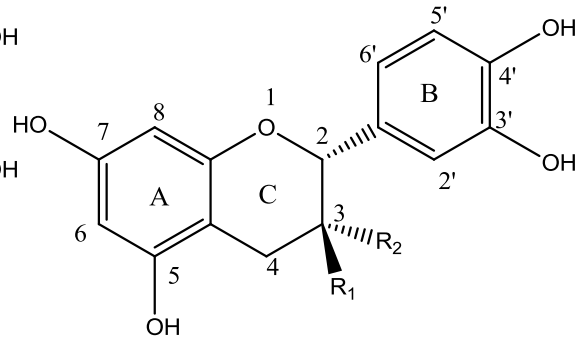


Figure 1–1 Structure of CT polymer (Meagher et al. 2004)

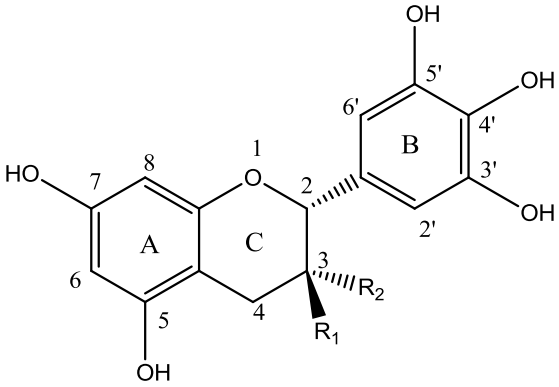
The oxygen and C-4 in the saturated heterocyclic ring (C-ring) lie in the same plane with the A-ring, while C-2 and C-3 lie either above or below the ring (**Figure 1-2**).



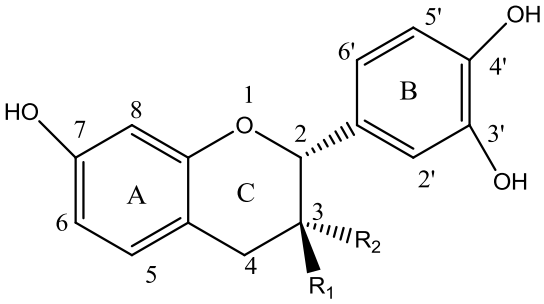
R_1= OH; R_2= H, Afzelechin
R_1=H; R_2= OH, Epiafzelechin
(Propelargonidin monomers)



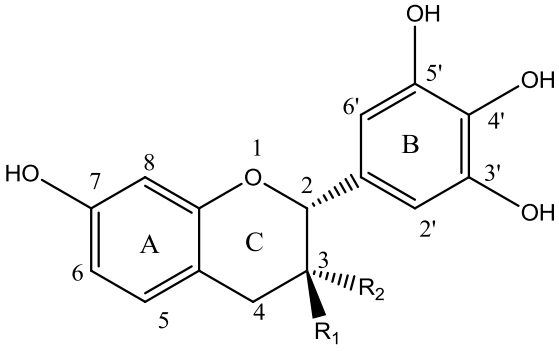
R_1= OH; R_2= H, Catechin
R_1=H; R_2= OH, Epicatechin
(Procyanidin monomers)



R_1= OH; R_2= H, Gallocatechin
R_1=H; R_2= OH, Epigallocatechin
(Prodelphinidin monomers)



R_1= OH; R_2= H, Fisetinidol
R_1=H; R_2= OH, Epifisetinidol
(Profisetinidin monomers)



R_1= OH; R_2= H, Robinitinidol
R_1=H; R_2= OH, Epigrobinitinidol
(Prorobinitidin monomers)

Figure 1–2 The monomeric units of different types of CT (Hummer and Schreier, 2008)

1.2.2. The chemical characteristics of condensed tannins

Condensed tannin (CT) polymers consist of a broad spectrum of structural differences. They vary in the mean degree of polymerisation (mDP), procyanidin (PC):prodelphinidin (PD) ratio, branching, and linkages between flavan-3-ol units (**Figure 1-2**).

There are two major types of CT monomer units prevalent in forages and plant-derived food substances: procyanidins and prodelphinidins (Meagher et al., 2004). The difference between PC and PD is exhibited in the hydroxylation pattern in the B-ring, which contributes to their classification as B-type CT (Haslam, 1989). In other words, the CT structure comprising of epicatechin and catechin is designated as procyanidin (PC), possessing the catechol B-ring. CT polymers with gallocatechin and epigallocatechin are designated as prodelphinidin (PD), possessing the pyrogalloyl B-ring (Hummer and Schreier, 2008). Pure PC are present in sorghum seeds (Watterson and Butler, 1983), willow leaves and pine bark (Jerez et al., 2007). A mixture of PC and PD CT polymers are present in *Lotus corniculatus* (Foo et al., 1996), grapes (Krueger et al., 2003) and wine (Fulcrand et al., 1999). In addition, this mixed type of CT have been isolated and characterised from *Lotus pedunculatus* (Foo et al., 1997), sainfoin (*Onobrychus viciifolia*) (Marais et al., 2000) and sulla (*Hedysarum coronarium*) (Meagher et al., 2006, Tibe et al., 2003). Pure PDs are very scarce and have only been isolated in white clover (*Trifolium ripens L.*) flowers (Foo et al., 2000).

CT can also vary in the stereochemistry at the three chiral centers (C-2, C-3 and C-4 positions) of the heterocycle C-ring. Thus, the stereochemistry of this ring can be 2,3-*cis* or 2,3-*trans*. The stereochemistry of a CT monomer unit involves the following absolute configuration: catechin (2R,3S), ent-catechin (2S,3R), epicatechin (2R,3R), ent-epicatechin (2S,3S). The stereochemistry at C-2 and C-3 carbons is mainly *cis* (2R,3R)-epicatechin and *trans* (2R,3S)-catechin configuration (Ferreira and Slade, 2002, Hummer and Schreier, 2008). Diastereoisomers-(2R,3S) catechin and (2R,3R) epicatechin are the most abundant in plants. A restricted rotation is found in the C-C interflavan linkages due to steric interactions. The molecular shape is dependent on the rotation and stereochemistry of the interflavanoid linkages. CT are a subclass of flavanoids with C_6-C_3-C_6 flavanol skeleton, and they differ from the low molecular weight phenolics by their polymeric structure (Ferreira and Slade, 2002, Haslam, 1989, Khanbabaee and Ree, 2001, Yoshida et al., 2000).

The monomer units in CT may be linked through C4→C8 for linear polymers and C4→C6 (B-type) for branched polymers (Haslam, 2002). The linkage between C-4 and C-8 is more stable than C-4 and C-6 and can only be cleaved under hot mineral acid. Location and stereochemistry of the dimeric substances are usually denoted with α and β, e.g., Procyanindin B-1 (epicatechin (4β→8)-catechin) and B-2 (catechin (4α→8)-epicatechin). The less common B-type CT are epi (afzelechin)-properlagonidins, epi (fisetinidol) profisetinidins, epi (robinetinidol)-prorobinetinidins (**Figure 1-2**).

There is some uncertainty in the literature on the mean degree of polymerisation or chain lengths or molecular weight of CT. They are highly diverse in chemical composition with molecular weights ranging from less than 100 Da for small phenolic molecules to greater than 30 000 Da for highly polymerised structures (Hummer and Schreier, 2008). They are usually classified as CT monomers (1 flavan unit), oligomers (2-10 units) and polymers (>10 units).

1.2.3. A-Type CT

A-type CT (4β-8, 2β-O-7) are characterised by an ether linkage between the C-5 or C-7 of the monomer unit of the A-ring and C-2 of the top monomer unit (Spencer et al., 2007). They lack two hydrogens compared to B-type linkages and are usually referred to as bridged linkages (**Figure 1-3**). The A-type interflavan linkages have been isolated from peanut skins and *Lotus americanus* (Sivakumaran et al., 2006). This type of CT is present in dock (*Rumex obtusifolius*) (Spencer et al., 2007), cranberry (Gu et al., 2002) and cinnamon (Anderson et al., 2004).

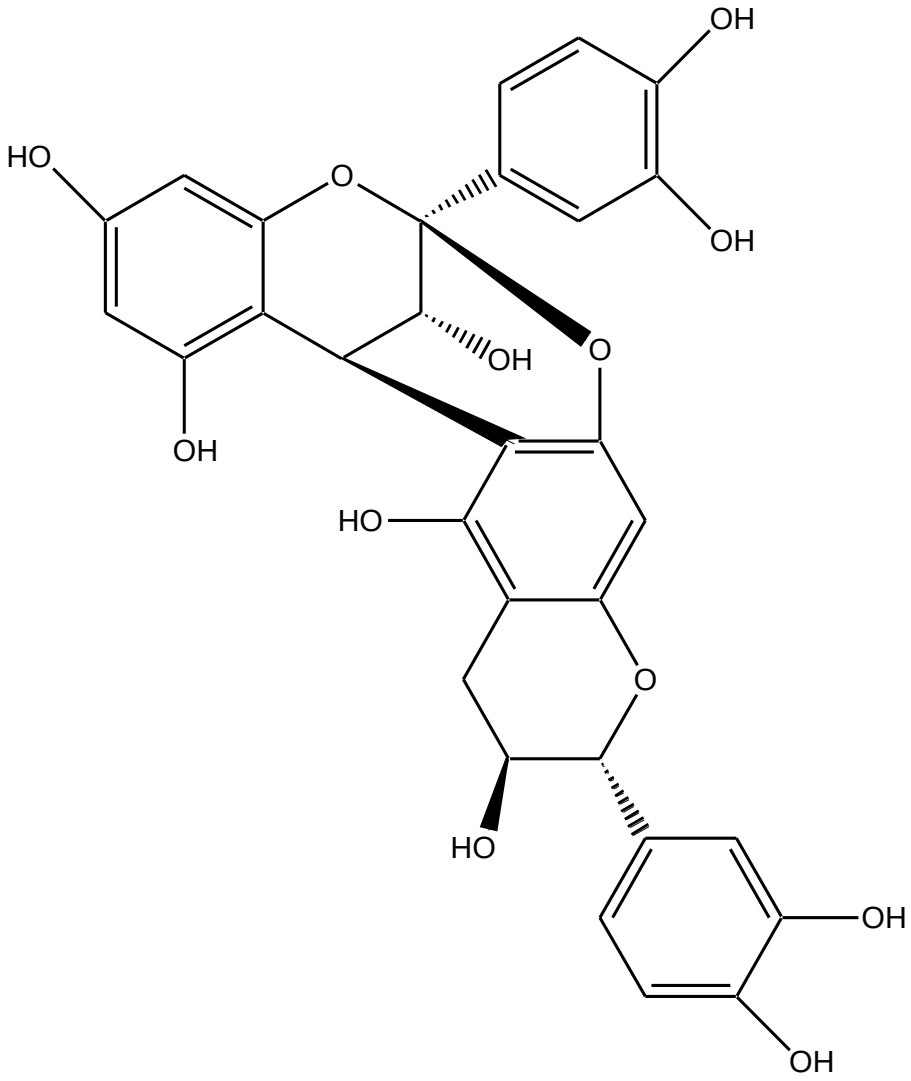


Figure 1–3 A-type CT structure

1.2.4. Galloylated CT

Functionalisation has a significant effect on increasing the biological activity of the CT. The most common substitution at C-3 position in the C-ring is esterification with gallic acid to form 3-O-gallates group (Da Silva et al., 1991). Glycosylation, attachment of sugar moieties, can also occur at the C-3 position. Catechin, epigallocatechin gallate and (-)-epigallocatechin have been isolated from green tea, grapes and red wine (Da Silva et al., 1991). Green tea polyphenols containing gallate are known to act as antioxidants and to lower blood cholesterol level (Chan et al., 2007, Molan et al., 2009). In summary, the higher the number of hydroxyl groups the greater the biological activity.

1.3. Sample preparation and purification

The most important step in phytochemical analysis is to preserve compounds of interest from degradation during different stages of processing. Any losses of compounds should be kept to a minimum. The compounds should be shielded from any chemical reactions with oxygen, light, water, and any biochemical decomposition (Hummer and Schreier, 2008). The effects of processing on the compounds have been examined and these include air drying at room temperature, oven-drying, sun-drying, fresh frozen and freeze-drying, freeze-drying with pre-freezing, freeze-drying without pre-freezing, desiccation with silica gel and molecular sieves (Hagerman, 1988, Julkunen-Tiitto and Sorsa, 2001).

1.3.1. Freeze-drying

The safest and most ideal method for the preservation of phenolic compounds of plant samples is freeze-drying (lyophilisation) prior to analysis (Wolfe et al., 2008). However, a freeze-dried plant material can be degraded if it is exposed to sunlight, humidity and air. Terrill et al. (1990) indicated that freeze-drying was superior to other forms of drying methods in preserving condensed tannins in *Serecia lespedeza*. Oven drying should be avoided as marked decrease of extractable CT was observed (Wolfe et al., 2008). On the other hand, this method may considerably elevate the levels of protein and fibre-bound CT and decrease the levels of free CT. For example, free CT in an oven-dried grape pomace peel were decreased considerably (Larrauri et al., 1997). Oven drying has also been found to decrease CT levels in *Eulalia villosa* Thunb (Nees) (Du Toit and Wolfson, 1996). Air-drying may be a suitable method for drying plant samples where freeze-drying facilities are not available. On the other hand, this drying method may result in increased depolymerisation. However, both air-drying and freeze-drying methods are the most ideal in preserving large molecular weight CT (Abascal et al., 2005). Another suitable preservation method is to collect plant samples from the field and store them in the freezer immediately. According to Tibe et al. (2003), the CT content in a freeze-dried and fresh frozen sulla plant were similar. Therefore, the extraction of the CT from a fresh frozen material without freeze-drying is also recommended.

1.3.2. Other drying methods

Drying of plant samples in a desiccator with molecular sieve and silica gel is safe and can be carried out in the field. It should be noted that phenolics and CT are heat labile, and an increase in temperature to above 60 ºC led to a significant reduction of CT by greater than 60 %. Sun-drying is also discouraged as it led to a decrease of the yield of procyanidins of up to 64% in pear (Ferreira and Slade, 2002). However, if analytical procedures necessitate sample preservation, then fresh frozen samples, desiccation at room temperature and freeze-drying at low temperatures, especially without pre-freezing, are the best and most ideal methods for sample preparation and handling (Dalzell and Shelton, 1997).

1.3.3. Extraction of CT

CT are commonly extracted from plant material using an acetone:water (7:3), and subsequently defatted with dichloromethane. Other solvents that are usually used for the isolation of CT are methanol, methanol/water mixtures, water and ethyl acetate (Meagher et al., 2004). Methanol/water mixtures and anhydrous organic solvents such as ethyl acetate mainly extract monomers and low molecular weight phenolics. These solvents usually recover a minimal amount of CT. The use of a boiled methanol in plant extraction is not ideal because CT are heat labile (Porter et al., 1986). Several trials have been employed by solvents with different concentrations, and optimum extraction is achieved by aqueous acetone:water (7:3) containing 0.1-5% ascorbic acid (Cork and Krockenberger, 1991) or 0.5-2% sodium metabisulfite (Amiot et al., 1995). Extraction periods are variable; however, longer extraction times may eventually oxidize phenolics. The use of an acid with acetone may not be ideal; as the carbon-carbon interflavan bonds may be cleaved in acidic conditions (Hummer and Schreier, 2008). Jones et al. (1976) extracted CT from legume pasture species using acetone:water mixture (7:3), which effectively extracted more than 95% of the CT. Overall, the use of acetone in plant extraction of freeze-dried plant material and fractionation of the extract is the most efficient in the CT recovery (Neves and Lourenco, 1998, Yu and Dahlgren, 2000). However, Koupai-Abyazani et al. (1992) used acetone:water (75:25) in extraction of CT from sainfoin leaves. It should also be noted that a high molecular weight (HMWCT) and medium molecular weight CT (MMWCT) may be recovered in the methanol:water (1:1) LH-20 fraction (Sivakumaran et al., 2006). In this study, an

initial wash with water followed by elution with aqueous methanol (1:1) produced a HMWCT and MMWCT. However, this trend has not been observed in other forages, so this is observed when there is a high molecular weight CT that cannot be eluted with acetone. This suggests that HMWCT may be eluted with low molecular weight phenolics in methanol:water (1:1).

Factors such as excessive temperature and light may affect the chemical structure of CT during extraction. On exposure to sunlight and increase in temperature, there is a significant reduction in amount of CT (Cork and Krockenberger, 1991). In addition to these factors, samples can be degraded by polyphenol oxidase, so CT should be protected from this enzyme, which may interact with them during processing (Ferreira and Slade, 2002). If the samples are stored at high temperatures, chemical reactions are likely to occur due to the activation by the enzyme. Therefore, plant samples should be stored in the freezer immediately after collection to inhibit the activity of the enzyme. Vitamin C (ascorbic acid), tert-butyl hydroquinone (BHT) and sulfites are commonly used as antioxidants.

1.3.4. Purification of CT

After the crude extract preparation, CT are isolated from low molecular weight (Mw) phenolics and sugars using Sephadex LH-20 chromatography (Jones et al., 1976). This purification process utilises size-exclusion chromatography (SEC). Although size-exclusion chromatography is used to separate CT in a preparative scale, it can also be used to determine the molecular weight of CT (Hummer and Schreier, 2008). Sephadex LH-20 gel is a cross-linked dextran (polysaccharide) which is derivatised with hydroxypropyl groups to produce chromatographic packing with lipophilic (hydrophobic) and hydrophilic properties (Bohm, 1998, Strumeyer and Malin, 1975). A dextran is a linear polysaccharide made up of glucose units by 1,6-α-glucosidic bonds. The beads swell well in water, mixtures of polar organic solvents, polar organic solvents and aqueous solvent mixtures. Sephadex gels are spherical in shape and contain a high percentage of solvent and less solid matter. These dextran gels are obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) marketed under the trade name of Sephadex (Kremer and Boross, 1979). The crude extract is dissolved in methanol:water (1:1), loaded and fractionated onto a Sephadex LH-20 column. The crude extract is first washed with methanol:water (1:1). The latter elutes low molecular weight phenolics and

sugars first, an indication that the solution has a high affinity for low molecular weight phenolics. After the elution of the latter, CT are eluted with acetone:water (7:3). This suggests that acetone has a high solvating power for the removal of the CT adsorbed on the Sephadex LH-20 column. The principle of separation involves adsorption chromatography on the CT polymer matrix (Bohm, 1998). However, the non-conventional method employed by Sivakumaran et al. (2006) demonstrated the separation of CT in a size-exclusion manner. Size-exclusion chromatography is also referred to as molecular sieve chromatography and separates analytes in order of decreasing molecular size (Harborne et al., 1975).

CT can also be purified by Toyopearl column chromatography. Souquet et al. (1996) applied this purification method to separate low molecular weight phenolics from polymerised CT, in which the low molecular weight phenolics were eluted earlier with ethanol-water-trifluoroacetic acid solution, and later elution of CT with acetone:water (7:3). Fractogel (Toyopearl) was also employed in the isolation and characterisation of phenolics and CT dimers and trimers in hops and malt (Naczk and Shahidi, 2004).

Other stationery phases that have been employed are reverse phase high performance liquid chromatography (RP-HPLC) and normal phase (NP) HPLC (Prior and Gu, 2005), and diol-bonded phase cartridge, C_{18} solid phase extraction or solid phase microextraction (SPME) (Mateos et al., 2001), molecularly imprinted polymers (MIP) (Ramstrom et al., 2001, Whitcombe et al., 1997), polyamide column chromatography (Escribano-Bailón et al., 1995) and Mitsubishi Company Ion Exchange-gel chromatography packing (MCI-gel CHP column 20) (Foo et al., 2000). CT have also been purified from grape seed and skin using inert glass powder (Pyrex microparticles), and based on their differences in the mean degree of poymerisation.

1.4. Qualitative and quantitative analysis of CT

Several methods have been employed for the qualitative and quantitative determination of the structure of flavan-3-ols and CT. Qualitative information is obtained using paper chromatography (PC), thin layer chromatography (TLC), countercurrent chromatography (CCC), centrifugal partition chromatography, several gel permeation techniques and high performance liquid chromatography (HPLC). Some of these chromatographic techniques are capable of detecting monomers, dimers and

trimers but are unable to detect polymerised structures. Quantitative analysis on the chemical composition of CT can also be determined by a nuclear magnetic resonance (NMR) and thiolytic degradation. In addition, quantitative information can be obtained from the butanol-HCl and vanillin-HCl colorimetric methods.

1.4.1. Thin layer chromatography

TLC is used to provide qualitative data on CT (Spencer et al., 2007). Plant extracts are often screened with TLC for the presence of phenolics and CT. TLC is only used to provide qualitative data on CT and the adsorbent used is silica gel, which is better than adsorption on paper chromatography. The CT are visualised by spraying a TLC chromatogram with vanillin-HCl reagent. CT binds with vanillin to form orange to reddish coloured spots, which are virtually immobile from the original spot, suggesting for the presence of high molecular weight substances. On the other hand, low molecular weight phenolics such as catechins and oligomeric CT are mobile and soluble in the mobile phase. The production of the red and orange coloured spots is an indication of the presence of CT and low molecular weight phenolics, respectively. This technique allows the separation of oligomeric proanthocyanindins up to heptamers (Lea and Arnold, 1978).

1.4.2. High Performance Liquid Chromatography (HPLC)

The coupling of HPLC and UV spectroscopic technology is an extremely powerful technique that is used for monitoring the presence of CT by photodiode array (PDA) of detectors (Bohm, 1998). The UV spectra for CT have two absorption maxima; the first is shown at 200-220 nm and the second at 278 nm (Bohm, 1998). A third absorption band can also be exhibited between the two absorption maxima provided a galloyl or glycosidic group is attached to the heterocylic ring.

Qualitative analysis of the purified CT in a reverse phase HPLC (silica column employing C-18, octadecyl silica, ODS) produces a broad unresolved hump in the chromatogram. The broad hump is attributed to the concentration of individual compounds that decreases with increasing molecular weight and a higher number of possible isomers. In addition, the peak capacity of the stationery phase is reduced in complex matrices. Thus, a shift in the baseline is obtained which suggests the presence of CT. The elution of the CT is carried out in acetonitrile/water, methanol/water in

acidic (formic and acetic acid) conditions (Robards and Antolovich, 1997). Quantitative information can be obtained after degradation of the CT polymer into monomeric units and subsequent analysis of the monomers with RP-HPLC.

1.4.3. Liquid Chromatography/Mass Spectroscopy

The composition of CT can be investigated by liquid chromatography electrospray ionisation-mass spectrometry (LC/ESI-MS). The CT eluting off an HPLC instrument can be ionised and subsequently detected by (ESI-MS). Other different MS interfaces employed include atmospheric chemical ionisation (APCI), electrospray ionisation (ESI), ion trap (IT), thermospray (TSP) and fast atom bombardment (FAB). ESI-MS is a soft ionization technique capable of providing very useful information in the characterisation of phenolics in foods and forages. However, with the increasing chain lengths multiply charged species are detected, which can complicate the interpretation of the spectra (Souquet et al., 2000a). The negative ionisation process involves deprotonation $[M-H]^-$ while positive ionisation involves protonation $[M+H]^+$.

1.4.4. Matrix Aided Laser Desorption/Ionisation-Time of Flight Mass Spectroscopy (MALDI-TOF-MS)

Another excellent and robust technique used in analysis of CT polymers is MALDI-TOF-MS, which can also provide quantitative information on the mean degree of polymerisation and the chemical composition of the CT polymer (Behrens et al., 2003). The main role of the mass spectrometer is to detect gas phase ions, either negative or positive, and to separate them based on their differences in mass per charge (*m/z*) ratio. This is also a soft ionisation technique because the nitrogen laser radiation is absorbed by the organic matrix, hence reducing fragmentation of the analyte molecules incorporated within the matrix. The matrix plays an integral role in promoting ionisation of the CT oligomers and polymers. The basic principle of the time of flight analyser involves the ions, irrespective of the molecular size, beginning their journey at the same time, and resulting in the lighter ions arriving at the detector before the heavier ones (Monagas et al., 2010).

An important factor in sample preparation and in acquisition of a quality MS spectrum is the proper co-crystallization of the analyte with the matrix and the

behaviour of the matrix during laser irradiation. The sample and the matrix should be mixed well to form a homogeneous mixture, and the solvent used should vaporise easily during co-crystallization. The matrix should also inhibit the analyte from degradation from the laser radiation. Dihydroxybenzoic acid (DHB) is the most ideal matrix in analysis of phenolics and CT by MALDI in a positive reflectron mode (Hummer and Schreier, 2008). In addition, other commonly used matrices are trans-3-indolacrlylic acid (IAA), R-cyano-4-hydroxycinnamic acid (CCA), sinapinic acid (SA), 9-nitroanthacene (9NA), 5-chlorosalicylic acid (5CSA). Although IAA provides a mass range similar to DHB, it has a high background noise in the mass range below 500, swamping out the signals from monomers (Es-Safi et al., 2006). For example, DHB was reported to produce two small signals for dimeric CT while IAA led to detection of monomers. Krueger et al. (2000) successfully used IAA for probing CT from grape seed extracts. This suggests that the choice of matrix is vital in characterisation of CT oligomers and polymers.

The MALDI-TOF-MS has enabled the analysis and detection of CT molecules from trimers up to undecamers in food and non-food plants (Hummer and Schreier, 2008). MALDI-TOF is complementary to ESI-MS which can only detect CT oligomers up to trimers. Polymeric molecules are not detected in MALDI-TOF due to longer flight paths but could be lost in the process due to post acceleration process (Monagas et al., 2010). The low molecular weight substances are ascertained to saturate the detector and inhibit the detection of high molecular weight substances. Polymers such as CT with the presence of electronegative elements, e.g. oxygen, are best cationized by Li^+, Na^+ or Cs^+. For example, the sample-matrix mixture is spiked with a solution of sodium chloride to enhance ionization. Actually, $[M-X]^-$ and $[M-2Z]^{2-}$ ions are not detected in MALDI-TOF-MS. Therefore, only cationized adducts $[M+Na]^+$, $[M+K]^+$ and $[M+Cs]^+$ ions are detected. Analytes with π electrons prefer large polarisable ions such as Cu^{2+} and Ag^+.

MALDI-TOF has been applied in the detection of CT polymers in forage plants such as *L. corniculatus* and *L. pedunculatus* (Sivakumaran et al., 2006), *O. viciifolia* (Marais et al., 2000), *H. coronarium* (Tibe et al., 2003), *R. obtusifolius* (Spencer et al., 2007). In the study conducted by Sivakumaran et al. (2006), CT compositrion varied in *Lotus* species, and homo and heteropolymers from trimer (DP 3) up to heptamer (DP 7) were detected in a positive linear mode. In analysis of CT from Lithorcarpus glaber leaves, MALDI-TOF-MS exhibited CT ranging from trimers to undecamers when

caesium trifluoroacetate was used as the matrix (Zhang and Lin, 2008). The MALDI-TOF mass spectra showed signals up to undecamers in analysis of CT in plant leaves and needles of willow and birch tree leaves in German forests (Behrens et al., 2003). Mixing of silver trifluoroacetic acid solution with IAA matrix with apple CT resulted in detection with good sensitivity of $[M+Ag]^+$ ions with a higher degree of polymerisation up to pentadecamer (Ohnishi-Kameyama et al., 1997).

1.4.5. Characterisation by Nuclear magnetic Resonance (NMR)

Nuclear magnetic resonance is a powerful analytical technique for the elucidation of CT structures. It is complimentary to the thiolysis degradation method. Data from ^{13}C-NMR analysis can provide information on the nature of the terminal and extender units in the CT polymer and on the presence of procyanidin or prodelphinidin units (Sivakumaran et al., 2004a).To ascertain the nature of the individual units that make up the polymer, the polymer chains are subjected to acid-catalysed cleavage in the presence of benzyl mercaptan or phloroglucinol. The thiolysis degradation reaction is discussed in the ensuing section.

^{1}H NMR is not useful in determining the structure of these polymeric substances because very broad signals are obtained, suggesting that the CT are of high molecular weight (Foo et al., 1982). In addition, the position of the interflavanoid bond (4β→8 or 4 β→6) cannot be identified. The broad signals are attributed to steric interactions in the vicinity of the carbon-carbon interflavan bond, which contributes to a free movement of the monomer units(Hummer and Schreier, 2008). However, the problem of free rotation can be reduced by derivatisation and subsequent analysis by NMR. A rapid, alternative method was developed and it used chemical degradation and ^{1}H NMR spectroscopy in analysis of CT. From the results, it was found that the resonances of a variety of flavan-3-ols and their thioether derivatives occur at different frequencies (Cai et al., 1991).

1.4.6. Thiolysis

The chemical composition of CT is characterised by acid catalysed degradation with benzyl mercaptan, and the corresponding free flavan-3-ols and thioether derivatives are subsequently analysed by reverse phase HPLC (**Figure 1-4**). This analysis can provide quantitative information on the mean degree of polymerisation (mDP), and chemical composition which includes *cis*/*trans* ratio, PC/PD ratio of the

terminal and extender units in the polymer (Foo et al., 1996). The use of a strong acid such as hydrochloric acid in a thiolysis reaction produces monomeric adducts. However, a weak acid such as glacial acetic acid produces oligomeric adducts. During the reaction, the terminal unit of the A-ring is protonated to form resonance stabilised carbonium ion. Electron release from the carbon-carbon interflavan bond stabilises the ion, and then cleaves the polymer to yield a resonance stabilised carbocation, which is susceptible for attack by the nucleophiles. The resonance structures for this reaction is shown in **Appendix 1-1**.

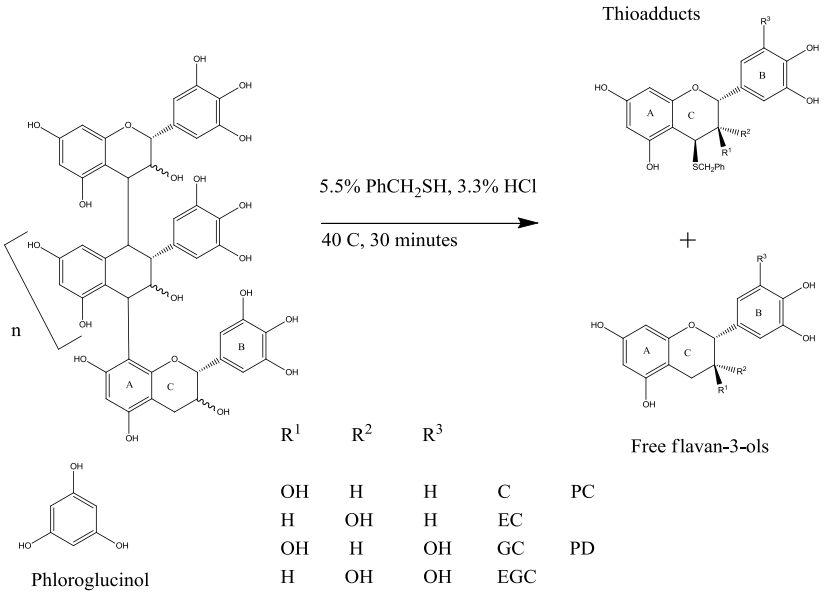


Figure 1–4 Acid catalysed thiolysis degradation of CT polymer using benzyl mercaptan to yield free flavan-3-ols and thioether derivatives. The *trans*-stereochemistry is associated with catechin and gallocatechin, while *cis*-stereochemistry is associated with epicatechin and epigallocatechin.

Phloroglucinol can also be used as a nucleophilic trapping reagent (Koupai-Abyazani et al., 1992) despite being unable to capture all the carbocations. This compound is also odourless and special safety requirements are not required. Although benzyl mercaptan is effective in capturing the carbocations, it has an unpleasant odour, and is toxic and special handling requirements are required. The chemical characteristics of CT can also be extrapolated in ^{13}C-NMR spectra (Koupai-Abyazani et al., 1992). Since CT are large molecules, long acquisition times are required.

1.4.7. Colorimetric assays

Many colorimetric protocols are widely employed in the estimation of extractable CT in food and forages. CT are estimated quantitatively after producing colours with different reagents and their absorbance measured by UV/Vis spectrophotometer.

1.4.7.1. Butanol-HCl method

CT are called proanthocyanidins because they produce red anthocyanidins- cyanidin (procyanidin) and delphinidin (prodelphinidin)- on heating in an acidic solution, which can be detected by UV/Vis spectrophotometer at 525 nm. These compounds are estimated colorimetrically by using the butanol-HCl method (95:5) (Mueller-Harvey, 2006). Butanol has been found as a more suitable solvent than water because it increases the yields of anthocyanidins markedly. The yields of anthocyanidins can also be increased by the addition of trace transition metal impurities, which accelerates autoxidation (Porter et al., 1986). The CT content has been conventionally investigated by degradation into anthocyanidins in the presence of a hot mineral acid solution (**Figure 1-5**). However, side reactions can result in the formation of phlobaphenes and tannin red polymers (Riberau-Gayon, 1972). These may contribute to low yields of CT. The degradation of the CT polymer is analogous to acid catalysed degradation of the CT polymer by thiolysis reaction, except that there is no nucleophile to attack the carbocation.

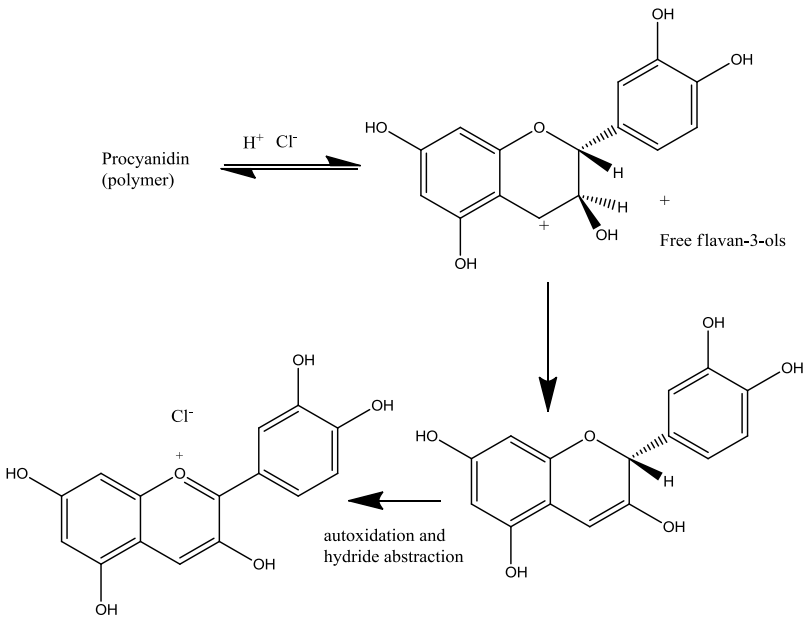


Figure 1–5 Formation of anthocyanidins for analysis of CT content

It was also observed that significant yields of anthocyanidins are achieved by the presence of aerial oxygen, and that the hydride abstraction step is a facile one (Foo et al., 1982). Therefore, the cleavage of the carbon-carbon interflavan bonds of the CT to anthocyanidins occurs by acid catalysis followed by autoxidation (Porter et al., 1986).

The butanol-HCl method cannot provide absolute concentrations but only estimates of CT. It is recommended that the purified CT from a plant being analysed is the ideal standard to estimate CT.

Jackson et al. (1996) indicated that CT can be free (extractable), protein-bound and fibre-bound, which may play a significant role in the health and nutrition of the animals. The interaction between tannins and protein or fibre is through hydrogen bonding and hydrophobic, and are only released when partial rehydration with the aqueous solvent mixtures breaks these bonds.

1.4.7.2. Vanillin-HCl method

The vanillin-HCl method is the most widely used for the estimation of CT. The reactant, vanillin, may interact with other low molecular weight phenolics such as dihydrochalcones and elevate the CT content. In this reaction (**Figure 1-6**), the protonated vanillin (vanillaldehyde) is added at the sixth position in the A-ring to yield a red coloured product, which is detected at 550 nm (Broadhurst and Jones, 1978).

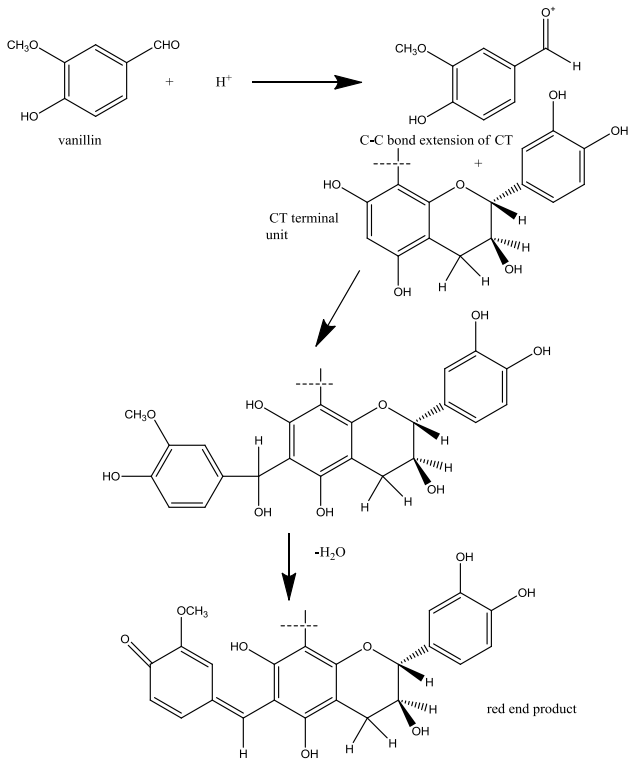


Figure 1–6 Reaction of vanillin with the A-ring of the CT polymer

This method is still widely used in CT research although it is not specific to CT. Some erroneous data could be obtained due to the standard chosen for the analysis of CT. For example, catechin, a monomeric unit of CT, is not an ideal standard for the estimation of CT, as it may lead to a marked overestimation.

1.4.7.3. Miscellaneous methods

Other colorimetric assays used in the determination of CT include the 4-(dimethylamino)-cinnamaldehyde (DMACA), Folin-Ciocalteu and Prussian blue reagents (FAO, 2000). The latter reagents are used for the estimation of total phenolics (Schofield et al., 2001). These reagents are not specific for CT, but they can detect all the phenolic substances present in the plant material, and antioxidant substances such as ascorbic acid may interfere in the process. DMACA reagent does not react with a variety of flavanoids, including dihydrochalcones, flavanones, flavonols and phenolic acids but mainly targets indoles and terpenes (Prior and Gu, 2005). In addition, the CT may be underestimated because this reagent reacts with only with the end unit of CT.

Another method that has been employed in CT determination is the trivalent ytterbium method (Giner-Chavez et al., 1997). This method involves precipitating CT from crude plant extracts using trivalent ytterbium (Giner-Chavez et al., 1997). The use of ytterbium in isolation of CT from plant species was found to be a more reliable alternative method to avoid inaccurate estimates realised with other methods (Giner-Chavez et al., 1997). One advantage with this method is that it is faster compared to the other colorimetric methods.

1.4.8. Other Pasture Phenolics

The biological effects of CT in forage plants have been discussed above. In addition, other potentially bioactive useful plant secondary metabolites which have been found in forage species include low molecular weight phenolics, carotenoids and sugars. It has been reported that sainfoin produces a broad spectrum of low molecular weight phenolic compounds, such as cinnamic acid derivatives, kaempferol, quercetin, afzelin and flavonoid glycosides (Lu et al., 2000). In addition to PD CT in white clover flowers, an abundance of phenolic substances have been found isolated and characterised (Foo et al., 2000). Despite multiple beneficial effects associated with plant secondary metabolites of forage species in animal health and nutrition, some can have adverse effects. For example, red clover is a source of oestrogenic isoflavones, formononetin, diadzein, biochanin-A and genistein(Klejdus et al., 2001). Although red clover isoflavones have adverse effects in animal production, products derived from red clover extracts are now available in market and can reduce pre- and post- menopause symptoms in women. More information on the characterisation of these pasture phenolics discussed in the introduction in **Chapter 3**.

1.5. BIOLOGICAL ACTIVITY

The biological activity of CT in forage plants depends on, among others, the concentration and the chemical structure. Numerous studies have shown that CT are widespread in parasitic and browse plants (Deeni and Sadiq, 2002, Madibela and Jansen, 2003), but their chemical composition (mean degree of polymerisation, PC/PD ratio, *cis/trans* ratio), biological (ethnopharmacological and ethnoveterinary) properties have not been extensively investigated. Thus, the structure-activity relationships may be unknown. Of great significance is the recent discovery of a broad spectrum of ethnoveterinary and ethnopharmacological effects such as nematicidal, immunostimulatory, antimicrobial, antioxidant, and radical scavenging in food and non-food plants (Deeni and Sadiq, 2002, Rochfort et al., 2008). In some cases, these effects are attributed to the presence of CT. Due to increasing development of drug resistance in gastrointestinal nematodes (GIN), and public outcry over synthetic chemical residues in meat and milk products, and the potential unavailability and unaffordability of drugs in certain areas, as well inappropriate and improper use (Sutherland and Scott, 2009), alternative control strategies are urgently needed. One approach is to assess the use of forage plants containing CT and with putative anthelmintic activity (Molan et al., 2003). A list of commercially available anthelmintic drugs is shown in **Table 1-1**.

Table 1-1 Broad spectrum anthelmintic drugs showing the active ingredients and mode of action

Chemical Family	Active ingredient	Mode of action
Benzimidazoles (white drenches or BZs)	Albendazole Fenbendazole Mebendazole Oxfendazole Ricobendazole	Binding to tubulin and causes depolymerisation of microtubules in cells
Levamisoles (clear drenches)	Levamisole Morantel	Acts as a cholinergic agonist and cause paralysis
Macrocyclic lactones (MLs) or 'Ectins'	Abamectic Avermectin Eprinomectin Ivermectin Moxidectin	Exert effect on muscle membranes by irreversible opening of chloride channels

(Leathwick et al., 2001, West et al., 2002)

1.5.1. Forage plants from Botswana

Browse and parasitic plants play a significant role in providing nutrients to the animals for growth and development, particularly when pasture grasses and fresh legumes are not available. Browse plants, tree foliage and shrubs are a major source of nutrients in many arid and semi-arid regions of the world, particularly in Africa. They compliment grasses during dry seasons. Some parasitic plants are used to provide essential minerals and proteins to animals (Mangan, 1988).

A parasitic plant (hemiparasite), commonly known as "mistletoe", is a plant that attaches and parasitises on the stems and branches of trees and shrubs (Madibela and Jansen, 2003, Visser, 1981), analogous to external parasites feeding on the blood of animals and humans. The plant is prevalent in the stems and branches of *Acacia* and *Boscia* trees and other small shrubs. Since the host plant has a long root system into the soil, it is known that parasitic plants continue to survive and remain green during dry

seasons. Domestication and commercialisation of the plants into Botswana farming systems through rotational grazing and cut and carry methods is highly recommended.

1.5.1.1. Parasitic plants description

Parasitic plants consist of small flat leaves, succulent stems, flowers, and roots. These plants are usually hosted in the branches of *Acacia tortilis* and different trees and/or shrubs. Some belong to the *Viscaecae* family. The *Viscaceae* is represented in Southern Africa by *Viscum*. Stems are mostly green and often swollen at the nodes or with ribbed flattened internodes twisted through 90º and resembling leaves (Visser, 1981). Flowers are exceedingly small, red in colour, and commonly about two millimetres in diameter. The calyx and corolla members are fused to form four valvate perianth segments.

1.5.1.2. Propagation

Pollination of the parasitic plants is by insects. The plant has seeded fruits that are sticky or smooth berry that are transculent, yellow, orange or red. The seeds can be eaten by a variety of birds. The seeds are propagated by birds on the stems and branches of trees, and some seeds are wind dispersed. The seeds can develop roots, feed on the nutrient from the host plant and continue to grow on exposure to sunlight (Visser, 1981).

1.5.1.3. Chemical composition

Animal nutrition studies have shown that parasitic plants and browses provide energy, proteins and minerals to ruminants. The mean levels of dry matter (DM), ash, crude protein, carbohydrates and CT of some parasitic and browse plants are presented in **Table 1-2**. The crude protein in the parasitic plants ranged between 10.4 and 20.6 g/kg DM and is usually lower than that of natural grasses of 70 and 40 g/kg (Madibela et al., 2000).

Table 1-2 Ash, dry matter (DM), calcium, acid detergent fibre (ADF), crude protein (CP) and condensed tannin content of parasitic plants and some browses in Botswana (g/100g DM)

Plant name	DM (%)	CP (%)	ADF (%)	Ca (ppm)	Ash (%)	CT (%)
T. lugardii	56.0	13.20	26.50	12.00	nd	5.60
E. ngamicum	nd	12.50	33.20	9.00	nd	6.50
V. verrucosum	61.0	15.80	27.70	11.00	nd	7.50
V. rotundifolium	nd	16.30	24.10	9.00	nd	3.10
Grewia flava	61.0	14.10	4.10	9.00	7.78	1.38
Acacia tortilis	50.0	16.60	2.70	15.00	3.98	1.71
Boscia albitrunca	61.0	19.73	2.60	nd	7.26	0.20

(Aganga et al., 2000, Aganga et al., 2005, Madibela et al., 2000, Phale and Madibela, 2006) nd-not determined

The total CT content in a range of grasses, legumes, trees, herbs and trees grown under temperate conditions is shown in **Table 1-3**.

Table 1-3 Condensed tannin concentration in temperate forage plants in New Zealand. CT was estimated by butanol-HCl method and is a sum of extractable, protein-bound and fibre-bound CT

Forage	Total condensed tannin (g/100g DM)
Grasses	
Lolium perenne (perennial ryegrass)	1.8
Legumes	
Lotus corniculatus (birdsfoot trefoil)	4.7
Lotus pedunculatus (big trefoil)	7.7
Hedysarum coronarium (sulla)	
Spring	8.4
Autumn	5.1
Trifolium repens (white clover)	
Normal	3.1
High CT selection	6.7
Trifolium pratense (red clover)	1.7
Medicago sativa (lucerne)	0.5
Herbs	
Chicorium intybus (chicory)	4.2
Plantago lanceolata (plantain)	14
Forage trees	
Willow (*salix spp*)	33
Poplar (*populus*)	14

(Mupeyo et al., 2010a)

1.5.2. Anti-parasitic activity

Infection with GIN, commonly called roundworms, significantly reduces protein utilisation and productivity in small ruminants (Dalton, 2006). The trichostrongylids are the most pathogenic species of small ruminants, and can cause irreversible damage and mortality in young sheep and goats (Hounzangbe-Adote et al., 2005).

1.5.2.1. Description and classification of gastrointestinal nematodes

A survey on the species of GIN of sheep and goats in Botswana indicated that *Haemonchus contortus* and *Trichostrongylus* species were the most prevalent and pathogenic in young sheep and goats (Carmichael, 1972). The economically important, free-living nematodes in sheep in New Zealand include *H. contortus*, *Ostertagia circumcincta* and *T. colubriformis* (Vlassoff and McKenna, 1994). These species were chosen and used in the study because goats and sheep share similar species of nematodes (**Table 1-4**). *H. contortus O. circumcincta*, and *T. colubriformis* species reside in the abomasum of sheep and goats. The nematode species *T. colubriformis*, *Nematodirus* and *Cooperia* are found in the small intestine. *H. contortus* spp, also called barber pole worm, are the main causes of diseases, and contribute enormous economic loss to the ruminant industry. *H. contortus* adult worms are red in colour, which is attributed to the sucked blood in the abomasum mucosa, which may result in anaemia. In addition, heavy infections may lead to death. Other symptoms associated with *H. contortus* are reduced feed efficiency, loss of bodyweight, poor reproductive efficiency, lowered production of wool and meat (Vlassoff and McKenna, 1994). *T. circumcincta* and *T. colubriformis* are also associated with weight loss, diarrhoea, dagginess, scour, inappetance, morbidity and motility (West et al., 2002).

Table 1-4 Important nematode parasites of sheep and goats

Location	Type
Abomasum	*Haemonchus contortus*
	Ostertagia trifucata
	Trichostrongylus axei
	Ostertagia (Telardorsagia) circumcincta
Small intestine	*Cooperia curticei*
	Cooperia oncophora
	Nematodirus fillicollis
	Mematodirus spathige
	Trichostrongylus colubriformis
	Trichostrongylus vitrinus
Large intestine	*Tricums ovis*
	Charbertia ovina

(Dalton, 2006, Vlassoff and McKenna, 1994, West et al., 2002)

1.5.2.2. Life cycle of gastrointestinal nematodes (GIN)

The *in vitro* anti-parasitic assays are derived from the free-living stages of the life cycle of GIN (**Figure 1-7**). The life cycle of gastrointestinal nematodes is relatively simple. The cycle begins with female and male adult worms mating in the gastrointestinal tract. The female worm then gives out eggs that are shed with the faecal matter on pasture and are hatched into first stage larvae (L1) depending on favourable environmental conditions. The first stage larvae feed on bacteria in the faeces, grow and moult into the second stage larvae (L2). The L2 larvae are the feeding stages that develop into ensheathed and infective third stage larvae (L3). The L3 larvae migrate into grass being motivated by warm conditions and a film of moisture. In this position, they can be ingested by the animal (cattle, sheep and goats) during grazing. The L3 larvae develop into L4 until reaching sexual maturity (Klion and Nutman, 2002). Egg hatch assays (EHA), larval development assays (LDA) and larval migration inhibition (LMI) assays are routinely used to evaluate the efficacy of plant extracts, purified fractions from plants and anthelmintic drugs on the viability of eggs and disruption of the life cycle of GIN.

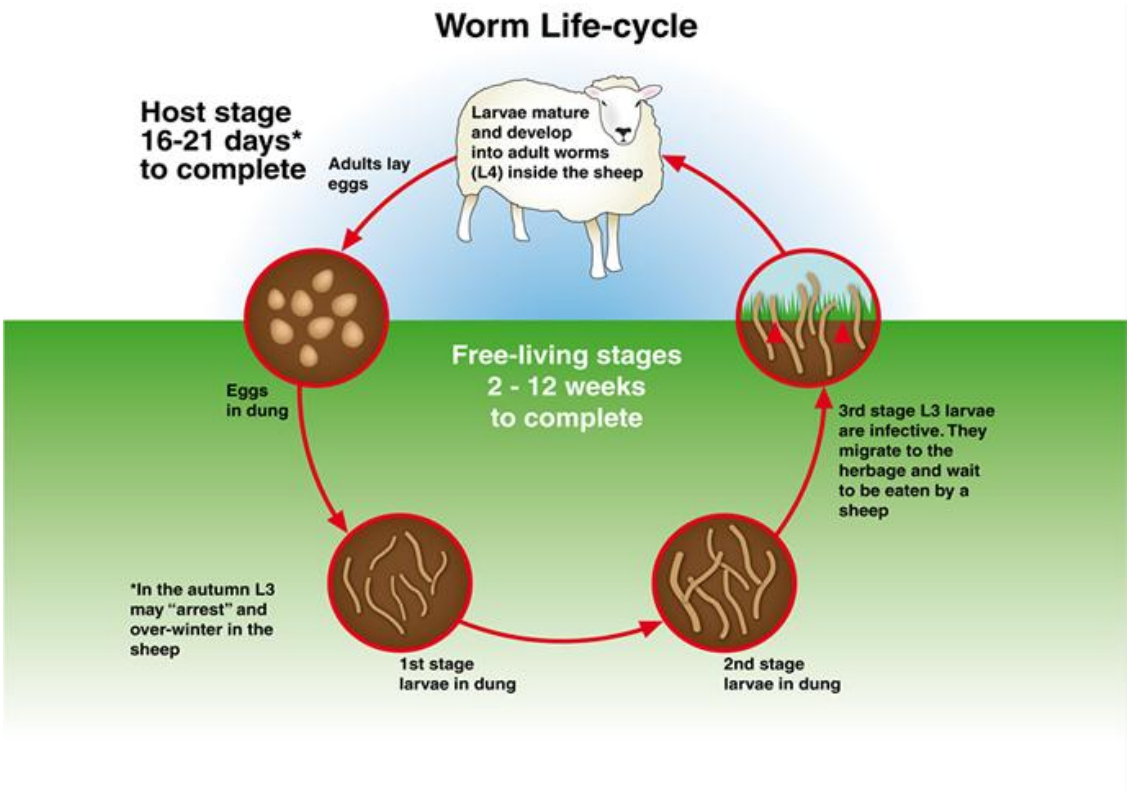


Figure 1–7 The basic life cycle of gastrointestinal nematodes in sheep and goats (http://www.scops.org.uk/endoparasites-worm-species.html downloaded on 14 August 2012).

1.5.3. Control of GIN in sheep and goats

Alternative sustainable non-conventional forages that can be integrated into farming systems to control GIN in sheep and goats are urgently needed. One such strategy is to include the use of forage plants with putative anthelmintic activity. CT in contrast to anthelmintic drugs are not absorbed in the gastrointestinal tract, but they are kept intact and eliminated with the faecal matter, although slight modification may take place. Terrill et al. (1994) indicated that CT are not absorbed in the gastrointestinal tract. Thus, eggs shed with faecal matter may be exposed to the negative effects of CT throughout their development.

1.5.3.1. Effects of plants and extracts on GIN *in vivo*

Parasitised lambs and goats have been found to have improved performance when given CT-containing forages relative to conventional forages. For example, an experimental trial demonstrated increased worm burdens of *T. colubriformis* and *O. circumcincta* in lambs grazing lucerne, while worm burdens and faecal egg counts were lowered in lambs grazing sulla (*H. coronarium*) (Niezen et al., 2002, Niezen et al., 1998). Initial results by Niezen et al. (1995) showed that worm numbers and faecal egg counts were reduced in both parasitised lambs and deer grazing sulla which contained CT. Further investigations when feeding infected animals with *L. pedunculatus* showed reduced worm numbers, egg excretion and increased live weight gain compared to those grazing perennial grass (Niezen et al., 1998). Forages grown under temperate conditions such as sainfoin (*O. viciifolia*), chicory (*Chicory intybus*), birdsfoot trefoil (*L. corniculatus*) and big trefoil (*L. pedunculatus*) reduced worm burdens and their establishment in sheep and goats (Marley et al., 2003, Molan et al., 2000b, Niezen et al., 1998, Paolini et al., 2003). Sainfoin reduced faecal egg counts in lambs infected with *H. contortus* and *T. colubriformis* nematodes (Carbonero et al., 2011). Paolini et al. (2005) also reported that goats infected with *H. contortus*, *T. circumcincta*, *T. colubriformis and* fed sainfoin hay had reduced faecal egg counts. In some *in vivo* studies, faecal egg counts reduction in sheep is associated with enhanced immune response (Mupeyo et al., 2010b, Ramírez-Restrepo et al., 2010).

To further assess *in vivo* studies in forage plants, the active ingredients in the plant material can be concentrated to produce crude extracts, which can then be administered as a drench to the animal. Quebracho (*Schinopsis*) bark extract containing CT has been reported to decrease the establishment of the third stage nematode larvae (L3) in sheep and goats (Athanasiadou et al., 2000). Paolini et al. (2003) reported that female worm fecundity and faecal egg counts were reduced in goats infected with *H. contortus* and receiving quebracho extracts. However, contradictory results were obtained in the case of incoming *H. contortus* L3 larvae subjected to quebracho extracts *in vivo* (Paolini et al., 2005).

Preliminary anthelmintic screening of both alternative forages and extracts has been widely carried out in many African countries, such as South Africa (Bizimenyera et al., 2006), Botswana (Aganga et al., 2006, Madibela and Kelemogile, 2008, Madibela and Jansen, 2003), Tanzania (Bogh et al., 1996), Kenya (Gathuma et al., 2004, Githiori

et al., 2006), Nigeria (Ademola et al., 2007), Benin (Hounzangbe-Adote et al., 2005) and Ivory Coast (Diehl et al., 2004). Lespedeza (*Sericea lespedeza*), prevalent in Asia, has been found to have a significant impact in reduction of nematode worm burdens in goats. In screening a total of 41 plants for anti-parasitic properties in Pakistan, twenty of them were potent in reduction of worm burdens in sheep (Hussain et al., 2008). In addition, non-forage sources such as green tea showed inhibition of motility of infective larvae of *T. circumcincta* and *T. colubriformis in vitro* (Molan et al., 2004). Plant-derived food substances have been used in treatment of internal parasitism and these include garlic, onion, mint, tamarind, chilli, ginger, apple and walnuts. Non-food tobacco leaves have been employed for the removal of external parasites in cattle, sheep and goats (Githiori et al., 2006, Hussain et al., 2008). Nevertheless, there is still a lack of information on the chemical characteristics of the active components present in the plants and responsible for this efficacy.

The mode of action can either be direct or indirect. CT can interact directly with internal and external surfaces of larvae and eggs, and can also disrupt the life cycle and normal physiological functions such as mobility, food absorption and normal physiological functions (Molan et al., 2002). Furthermore, CT can disrupt the physical interference with adult feeding and fecundity (Mupeyo et al., 2010a). However, CT may indirectly reduce the effects of gastrointestinal nematodes by the stimulation of the immune system (Bonanno et al., 2011), and this area will also be addressed in the study The reduction of worm burdens in the animals is attributed to the protection of the protein from degradation in the rumen leading to an increase in protein outflow, which can stimulate the immune system (Mupeyo et al., 2010a). CT complex with proteins in the rumen, and the complexes dissociate in the abomasum and release protein for absorption in the small intestine. Increased flow of amino acids is known to enhance immunity to gastrointestinal nematodes. The presence of CT does not always guarantee anti-parasitic activity. For example, in a study conducted by Whitley et al. (2009), grain sorghum containing CT given to goats did not positively influence faecal egg counts. Other plant species such as peanut skins, although they are a rich source of CT, were investigated for anthelmintic properties but did not show any activity (MacKown et al., 2011). However, it was ascertained that the low prodelphinidin content in the peanut skins contributed to the lack of anthelmintic effects.

1.5.3.2. Effects of purified CT on GIN

The *in vitro* assays are widely used for the high throughput screening of plant extracts for anti-parasitic effects, although validation with *in vivo* studies is also vital. For example, purified CT from seven herbages exhibited anti-parasitic effects against *T. colubriformis in vitro* (Molan et al., 2002). In this study, complete inhibition of larval development at concentrations ranging between 200 and 400 µg/mL was observed. Dose-dependent relationship is usually observed. Actually, anti-parasitic activity of the purified CT is observed between 100-1200 µg/mL (Githiori et al., 2006). However, anthelmintic activity may not be attributed to CT only. Lipids (fatty acids to complex derivatives such as tetrahydrofurans), simple phenolics, alkaloids, terpenes (ranging from essential oils to glycosylated triterpenes-saponins) are also involved in reduction of GIN establishment and fecundity (Rochfort et al., 2008). In addition, another group of compounds, the cysteine proteases, present in papaya, pineapple and figs have been reported as having potential for a new group of antiparasitics, as they might destroy the cuticle of the nematode (Stepek et al., 2004). The cyclotides-natural, circular plant peptides-have showed significant anthelmintic activity in inhibiting larval development and adult worm motility, but with a minimal effect on the inhibition of egg hatching in sheep (Colgrave et al., 2008). More information on the characterisation of these pasture phenolics discussed in the introduction in **Chapter 4**.

1.5.4. CT extracts with immunostimulatory properties

Young cattle, sheep and goats possess relatively high proportions of gamma delta (γδ) T cells, a subset of T lymphocytes, which play a major role in host innate immune response. These cells express γδ T cell receptors (TCR) which recognise an array of antigens (pathogens and gastrointestinal nematodes) and ligands (Hedges et al., 2005). Furthermore, they guard the extracellular environment by ensuring tissue integrity of the damaged epithelial surface (Nath, 2008). While γδ T cells represent a small fraction of T-lymphocytes in the human peripheral blood (0.5-5%), they can be present in much higher proportions (30-70%) in the blood stream of young ruminants, as well as in chickens. They are known to migrate from the thymus and become predominant in the intestinal mucosal linings, reproductive and respiratory tract of all animal species (Gertner et al., 2007, Hedges et al., 2005, Nath, 2008). They are called T

cells because the thymus is the main organ where their maturation takes place (Ferrero et al., 2007).

In contrast to αβ T cells, which recognise processed antigens presented on major histocompatibility complex (MHC), γδ T cells recognise antigens independent of MHC. As such, γδ T cells can recognize low molecular weight compounds, such as phosphoantigens and aminobisphosphanates. In addition, high molecular weight compounds such as CT can be recognised (Gertner et al., 2007). According to Holderness et al. (2007), CT isolated from *Uncaria tomentosa* (Cats claw) and *Malus domenstica* (apple) demonstrated immunostimulatory activity in both human and bovine cells. Moreover, ethanolic extracts of some Sudanese medicinal plants exhibited immunostimulatory effects *in vitro* (Koko et al., 2008). The effect of CT on antioxidant and antimicrobial properties is discussed in the ensuing sections, including the structure-activity relationships. More information on the characterisation of these pasture phenolics discussed in the introduction in **Chapter 3**.

1.5.5. Antioxidant activity

Plant CT and low molecular weight phenolics can act as good antioxidants through several mechanisms such as free radical scavenging, chelation of transition metals, quenching of singlet oxygen or reducing agents, and inhibiting enzymes (Haslam, 1989). An antioxidant is a substance that inhibits the oxidation of another substance (Gordon, 2003). The production of free radicals (reactive oxygen/nitrogen species) by living systems may potentially cause irreversible oxidative damage to the DNA and other cellular components including fats/lipids, carbohydrates and proteins. This may eventually disrupt structural and functional integrity of the cell and consequently result in chain reactions that may cause ageing and onset of diseases (Astley, 2003a).

Free radicals are produced by several conditions such as solar radiation and photosensitivity, food additives, pollution, pesticides, ozone and stroke (Astley, 2003b). Living systems have the potential of terminating produced free radicals by superoxide dismutase enzyme produced endogeneously, or nutrients such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoid (lycopene) produced exogeneously in dietary substances (Haslam, 1989), or elements such as selenium (Molan et al., 2009). Free radicals or reactive oxygen species are oxygen or nitrogen radicals, or non–radicals that

either convert to or easily converted to oxidizing radicals. Types of radicals or anions include the following: superoxide anion (O_2^{-}), hydroxyl radical ($OH^{\cdot}$), alkoxyl ($CH_3O^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), nitric oxide ($NO^{\cdot}$), lipid peroxyl ($LOO^{\cdot}$). Flavan-3-ols electron configuration permits the transfer of an electron to a free radical, and can accept the radical character of the free radical. Conversely, antioxidants such as α-tocopherol can donate an electron to a lipid peroxyl radical and make it return to its original state. Antioxidants are reducing agents because they can terminate chain reactions by stabilising intermediate radicals and become oxidised themselves. However, flavan-3-ols radicals are generally more stable and less harmful to other substances essential for life in living systems (Motlhanka et al., 2008). The free flavan-3-ols have the ability to complex with iron and copper and inhibit the occurrence or formation of hydroxyl radicals (Aron and Kennedy, 2008).

1.5.6. Antimicrobial activity

Plants are traditionally used to treat various diseases in many developing and some developed countries because they exhibit antimicrobial properties. Antimicrobial (antibacterial) are those substances that kill bacteria (bactericidal) and those that inhibit the growth of bacteria (bacteriostatic). *Tapinanthus dodoneifolius*, an African mistletoe, is used as a remedy for various human and animal ailments including stomach ache, diarrhoea and wounds (Deeni and Sadiq, 2002). Screening assays of this plant showed that the parasitic plant had antimicrobial activity against certain multiple drug resistant bacterial and fungal isolates of farm animals. The activity in the plant was attributed to the phytochemical constituents such as CT, saponins, anthraquinones, alkaloids, phlobatannins and other phenolic substances. The CT and phenolic substances of some medicinal plants in Mauritius were linked to the antifungal and antimicrobial properties (Mahomoodally et al., 2005).

1.6. Structure-activity relationships

CT are characterised by their ability to precipitate proteins, alkaloids, carbohydrates and other large bio-molecules. The affinity of CT for protein, due to the hydrogen bonding and hydrophobic interactions, defines their complexing characteristics, which is the basis for most of their biological activities (Hagerman and

Butler, 1980). Proteins and CT interact during harvesting, grinding, extraction, and mastication and digestion by grazing animals, in which the cell structures are ruptured. Terrill et al. (1992b) indicated that in the determination of the extractable CT, the protein and fibre-bound CT should also be estimated. The formed complex is pH-dependent and reversible (Jackson et al., 1996). For example, CT are known to dissociate from proteins in the abomasum (pH<3.5) and in the small intestine (pH>7.0) (Jackson et al., 1996), leading to an increase in the absorption of amino acids. The aromatic regions in CT form hydrophobic interactions with the hydrophobic regions of the protein molecule, and hydrogen bonding regions are attributed to the presence of many hydroxyl groups. The affinity of proteins to CT is influenced by high molecular weight, open and flexible tertiary structures of proteins, including high contents of proline and hydrophobic sites in some amino acids. Irreversible covalent complexes are also likely to occur especially when the polyphenols are oxidized to quinones and their reactions with the nucleophilic groups of proteins (Frutos et al., 2004).

In addition, there is a significant difference in the structural variation in types of CT and concentrations within and between species, and this has a significant effect on the biological activity. Plants may contain a mixture of PD and PC CT, which may contribute to variation in the structure-activity relationships. Significant differences in the chemical structure and biological activity among the same species have been reported in *Lotus* species. For example, the CT structure of *L. pedunculatus* is different from *L. corniculatus* (Foo et al., 1997, Foo et al., 1996). Furthermore, Sivakumaran et al. (2006) reported considerable differences in CT composition in 12 temperate *Lotus* species. A wide variation in biological activity is attributed to the mean degree of polymerisation (mDP), polydispersity of CT, PC:PD ratio and *cis/trans* ratio among the *Lotus* spp. The variation in the chemical composition within species has been evidenced in sainfoin (*O. viciifolia*) (Koupai-Abyazani et al., 1992, Lu et al., 2000). Sainfoin has been reported to have a variable composition with *cis:trans* ratios ranging between 47:53 to 90:10 and delphinidin:cyanidin ratios ranging from 36:64 to 93:7. The differences in the CT composition and concentration could be influenced by cultivars, age of the plant, different stages of development, soil fertility, temperature, drought and seasonality. Furthermore, the hydroxylation pattern may have a strong influence on the activity. PD-type CT have three phenolic groups in the B-ring in contrast to PC-type with two phenolic groups. Thus, CT with higher proportions of PD react more strongly with proteins (Guimarães-Beelen et al., 2006). In addition, the spatial distribution of the

monomer units in the CT molecule contributes to their protein binding capacity. The PC:PD ratio plays a significant part in the antioxidant activity of the CT molecule, and the activity increases with increasing proportions of PD (Heim et al., 2002). This study confirmed that antioxidant activity is dependent on the hydroxylation pattern in the B-ring. Flavonoids with three phenolic groups in the B-ring are more potent than those with two phenolic groups in the B-ring (Motlhanka et al., 2008). The biological activity is dependent on the chemical characteristics in the terminal units or in the extender units of the polymeric CT. The activity of CT can be removed using polyethylene glycol (PEG) in *in vitro* and *in vivo* studies, because PEG has a high affinity for CT. CT preferentially binds to PEG, causing an exchange reaction where protein is released from the CT-protein complex (Holderness et al., 2008). In *in vivo* studies, this process releases protein in the plant for normal rumen degradation. In addition to the chemical characteristics of CT and their biological activity, the concentration of CT may determine the mechanism of action of the CT molecule. According to Hoste et al. (2006), anti-parasitic effects in sheep, goats and deer were realised at a concentration level of 30-40 g CT/kg DM.

1.7. PhD project objectives

In this study, phenolics and condensed tannins (CT) were isolated and characterised from five Botswanan forage plants (*Viscum rotundifolium*, *Viscum verrucosum*, *Tapinanthus oleifolius*, *Grewia flava* and *Ipomoea sinensis*). The anti-parasitic effects of the active components were investigated using *in vitro* parasite assays. The immunostimulatory properties of CT from forage plants in stimulation of the innate immunity were also investigated. The variability of anti-parasitic and priming of the γδ T cells via CD25 up-regulation in CT-containing fractions from plants collected in different years, i.e in 2009 and 2010 was investigated. A flow chart of the experimental plan of the whole project is shown in **Figure 6.1** under summary and future directions (**Chapter 6**).

CHAPTER TWO:

ISOLATION AND CHARACTERISATION OF CONDENSED TANNINS FROM BOTSWANAN FORAGE PLANTS

2.1. Introduction

Polyphenolic compounds such as condensed tannins (CT) or proanthocyanidins (PAs) are widely distributed in the plant kingdom, and are ubiquitous in leaves, stems, barks, roots and seeds (Bravo, 1998). CT are present in plant-derived food substances such as cocoa (Adamson et al., 1999), red wine (Fulcrand et al., 1999), green tea (Paquay et al., 2000), grapes (Souquet et al., 2000b), fruit and vegetables (Scalbert, 1991). They exhibit antiviral, superoxide radical scavenging (De Bruyne et al., 1999), antioxidant and chelating (Heim et al., 2002), and antimicrobial properties (Scalbert, 1991).

Previous studies have shown that CT (2-6% DM) from forage plants exhibit beneficial effects on the ruminant health and productivity of ruminants. However, forages containing traces of CT such as grasses, lucerne, and clover pastures are associated with negative effects in ruminant nutrition. For example, rapid fermentation in the rumen results in protein loss in the form of ammonia and increased urinary nitrogen excretion to the environment. In addition, the ingestion of legumes may increase the occurrence of bloat, which can cause death to the animal. Similarly, forages containing high concentrations of CT have adverse effects on ruminants, such as inhibition of protein and fibre digestion, amino acids absorption, inhibition of digestive enzymes, depression of voluntary feed intake, and depression of mineral bioavailability. To improve productivity and protein utilization in ruminants, non-conventional forages are urgently required. One approach is to assess forage species that contain low to moderate CT. Although condensed tannins are often considered anti-nutritional, some studies have shown that these substances in low to moderate concentrations (2-6% DM) have beneficial effects in ruminants. They can protect dietary protein from degradation in the rumen (pH 6.0 to 7.0) micro-organisms and dissociate in the abomasum (pH<3.5) and the small intestine (pH>7.0), resulting in increased absorption of amino acids in the small intestine. Consequently, multiple beneficial effects such as increase in milk yield, and liveweight gain, and ovulation rate, and wool growth, and enhancement of resistance to infections, and reduction of worm burdens, and dagginess and flystrike, and methane emissions in ruminants are realized (Aerts et al., 1999).

Condensed tannins are oligomers and polymers of flavan-3-ol units. CT structures may differ at many levels. The flavan-3-ol units in several forage species consist of procyanidin (PC) monomers, catechin (C, 2,3-*trans*) and epicatechin (EC, 2,3-*cis*) and/or prodelphinidin (PD) monomers, gallocatechin (GC, 2,3-*trans*) and epigallocatechin (EGC, 2,3-*cis*) isomers. Many polymers are mixtures containing prodelphinidin and procyanidin units in differing ratios. The flavan-3-ol units are usually identified with the use of the alphabets A, B and C rings (**Figure 1-1 Section 1.2.1 under Literature Review**). In addition, CT may differ in the degree of polymerisation, carbon-carbon interflavan linkages, and galloylation (Spencer et al., 2007). Condensed tannins from Botswanan forage plants such as *V. verrucosum* have been demonstrated to reduce the effects of parasitism in goats (Madibela and Jansen, 2003) . Smallholding farmers in Botswana have reported feeding these plants as supplement to cattle, sheep and goats during the dry season to meet their nutritional requirements and to improve their health (Madibela et al., 2000). The effects of CT on ruminants depend not only on the concentration, but also on chemical structure and other factors such as different ruminant species, physiological state of the animal and composition of the diet. The butanol-HCl colorimetric assay is widely used to quantify condensed tannin concentrations in diets from plant species, but data on the composition of the condensed tannin fraction or other phenolic components is often not available. While *in vivo* and *in vitro* effects of CT from these forages in Botswana have been studied, their phenolic chemistry has not been studied in detail. Hydroxylation, galloylation and the extent of oligomerisation (n=2 to 10) and stereochemistry are known to play a significant role in the structure-activity relationships. Hence, the aim of this study was to isolate and characterise CT from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis*. The first three plants above are called parasitic plants because they attach to the stems of *Acacia* and *Boscia* species, deriving nutrients and water from the host plant. *G. flava* is a browse while *I. sinensis* is a legume.

2.2. Materials and Methods

2.2.1. Materials

Sephadex LH-20 matrix was purchased from GE Healthcare, New Zealand. Silica gel matrix (C-18) was obtained from Grace Davison Discovery Sciences, New

Zealand. Acetone, methanol, acetonitrile, hydrochloric acid, ethyl acetate, dichloromethane, formic acid, benzyl mercaptan, acetone-d_6, methanol-d_4, D_2O and all other reagents were of analytical grade unless otherwise specified and were used as received. Ascorbic acid was purchased from BDH Chemicals, Auckland, New Zealand. Catechin (C), epicatechin (EC) and epigallocatechin (EGC) were obtained from Sigma Chemicals, New Zealand. Solvent % (v/v) is for % solvent in water unless otherwise stated.

2.2.2. Equipment

A rotary evaporator (Rotavapor, Buchi, Switzerland) was used for the removal of solvents under vacuum unless otherwise stated. Thin-layer chromatography was done on silica gel TLC plates 60 F_{254} (Merck). UV/Vis (GBC UV/Vis 918) spectrophotometer was used in the determination of CT content (free, protein and fibre-bound CT). Sephadex LH-20 column (XK 26/40 and XK 26/100) was connected to a Fast Protein Liquid Chromatography (FPLC, LCC-500, Uppsala, Sweden) for the fractionation of the extracts. A reverse-high performance chromatography RP-HPLC (Alliance HT Waters 2790 Separation mode) which was coupled to a photodiode array detector-PDA (Waters 996A, Milford, MA, USA) was used for screening of the fractions for the presence of CT and flavonoids. Elution was performed with columns of the following dimensions: reverse phase (C-18) Phenomenex Jupiter RP column (250 x 4.6 mm, particle size: 5 µm), RP (C-18) Alltima column (250 x 2.1 mm, Alltech, particle size: 5 µm). ESI-MS (Waters ZMD, Milford, MA, USA) and (Micromass MALDI TM, Milford, MA, USA) were used for the determination of the molecular weight of the phenolic compounds. In addition, the ^{1}H and ^{13}C-NMR were recorded on a Bruker Avance 500 and 700 MHz NMR spectrometer (Rheinstetten, Karlsruhe, Germany).

2.2.3. Methods
2.2.3.1. Plant collection

Fresh five different forage plants, consisting of stems and leaves, were harvested from the Botswana College of Agriculture (BCA) farm in Gaborone in summer (February 2009 and 2010) and stored in the freezer at -20 ºC prior to freeze-drying and grinding (**Table 2-1**). The pictures of the forage plants are shown in **Appendix 2-1.**

Table 2-1 Plant samples collected at the BCA farm in February 2009 and 2010

Sample number	Voucher specimen number	Date of collection	Local name	Scientific name	Local name and host plant
OTC00101	BT/08/05	27/02/09	Boletswa ja mosu (PP)	*Viscum rotundifolium*	Mosu (*Acacia tortilis*)
OTD00101	BT/08/05	10/02/10	Boletswa ja mosu (PP)	*Viscum rotundifolium*	Mosu (*Acacia tortilis*)
OTC00102	BT/08/01	27/02/09	Boletswa ja motlopi (PP)	*Viscum verrucosum*	Motlopi (*Boscia albutrunca*)
OTD00102		12/02/10	Boletswa ja motlopi (PP)	*Viscum verrucosum*	Motlopi (*Boscia albutrunca*)
OTC00103	BT/08/04	27/02/09	Boletswa ja mongana (PP)	*Tapinanthus oleifolius*	Mongana (*Acacia mellifera*)
OTD00103	BT/08/01	02/02/10	Boletswa ja mongana (PP)	*Tapinanthus oleifolius*	Mongana (*Acacia mellifera*)
OTC00104	BT/08/02	27/02/09	Moretlwa (shrub)	*Grewia flava*	-
OTD00104	BT/08/02	02/02/10	Moretwa (shrub)	*Grewia flava*	
OTC00105	BT/08/06	27/02/09	Motantanyane (legume)	*Ipomoea sinensis*	-
OTD00105	BT/08/06	02/02/10	Motantanyane (legume)	*Ipomoea sinensis*	

PP- parasitic plant

2.2.3.2. Determination of nutrient composition

Freeze-dried and ground plant samples were submitted to Mr. Grant Taylor at Feedtech, AgResearch Grasslands for nutritional composition determination using near infrared reflectance spectroscopy (NIRS systems 6500). The dry matter (DM), crude protein (CP), crude fat, metabolizable energy (ME), soluble sugars and starches (SSS), neutral detergent fibre (NDF) and acid detergent fibre (ADF), and condensed tannins (free, protein and fibre bound) of each plant sample were determined.

2.2.3.3. Determination of CT content

The extractable, protein and fibre-bound CT concentrations of the individual plants were determined by the butanol-HCl colorimetric method described by Jackson et al. (1996). Measurements were performed in duplicate and the mean value presented as a percentage (%) of DM. All CT contents were determined using CT extracted from *Lotus pedunculatus* as a reference standard for calibration. This is the only standard that was available when this experiment was carried out.

2.2.3.3.1. Free CT

For each forage species, freeze-dried plant sample (500 mg) was weighed into 50 mL screw-top Falcon tubes (Falcon, USA) in duplicate. The samples were extracted

with a mixture of acetone:water (7:3, 20 mL) containing 1 g/L ascorbic acid and diethyl ether (10 mL). The purpose of the ascorbic acid was to prevent oxidation. The tubes were placed on a vortex mixer and centrifuged at 3000 g for 15 minutes to separate liquids and solids. The extraction was repeated two times and the supernates were pooled in a conical flask (100 mL). Additional diethyl ether was added to improve separation of the aqueous layer containing CT from lipids and chlorophyll. At this ratio of diethyl ether to acetone and water, a two phase separation occurred with an upper phase of organic and a lower aqueous phase. The upper phase solvent was removed and discarded. The aqueous phase, containing CT, was rotary evaporated to remove the residual acetone and diethyl ether solvent at 40 ºC. The aqueous phase was centrifuged at 15000 rpm for 15 minutes at 20 ºC to remove non-tannin debris. The supernate above the debris was transferred into 50 mL measuring cylinders and increased to 45 mL with distilled water, and the remaining extract was later stored in Falcon tubes and kept in the refrigerator. For analysis of CT, each extract (1 mL) was combined with butanol-HCl (6 mL) in duplicate. The other tube containing 1 mL of each supernate and 6 mL of butanol-H_2O was used as the blank. For the standards, each *Lotus pedunculatus* standard dissolved in water in duplicate was combined with butanol-HCl (6 mL) and used for free CT. The samples were then placed in a boiling water bath for 75 minutes. The same treatment was applied to all the standards. The stock solution was prepared by using purified CT from *Lotus pedunculatus* (5 mg) in 10 mL volumetric flask and diluted to the mark with distilled water. The samples were allowed to cool, and centrifuged at 3000 g for 15 minutes and the supernatant analyzed using UV/Vis spectrophotometer (GBC UV/Vis 918) at 550 nm.

2.2.3.3.2. Protein-bound CT

In order to extract protein-bound CT, a sodium dodecyl sulfate (SDS) solution (15 mL) in trisaminomethane and 2-mercaptoethanol was added to the solid residue which was obtained after the removal of the supernate for analysis of free CT. The SDS solution contained SDS (1%) and 2-mercaptoethanol (5%). The tubes were vortexed and placed in a constant-level boiling water bath at 100 °C for 45 minutes. The extraction was repeated with the addition of 15 mL SDS solution. The extracts were allowed to cool, then centrifuged and the supernates were placed in Oakridge tubes. The combined supernates were centrifuged at 15000 rpm to remove any non-tannin debris.

The supernates were increased to 35 mL with SDS solution. The unused supernates were placed into Falcon tubes. Next the extracts (1mL of extract and 6 mL of butanol-HCl) were placed in a water bath for 45 minutes, then allowed to cool and analyzed with UV/Vis at 550 nm. The other tube containing 1 mL of each supernate and 6 mL of butanol-H_2O was used as the blank and placed in a water bath and boiled in the same manner. For analysis of standards, each SDS standard (1 mL) was mixed with butanol-HCl (6 mL) in duplicate and used for protein-bound CT evaluation.

2.2.3.3.3. Fibre-bound CT

Fibre-bound CT was determined directly from the residue remaining from the extraction of protein-bound CT. To extract the fibre-bound CT, 5 mL of SDS solution in Tris and 2-mercaptoethanol, 30 mL of butanol-HCl, and 30 mL butanol-H_2O were added directly to the residue remaining from the extraction of protein-bound CT. An SDS solution (1 mL), and butanol-HCl (30 mL), and butanol-H_2O (30 mL) were used as the blank. The tubes were placed in a boiling water bath for 75 minutes. The tubes were allowed to cool and centrifuged for 15 minutes at 20 ºC at 15,000 rpm using Sorvall Centrifuge (Sorvall RC-5 Superseed Refrigerated). The supernates were then analysed using UV/Vis at 550 nm. Standards used for the determination of protein-bound CT were used for fibre-bound CT.

2.2.3.3.4. Preparation of CT standards

Standards were analysed concurrently with each batch of samples analysed for free, protein and fiber bound CT determination. The CT standard was made up in water to give a standard for the estimation of extractable CT, whilst CT was made up in SDS-2-mercaptoethanol ethanol reagent for the estimation of protein and fibre-bound CT. All standards were treated identically to the samples by addition of butanol-HCl and butanol-H_2O. Thawed *Lotus pedunculatus* CT extract (5 mg) was dissolved in 10 mL of water. The stock solutions were used for the preparation of working standards for analysis of free CT. For protein-bound CT, *Lotus pedunculatus* CT extract (5 mg) was dissolved in 10 mL of SDS solution. The stock solution was used for preparation of working standards for analysis of protein-bound CT and fibre-bound. The standards were placed in a water bath, allowed to cool and read in UV/Vis spectrophotometer. Working standards from the stock solution were prepared as shown in **Table 2-2** below.

Table 2-2 The working standards prepared from the stock solution of *Lotus pedunculatus* CT

Amount in mL taken from stock solution	Water (mL)	Concentration (ppm)
0.00	5.00	0
0.25	4.75	25
0.50	4.50	50
1.00	4.00	100
1.50	3.50	150
2.50	2.50	250
3.75	1.25	375

2.2.3.4. Plant extraction

The low molecular weight (Mw) phenolics and condensed tannins (CT) from the forage plants were extracted according to the method described by Meagher et al. (2004). Each freeze-dried and ground plant sample (100 g), shown in **Table 2-1**, was stirred for 2 hours in 2 L acetone (AR)-water mixture (7:3, v/v) containing 1g/L ascorbic acid. Each extract was strained through three layers of cheesecloth to remove plant debris. The extract was concentrated under reduced pressure at 40 ºC to remove acetone. Chlorophyll and lipids were removed from the extracts using dichloromethane (DCM). The defatted aqueous layer was concentrated under vacuum to remove residual dichloromethane (DCM). The aqueous layer was partitioned three times with ethyl acetate (3x200 mL). The ethyl acetate was concentrated under reduced pressure to yield an ethyl acetate crude extract, while the aqueous layer yielded a brown aqueous acetone crude extract solution. The crude extracts were freeze-dried and their weights were recorded (**Table 2-3**).

Table 2-3 The weights of freeze-dried crude and ethyl acetate extracts from parasitic plants

Sample name	Sample code	Voucher specime number	Date of collection	Aqueous crude extract	Amount (g)	Ethyl acetate	Amount (g)
Viscum	OTC00101	BT/08/05	27/02/2009	OTC00301	20.47	OTC00302	2.045
rotundifolium				OTC00501	24.70	OTC00502	0.444
	OTD01001	BT/08/05	10/02/2010	OTD01801	20.39	OTD01802	0.545
Viscum	OTC00102	BT/08/01	27/02/2009	OTC00601	23.49	OTC00602	0.458
verrucosum				OTC00701	25.12	OTC00702	0.336
	OTD01002	BT/08/01	12/02/2010	OTD01901	21.88	OTD01902	0.309
Tapinanthus	OTC00103	BT/08/04	27/02/2009	OTC00801	28.47	OTC00802	0.882
oleifolius	OTD00103	BT/08/04	02/02/2010	OTD02001	17.89	OTD02002	0.900
Grewia flava	OTC00104	BT/08/02	27/02/2009	OTC00901	16.73	OTC00902	1.140
				OTC01001	15.55	OTC01002	1.180
	OTD00104	BT/08/02	02/02/2010	OTD02101	13.15	OTD02102	0.720
Ipomoea	OTC00105	BT/08/06	03/03/2009	OTC01101	16.92	OTC01102	0.740
sinensis				OTC01201	11.51	OTC01202	0.587
	OTD00105	BT/08/06	02/02/2010	OTD02201	11.58	OTD02202	0.000

2.2.3.5. Packing of the column

The media, Sephadex LH-20 gel, was allowed to swell overnight before it was packed into the column. The swelling capacity of the Sephadex LH-20 powder (Uppsala, Sweden) in methanol is 3.9-4.1 mL per 1 g dry powder (**Appendix 2-2**). Therefore, dry powder (40 g) was allowed to swell in 180 mL of methanol-water (1:1, v/v). Then the entire fairly thick slurry was applied carefully into an XK 26/40 column (bed height 12-37 cm, bed volume 60-200 mL, mesh size: 45 μm) in a single operation along a glass rod. The media was then packed using a FPLC pump at a flow rate of 10.0 mL/min and at 1.0 MPa. The maximum constant height of the packed slurry was 32 cm (160 mL) and the average column (bed) volume was 160 mL based on two column volumes collected for sixty four minutes. The bed volume was also calculated using the formula for calculation of the volume of a cylinder ($V=\pi r^2L$). It is convenient when comparing results for columns of different sizes to express flow as linear flow (cm/hour). However, the volumetric flow rate (mL/min) was used. To convert between linear flow rate and volumetric flow rate, the formula in **Appendix 2-3** was used. The FPLC running methods designed for purification of CT using aqueous methanol and aqueous acetone are shown in **Appendix 2-4 and 2-5**, respectively. For the purification

of the extracts using a linear gradient method, an XK26/100 column (bed height 68.5-95 cm, bed volume 363-504 mL, mesh size: 45 μm) and a Sephadex LH-20 powder (120 g) was used.

2.2.3.6. Step (isocratic) fractionation

The fractionation of aqueous acetone crude extracts on Sephadex LH-20 column (XK 26/40) was carried out with a slight modification of the conventional (step) method described by (Meagher et al., 2004). Each freeze-dried aqueous acetone crude extract (6 g) from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis* plants collected in 2009 and 2010 (**Table 2-3**) was dissolved in methanol:water (1:1, v/v; 30 mL). The extracts were then centrifuged at 4500 g for 10 min and loaded onto a Sephadex LH-20 column packed in methanol:water (1:1, v/v) and connected to an FPLC system. The column was pre-equilibrated with two column volumes (2x160 mL) of methanol:water (1:1, v/v) before the extract was loaded. After loading, the column was eluted with methanol:water (1:1, v/v) at a flow rate of 5 mL/min, and four fractions with a total volume of about 1000 mL were collected (fraction 1-100 mL, fraction 2-200 mL, fraction 3-300 mL and fraction 4-400 mL). The adsorbed brown band containing CT was eluted exhaustively with acetone:water (7:3, v/v), and three fractions with a total volume of 800 mL (fraction 5-100mL, fraction 6-400 mL, fraction 7-300 mL) were collected. This procedure was repeated to obtain sufficient amounts of the purified extracts for further purification and analysis. Therefore, two batches for step fractionation were obtained for each crude extract and the weights of the freeze-dried fractions were recorded (**Appendix 2-6 and 2-7**). Aliquots of each fraction were retained and screened for the presence of CT by RP-HPLC-PDA at 280 nm. The scheme of separation is shown in **Figure 2-1.**

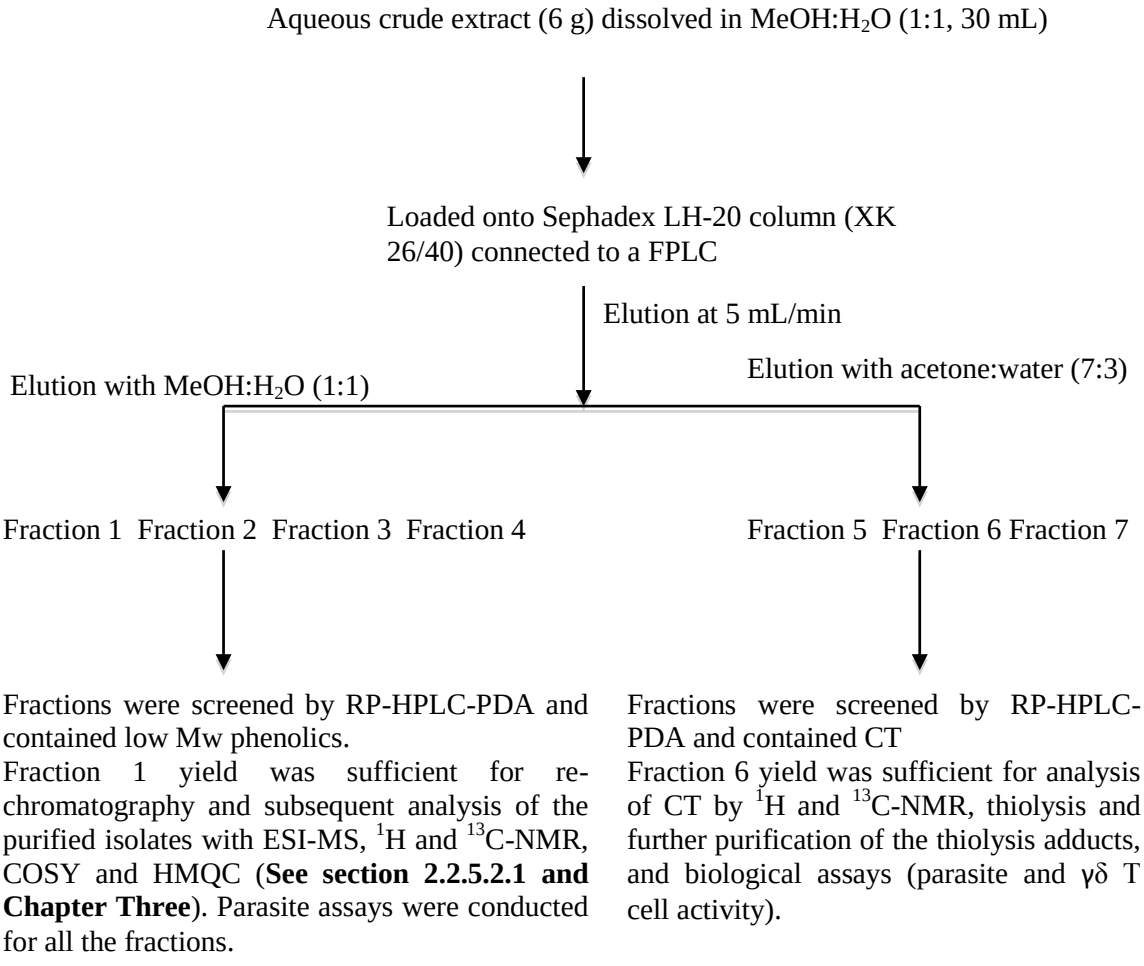


Figure 2–1 The schematic representation of the separation of the aqueous crude extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis* by the step Sephadex LH-20 column (XK26/40)

2.2.3.6.1. Re-chromatography of methanolic LH-20 fractions containing CT

A portion of *V. rotundifolium* CT extracts was not adsorbed on Sephadex LH-20 but was eluted in the early aqueous methanol (1:1, Fraction 1) fraction. The extract was characterised as containing CT by the presence of a broad unresolved hump in the HPLC-PDA at 280 nm. This CT extract also contained sugars and low molecular weight phenolics, and hence CT were separated using Sephadex LH-20 column (XK26/40, column volume: 160 mL). Thus, the following CT containing fractions eluted in the early MeOH:H_2O (1:1) were pooled and re-chromatographed: OTC02101 (first batch) and OTC02201 (second batch) from *V. rotundifolium* plant samples (2009) gave fraction OTE09201. Fractions OTD02901 and OTD03001 from *V. rotundifolium* (2010) plant samples were pooled to give OTE09202. These initial aqueous methanolic eluates from *V. rotundifolium*, which contained CT as evidenced from RP-HPLC PDA, were re-chromatographed on Sephadex LH-20 (XK 26/40) at a flow rate of 5 mL/min. An initial elution with water (100 mL) was followed by a wash with aqueous methanol (1:1, v/v; 200 mL) and lastly with acetone:water (7:3, v/v, 200 mL). The following fractions in order of elution were obtained from the purification of OTE09201: OTE09501 (water, 696 mg), OTE09502 (MeOH:H_2O 1:1, 289 mg) and OTE09503 (MeOH:H_2O 1:1, 30 mg). Fractionation of OTE09202 yielded the following fractions: OTE09701 (water, 999 mg), OTE09702 (MeOH:H_2O 1:1, 280 mg), OTE09703 (MeOH:H_2O 1:1, 73 mg) OTE09704 (MeOH:H_2O 1:1, 16 mg) from the 2010 samples. Aliquots of each fraction were monitored by RP-HPLC PDA. Elution with acetone:water (3:7) was not carried out because CT were not adsorbed to the column after elution with water and methanol.

2.2.3.7. Linear gradient fractionation

To improve the separation of CT oligomers from polymers, a linear gradient method was employed. The fractionation of extracts on Sephadex LH-20 was carried out with a slight modification of this method described by Meagher et al. (2004). This method was employed for the fractionation of the crude extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *G. flava*, which contained CT. To improve the separation of CT oligomers from polymers, a linear gradient method was employed. To improve the separation of CT oligomers from polymers, a linear gradient method was employed. Each freeze-dried crude extract (17 g) (**Table 2-3)** from the plant samples collected in 2009 was dissolved in 100 mL water, centrifuged at 4500 g for 10 minutes.

The extracts were loaded onto a Sephadex LH-20 column (XK 26/100, column volume: 400 mL) and coupled to a FPLC system. The column was eluted first with water (two fractions, 500 mL each), then with methanol-water (1:1, v/v, 250 mL), followed by MeOH:H_2O (1:3, 250 mL), MeOH (1:1, 250 mL), MeOH (3:1, 250 mL), 100% MeOH (four fractions, 250 mL each), and finally with acetone:H_2O (2 fractions, 500 mL each) at a flow rate of 7 mL/min. An adsorbed brown containing CT was eluted exhaustively with acetone-water (7:3, v/v) and three fractions with a total volume of 1300 mL (fraction 10-500 mL, fraction 11-500 mL, fraction 7-300 mL) were obtained. Small volumes of the fractions were retained and monitored by RP-HPLC-PDA. The weight of each fraction was recorded (**Appendix 2-8).** The crude extracts from plants collected in 2010 were not separated due to time constraints and insufficient samples. The schematic diagram for the purification is shown in **Figure 2-2** and is continued in **Section 2.2.3.7.1** for further separation of the initial water fractions.

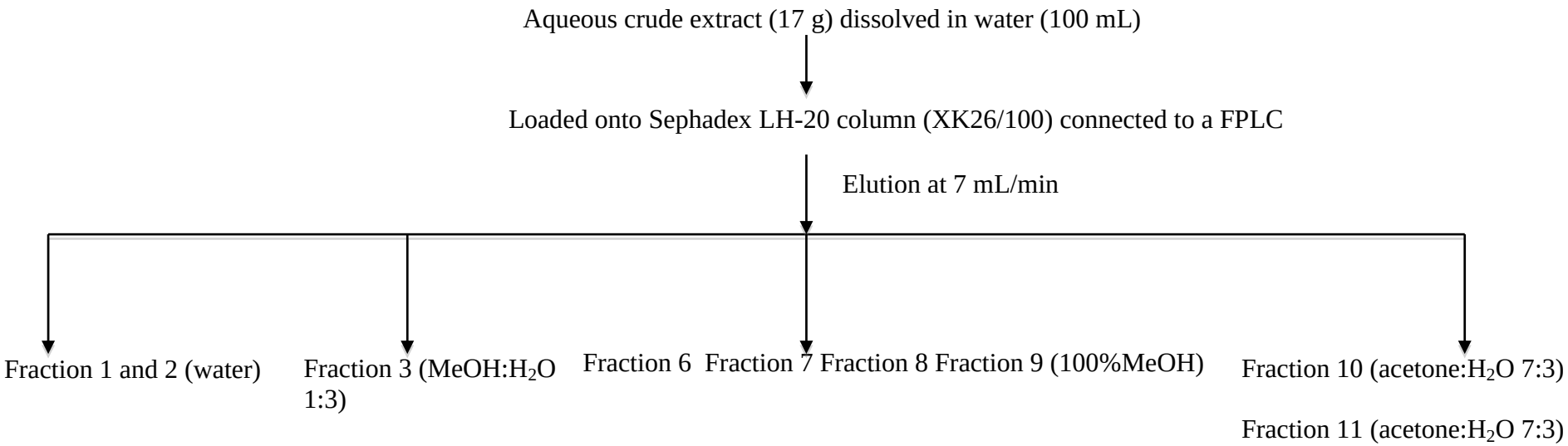


Figure 2–2 The schematic representation of the separation of the aqueous crude extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* by the gradient Sephadex LH-20 column (XK26/100)

2.2.3.7.1. Re-chromatography of linear gradient LH-20 fractions (water)

The presence of CT (broad absorption peak at 280 nm) of the initial water gradient fraction from *G. flava* collected in 2009 was detected by RP-HPLC-PDA. However, CT were not present in the water fractions from *V. verrucosum* and *T. oleifolius* although re-chromatography was still conducted. The following fractions were re-chromatographed on Sephadex LH-20 column (XK26/40, column volume: 160 mL): OTD04201 (water), OTD04401 (water) and OTD04601 (water). An initial fraction eluted with water (100 mL) was followed by a wash with MeOH:H_2O (1:1, 200 mL) and lastly with acetone:water (7:3, v/v, 200 mL). Further purification of OTD04201 isolated from *V. verrucosum* yielded OTE12201 (water, trace), OTE12202 (MeOH:H_2O 1:1, 844 mg) and OTE12203 (MeOH:H_2O 1:1, 120 mg). Further fractionation of OTD04401 (water, 496 mg) which was isolated from *T. oleifolius* produced the following fractions: OTE11002 (water, 396 mg), OTE11003 (water) fractions, while purification of OTD04601 which was isolated from *G. flava* yielded the following fractions: OTE11601 (water, 370 mg), OTE11602-4 (MeOH:H_2O 1:1, trace) and OTE11605 (acetone:water 7:3, 330 mg). Small volumes of the fractions were retained and screened with HPLC-PDA. A ^{13}C-NMR spectrum was obtained for fraction OTE11605 (acetone:water 7:3). No ^{13}C-NMR spectra showing the presence of CT were obtained for the re-chromatographed fractions from *T. oleifolius* and *V. verrucosum* above.

2.2.3.8. Phytochemical screening of step, gradient and re-chromatograhed LH-20 fractions

Phytochemical screening of the purified LH-20 fractions (step, linear gradient and re-chromatographed) was performed by RP-HPLC-PDA which was equipped with a PDA detector. The PDA detector was set to monitor the chromatogram at 280 nm and the UV spectra were acquired between 240 and 360 nm. The following chromatographic conditions were used: mobile phase: A= 0.1% formic acid in H_2O, B= 0.1% formic acid in CH_3CN, acetonitrile, gradient elution conditions: 0-7 min; 10% B, 7-23 min; 10-18% B, 23-28 min; 18-23% B, 28-69 min; 23-41%, 69-72 min; 41-95% B, 72-80 min; 95-10%, 80-95 min; 10-10%. The samples were analysed by elution of a 25 µL sub-sample on a reverse phase (C-18) Phenomenex Jupiter RP column (250 x 4.6 mm, particle size: 5 µm) at a flow rate of 0.3 mL/min.

2.2.3.9. Thin Layer Chromatography (TLC)

Crude extracts and fractions containing CT and low molecular weight phenolics were screened by thin layer chromatography. The fractions (2 mg) were dissolved in methanol (0.5 mL). Elution of the components was carried out using a mixture of ethyl acetate:formic acid:water (18:1:1). After separation, the solvent front was quickly marked and the chromatograms were viewed in UV-light at 356 nm. The detection of CT and other phenolics was visualised by spraying the plates with vanillin-HCl spray reagent (0.8 g vanillin in 20 mL methanol and 5 mL of concentrated HCl in 100 mL Erlenmeyer flask) and warming.

2.2.3.10. Qualitative and quantitative analysis and characterisation of CT

2.2.3.10.1. Analysis of CT polymer containing fractions by using ^{1}H and ^{13}C-NMR

The three species with the highest CT content were selected for further investigation. In addition, the fractions had broad humps in the chromatograms which were suggestive of the presence of CT. The following step fractions isolated with acetone:water (7:3) were probed with NMR: OTC02906 from *V. verrucosum*, OTC03406 from *T. oleifolius*, and OTC03706 from *G. flava* collected in 2009, and OTD03106 from *V. verrucosum*, OTD03206 from *T. oleifolius*, OTD03406 from *G. flava* collected in 2010. Each CT fraction (50 mg) was dissolved in acetone-d_6 (0.6 mL) and in a minimum amount of D_2O (0.1 mL). In the other experiments, particularly for analysis of fractions containing low molecular weight phenolics, the fractions were dissolved in d_4-methanol. Each sample filtered through a glass micro-fibre filter and analysed overnight by ^{13}C-NMR (700 MHz). Due to the polymeric nature of CT, long acquisitions times were required. The chemical properties of the following linear gradient fractions (OTD04210, OTD04410 and OTD04610) isolated with acetone:water (7:3) were also investigated. These fractions were selected based on the evidence of the presence of CT on the chromatograms. An initial or first water fraction from *G. flava*, OTD04601, was purified and yielded a CT-containing fraction OTE11605 (acetone:water 7:3) which was also analysed.

2.2.3.10.2. Analysis of CT from linear gradient fractions with MALDI-TOF

The following fractions containing CT oligomers were selected for MALDI-TOF and were analysed: OTD04206 (100% MeOH), OTD04207 (100% MeOH),

OTD04208 (100% MeOH), OTD04209 (100% MeOH), OTD04210 (acetone:water 7:3) from *V. verrucosum*, and OTD04406 (100% MeOH), OTD04407 (100% MeOH), OTD04408 (100% MeOH), OTD04409 (100% MeOH) and OTD04410 (acetone:water 7:3) from *T. oleifolius*, and OTD04607 (100% MeOH), OTD04608 (100% MeOH), OTD04609 (100% MeOH), OTD04610 (100% MeOH) and OTD04611 (acetone:water 7:3) from *G. flava*.

Each fraction was re-constituted in acetone:water (8:2, v/v; 0.5 mg/mL) and mixed with a matrix solution of 2,5-dihydroxybenzoic acid (DHB) in acetone:water (8:2, v/v;10 mg/mL) at a volumetric ratio of 1:1 (50 µL:50 µL). The CT-matrix solution was deionized on a cation exchange cartridge (Phenomenex Strata SCX, 100 mg, 1 mL) pre-conditioned with HCl (1 mL, 0.1 M), Milli-Q water (5 mL), and finally with acetone:water (8:2, v/v; 2 mL). The deionized CT-matrix solution was doped with a NaCl solution (0.1 M, 0.5 µl) to promote the formation of single ion adducts $[M+Na]^+$. The mixture (1 µL) was applied to a stainless steel target plate and left to co-crystallize at room temperature prior to analysis. The MALDI-TOF mass spectra were recorded using a Bruker MALDI-TOF in the positive ion reflectron mode. The nitrogen laser was set at 337 nm, source voltage; 15 kV, pulse voltage; 2470 V and reflectron voltage 2kV. Sensitivity was improved by taking multiple laser shots. Different matrices such as α-cyano cinnamic acid (CCA) and α–cyano-4-hydroxycinnamic acid (CHCA) were employed in the analysis but no spectra were obtained. In addition, different concentrations of matrix to sample (1:1, 1:2, 1:3, 1:4, 2:1, 3:1and 4:1) were also tried. However, a series of CT oligomers were not detected as expected and hence no results were discussed for this section.

2.2.3.10.3. Analysis of CT oligomers from linear gradient fractions with ESI-MS

The following fractions were selected for ESI-MS analysis: OTD04206 (100% MeOH), OTD04207 (100% MeOH), OTD04208 (100% MeOH), OTD04209 (100% MeOH), OTD04210 (acetone:water 7:3) from *V. verrucosum*, and OTD04406 (100% MeOH), OTD04407 (100% MeOH), OTD04408 (100% MeOH), OTD04409 (100% MeOH) and OTD04410 (acetone:water 7:3) from *T. oleifolius*, and OTD04607 (100% MeOH), OTD04608 (100% MeOH), OTD04609 (100% MeOH), OTD04610 (acetone:water 7:3) and OTD04611 (acetone:water 7:3) from *G. flava*.

Two methods were used for syringe injection of samples into the ESI-MS probe; HPLC (chromatographic conditions for the RP-HPLC PDA were used-**section 2.2.3.8**). For syringe injection, each fraction (5 mg) were dissolved in 1 mL methanol, loaded into a syringe and injected directly into the ESI-MS at a flow rate of 0.15 mL/min. The following ESI-MS conditions: source block temperature- 95 °C and desolvation temperature- 190 °C, run time- 3.50 min, capillary voltage- 3.49 kV, cone voltage 35 V, mass range- scan mode 250-1500 m/z, desolvation gas 200 L/hr and cone gas flow- 50L/hr. Detection of the ions was carried out both in in the negative and positive ion mode. Surprisingly, the ions were not detected and a series of CT oligomers were not detected as expected and hence no results were discussed for this section.

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2.2.3.10.4. Quantitative analysis of thiolysis adducts

Thiolysis was carried out for the quantitative determination of the individual units in the CT polymers from the fractions of plants collected in 2009 and 2010 (*T. oleifolius, V. verrucosum* and *G. flava*). These plants were selected because of their high CT content and broad humps in the HPLC chromatograms suggesting the presence of CT. This reaction was carried out on the following fractions: OTC02906 from *V. verrucosum,* OTC03406 from *T. oleifolius* and OTC03706 from *G. flava* collected in 2009, and OTD03106 from *V. verrucosum*, OTD03206 from *T. oleifolius*, OTD03406 from *G. flava* collected in 2010. The following linear gradient fractions were also analysed: OTD04208 (100% MeOH), OTD04209 (100% MeOH), OTD04210 (acetone:water 7:3) from *V. verrucosum*, and OTD04406 (100% MeOH), OTD04407 (100% MeOH), OTD04408 (100% MeOH), and OTD04410 (acetone:water 7:3), OTD04411 (acetone:water 7:3) from *T. oleifolius*, and OTD04607 (100% MeOH), OTD04608 (100% MeOH), OTD04609 (100% MeOH), OTD04610 (acetone:water 7:3) and OTD04611 (acetone:water 7:3), OTE11605 (Re-chromatographed, acetone:water 7:3) from *G. flava*, OTD04805 (MeOH:H_2O 3:1) and OTD4806 (100% MeOH).

A condensed tannin solution (4 mg/mL in methanol) was prepared for each fraction. A sub-sample (50 µL) and benzyl mercaptan (5 %; v/v 50 µL) were placed into a vial and to this was added concentrated hydrochloric acid in methanol (3.3 %; v/v 50 µL). The solution was heated to 40 ^{o}C for 30 minutes in a heating block and cooled to room temperature. An internal standard (IS), dihydroquercetin in water (100 µL, 5.2 x 10^{-2} mg/mL solution), was added and a 25 µL sub-sample was analysed immediately by

RP-HPLC. The following chromatographic conditions were used: mobile phase: A= 0.1% formic acid in H_2O, B= 0.1% formic acid in acetonitrile, gradient elution conditions: 0-7 min; 10-18% B, 7-23 min: 18-23% B, 28-44 min; 23-31% B, 44-53 min; 31-85% B, 47-53 min; 85-85% B, 53-58 min; 85-10% B, 58-70 min; 10-10% B, stationery phase: a RP (C-18) Alltima column (250 x 2.1 mm, Alltech, particle size: 5 µm with 25 µL injections), flow rate: 0.3 mL/min. The UV absorbance was monitored between 240 and 360 nm and detection at 280 nm using a Waters PDA detector.

2.2.3.10.4.1. Preparation of standards

Standards (catechin, epicatechin and epigallocatechin) were obtained from Sigma Chemicals, New Zealand. The thiolysis adducts (flavan-3-ol benzyl thioethers) were kindly supplied by Dr Mike Tavendale (AgResearch, New Zealand) and the stock solutions were prepared as shown in **Table 2-4**.

Table 2-4 Authentic standards used in analysis of the thiolysis adducts and free flavan-3-ols.

Authentic standards	Amount per mL of solvent
Catechin (C)	2.6 mg/ 5mL MeOH
Epicatechin (EC)	2.6 mg/5 mL MeOH
Epigallocatechin (EGC)	1.1 mg/2 mL100% MeOH
Epigallocatechin-thio (EGC-thio)	1.1 mg/2 mL100% MeOH
Catechin-thio (C-thio)	1.1 mg/2 mL100% MeOH
Epicatechin-thio (EC-thio)	1.1 mg/2 mL100% MeOH
Dihydroquercetin (DHQ)	4.8 mg/50 mL milli-Q water

Mixed standards consisting of catechin (100 µL), epicatechin (100 µL), epigallocatechin (100 µL), epigallocatechin-4β-benzyl thioether (100 µL), catechin-4β-benzyl thioether (100 µL), epicatechin-4β-benzyl thioether (100 µL), DHQ (100 µL) and 200 µL of milli-Q water were prepared in duplicate and were analysed as illustrated in **Figure 2-3**.

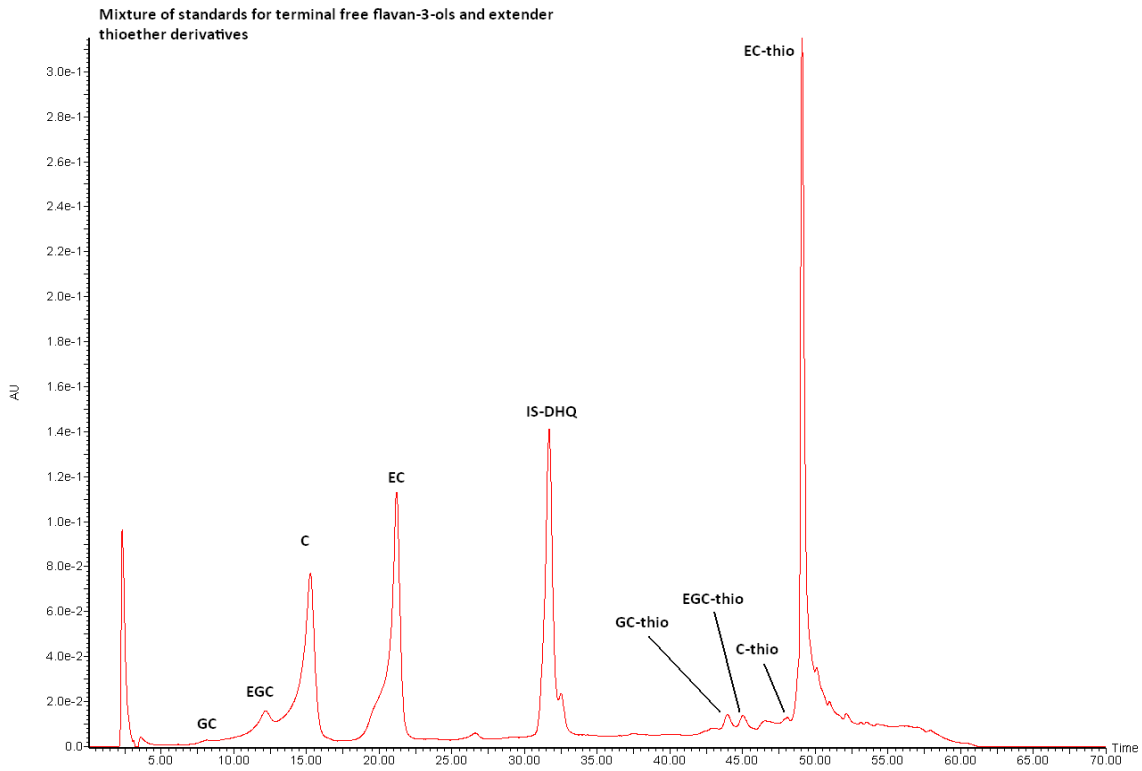

Figure 2–3 HPLC-PDA chromatogram of the free flavan-3-ols and thioether derivatives from authentic standards at 280 nm. Abbreviations: GC = gallocatechin. EGC = epigallocatechin. C= catechin. EC = (epi) catechin. IS = internal standard, thio = thiolysis adduct.

Concentrations of the terminal flavan-3-ol units and extender flavan-3-ol adducts from full thiolysis were estimated by peak area integration at 280 nm relative to dihydroquercetin (DHQ) as the internal standard. Responses relative to DHQ determined from standards were 0.09 for the terminal PCs and 0.29 for the extender PCs. Response factors of the extender units agreed with those of Sivakumaran et al. (2006), but the response factors of the terminal units were lower, which could have been caused by the low signal intensity of the standard.

2.2.3.10.4.2. Purification of thiolysis adducts for qualitative analysis

Thiolysis adducts were purified to obtain qualitative information on the identity of the individual units in the CT polymer by column chromatography (silica gel matrix). The following fractions which were found to contain CT were used: OTC02906 from *V. verrucosum*, OTC03406 from *T. oleifolius* and OTC03706 from *G. flava* collected in

2009. Each CT containing fraction (240 mg) was dissolved in MeOH (30 mL) and benzyl mercaptan (5 %; v/v 60 mL) and reacted with HCl in MeOH (3.3%, 30 mL). The solution was heated at 40 ^{o}C for 30 minutes in a heating block and cooled to room temperature. The reaction mixture (25 µL) was analysed by RP-HPLC-PDA. The solvents were removed by a rotary evaporator. The residue was dissolved in milli-Q water (75 mL) and partitioned with diethyl ether (25 mL x 6). The aqueous extract was concentrated under reduced pressure and dissolved in water, while the diethyl ether was concentrated under reduced pressure. The oil residue from the diethyl ether extract was dissolved in methanol and loaded onto a C-18 column (12 x 3 cm, 50 g silica packing material, 5 µm particle size) pre-conditioned with MeOH:H_2O (1:1) at a flow rate of 2.5 mL/min.

For pilot investigations, fractions (100 mL) were collected, but they were not well separated (data not shown). As a result, smaller volumes (about 50 mL) were collected and pure isolates were obtained. The thiolysis of CT of OTC03706 from *G. flava* with gradient elution from MeOH:H_2O (1:1) to MeOH:H_2O (7:3) yielded the following: OTE18301 and OTE018302 (50% MeOH, 50 mL, trace), OTE018303 (50-55% MeOH, 50 mL, trace), OTE018304 (55% MeOH, 50 mL, 6.8 mg), OTE18305 (55% MeOH, 20 mg), OTE018306 (55% MeOH, 50 mL, 43 mg), OTE18307 (55-60% MeOH, 50 mL, 60 mg) and OTE18308 (60% MeOH, 50 mL, 10.6 mg). The purified adducts were then characterised by ESI-MS, ^{1}H NMR and ^{13}C-NMR. The above procedure was repeated to purify the thiolysis adducts of CT from *V. verrucosum* (OTC02906) and *T. oleifolius* (OTC03406). *T. oleifolius* fractions were obtained with gradient elution carried out from MeOH:H_2O (1:1) to MeOH:H_2O: OTF02001 and OTF02002 (50% MeOH, 50 mL, traces), OTF02003 (50-55% MeOH, 50 mL, traces), OTF02004 (55% MeOH, 50 mL, 6.8 mg), OTF02005 (55% MeOH, 20 mg), OTF02006 (55% MeOH, 50 mL, 43 mg), OTF02007 (55-60% MeOH, 50 mL, 60 mg) and OTF02008 (60% MeOH, 50 mL, 10.6 mg). The schematic representation for the preparation of monomer units from CT of *V. verrucosum*, *T. oleifolius*, *G. flava* by column chromatography (silica gel) is shown in **Figure 2-4.**

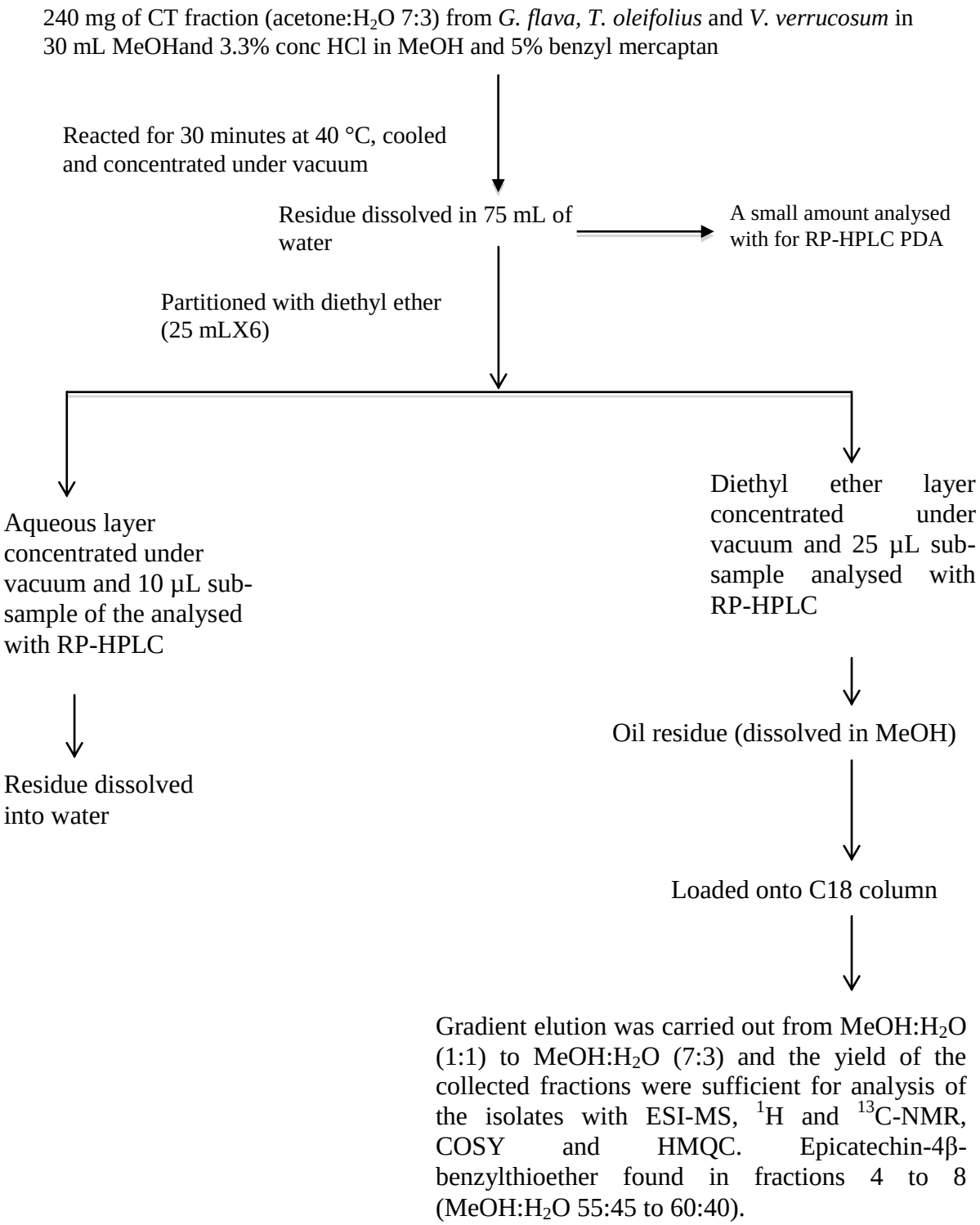

Figure 2–4 The schematic representation for the preparation of epicatechin-4β-benzylthioether monomer units in CT of *V. verrucosum*, *T. oleifolius*, *G. flava* using open column chromatography (silica gel matrix)

The thiolysis reaction of the CT from *V. verrucosum* yielded the following fractions: OTF02702 and OTF02702 (50% MeOH, 50 mL, traces, OTF02703 (50-55% MeOH, 50 mL, traces), OTF02704 (55% MeOH, 50 mL, 6.8 mg), OTF02705 (55% MeOH, 20 mg), OTF02706 (55% MeOH, 50 mL, 43 mg), OTF02707 (55-60% MeOH, 50 mL, 60 mg) and OTF02708 (60% MeOH, 50 mL, 10.6 mg). Fractions OTF02705 and OTF02706 with the same HPLC-PDA profile were pooled to give fraction OTF02901 and characterised with ^{1}H and ^{13}C NMR.

2.2.3.10.4.3. Characterisation of the thiolysis adducts using RP-HPLC-PDA, ESI-MS, ^{1}H NMR and ^{13}C-NMR

The HPLC-PDA chromatographic conditions in **section 2.2.3.8** were used. Each fraction was analysed by RP-HPLC-PDA. The purified adducts were also characterised by ^{1}H and ^{13}C-NMR and ESI-MS using the same conditions shown in sections **2.2.3.10.1 and 2.2.3.10.3** above.

2.3. Results and Discussion

2.3.1. Determination of free, protein and fibre bound CT content

The CT content from the five forage plants which was estimated by the butanol-HCl method is shown in **Table 2-5**. There were variations in the CT content between the plant species. The total CT concentrations ranged from 0.2 to 12.7 (g/100 g DM). *V. rotundifolium* and *I. sinensis* plants collected in summer (2009 and 2010) contained traces of CT (<0.5 w/w DM). *V. verrucosum* leaves and small stems collected in 2009 and 2010 contained condensed tannins 6.0 and 5.3% w/w DM, respectively. *T. oleifolius* leaves and small stems collected in 2009 contained 5.1% CT while for those collected in 2010 contained 11.8% CT. *G. flava* leaves collected in 2009 had the highest CT concentration of 12.7 (g/100 g DM) while in those collected in 2010 CT content was 9.7%. The results indicated that significant amounts of CT was found in *V. verrucosum*, *T. oleifolius* and *G. flava*, and these results were in agreement with those from RP-HPLC-PDA.

Table 2-5 Condensed tannin concentration (g/100 g DM) determined by the butanol-HCl colorimetric assay

Plant name	Year	Free	Protein-bound	Fibre-bound	Total
V. rotundifolium	2009	0.1	0.4	<0.1	0.5
V. rotundifolium	2010	0.1	0.3	<0.1	0.4
V. verrucosum	2009	3.9	2.0	0.1	6.0
V. verrucosum	2010	3.2	2.0	0.2	5.3
T. oleifolius	2009	2.0	2.9	0.2	5.1
T. olefolius	2010	9.2	2.4	0.3	11.8
G. flava	2009	6.9	5.0	0.7	12.7
G. flava	2010	5.0	4.1	0.5	9.7
I. sinensis	2009	<0.1	0.1	0.1	0.2
I. sinensis	2010	<0.1	0.1	0.1	0.2

The protein-bound CT content ranged from 0.1 to 5.0 (g/100g DM). The lowest protein bound was found in *I. sinensis* (0.1 %), while the highest protein-bound CT was

found in *G. flava* (5.0%). The concentrations of protein-bound CT from *V. verrucosum*, *T. oleifolius* and *G. flava* may have profound implications in ruminant nutrition. They may be nutritionally beneficial to ruminants and may increase the absorption of amino acids in the small intestine. These complexes are stable at ruminal pH and dissociate in the small intestine (pH>8.5) and abomasum (pH<3.5), thus leading to an increase in protein supply and absorption of amino acids.

The free CT, in contrast, can be detrimental to ruminants especially in high concentrations. The free CT ranged from 0.1 to 9.2%, with the highest free CT content obtained in *T. oleifolius* which was collected in 2010. Moderate concentrations of CT in *V. verrucosum*, *T. oleifolius* and *G. flava* (2.0-6.0%) may complex with plant proteins in the rumen and dissociate in the small intestine, and may in turn increase the absorption of amino acids. When free CT content is above 6%, animal performance might be impaired due to reduction in rumen carbohydrate digestion (Barry and Manley, 1986), protein digestibility and reduced feed intake (Kumar and Singh, 1984). However, the CT threshold may not apply to all CT because of other factors such as the CT structure and the physiological status of the animal. Higher concentrations of CT were observed in *G. flava* (6.9%) and *T. oleifolius* (9.2%), and these concentrations may be detrimental to the animals. However, further *in vivo* studies to ascertain the effects of CT from these plants in ruminant nutrition need to be conducted.

The fibre-bound CT content was relatively lower and might not play any significant role in animal nutrition. *Lotus pedunculatus* CT were used as the standard. Overall, CT content from these plants were in agreement with that from *Lotus pedunculatus*, sulla (*Hedysarum coronarium*) and white clover (*Trifolium repens* L.) flowers (**Table 1-3, Chapter 1**) (Mupeyo et al., 2010a). However, the CT content from the plants in our study were also in agreement with the reported values in the literature (**Table 1-2, Chapter 1**). However, the *G. flava* CT content values were higher than the literature values. The differences in the CT content within and between plant species could be attributed to the calibration standards, methods used for the determination of the CT content, age and seasonality.

2.3.2. Determination of nutrient composition

Plant samples were run under a number of NIRS calibration curves for nutritional analysis but no results were obtained. These samples gave too many values that were outliers to the calibration curves. This study along with other quality assurance (QA) deficiencies meant that the analysis could not be completed. It was concluded that the samples were not suitable for NIRS analysis because the sample types were too different from the validation points used in the calibration curves made available for each component. The calibration curves have been prepared using forages grown under temperate conditions, so the samples analysed were completely different from the temperate forages, as the plant samples were collected in Botswana.

2.3.3. Plant extraction

The extraction of each plant sample (collected in 2009 and 2010) with acetone:water (7:3) and subsequent partitioning of the extracts with ethyl acetate yielded a brown aqueous acetone extract and ethyl acetate extract, respectively. The weight of each extract obtained from the plant samples is shown in **Table 2-3**. The weights of acetone:water (7:3) crude extracts ranged between 11.51 g and 28.77 g, while the ethyl acetate extracts ranged between 0.309 g and 2.045 g. The extraction was conducted on the plant samples collected in different years, so that variability of the biological activity with the chemical structure could be investigated.

2.3.4. Crude extract fractionation

Step and linear gradient methods were employed for the fractionation of the aqueous crude extracts obtained from the plants collected in 2009 and 2010. The amount of methanol-water (1:1, v/v) and acetone:water (7:3) fractions are shown in **Appendix 2-6 and 2-7**. A brown band was adsorbed at the top of the column and was successfully eluted with aqueous acetone, suggesting that Sephadex LH-20 was operating in an adsorption manner. This phenomenon was observed for crude extracts from *V. verrucosum*, *T. oleifolius* and *G. flava*. However, for the fractionation of *V. rotundifolium* extracts, the brown band was not adsorbed at the top of the column. The amount of water, methanol:water (25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH, v/v) and acetone:water (7:3, v/v) fractions obtained from the linear gradient

fractionation are shown in **Appendix 2-8**. A brown band was also adsorbed at the top of the column and was successfully eluted with aqueous acetone, suggesting that Sephadex LH-20 was operating in an adsorption manner. This phenomenon was found in crude extracts from *V. verrucosum*, *T. oleifolius* and *G. flava*. However, for the fractionation of *V. rotundifolium* extracts, a brown band was not adsorbed at the top of the column, but it was distributed in water, MeOH:H_2O (1:3), MeOH:H_2O (1:1), MeOH:H_2O (3:1) and 100% MeOH fractions. Elution with acetone: H_2O (7:3) yielded trace amounts of the fractions.

2.3.5. Phytochemical screening of step LH-20 fractions

The step LH-20 fractions obtained from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava*, and *I. sinensis* plants were screened for the presence of CT by RP-HPLC. The HPLC-PDA chromatograms of the LH-20 fractions are shown in **Appendix 2-9 to 2-13**. Generally, the step LH-20 methanol:water (1:1) fractions exhibited sharp peaks in contrast to the acetone:water fractions which exhibited broad peaks (**Figure 2-5**). The sharp peaks might be indicative of the presence of the low molecular weight phenolics, while the broad humps could be indicative of the presence of unresolved and polymeric CT. The latter is observed because the concentration of individual components decreases with increasing mDP and a higher number of possible isomers.

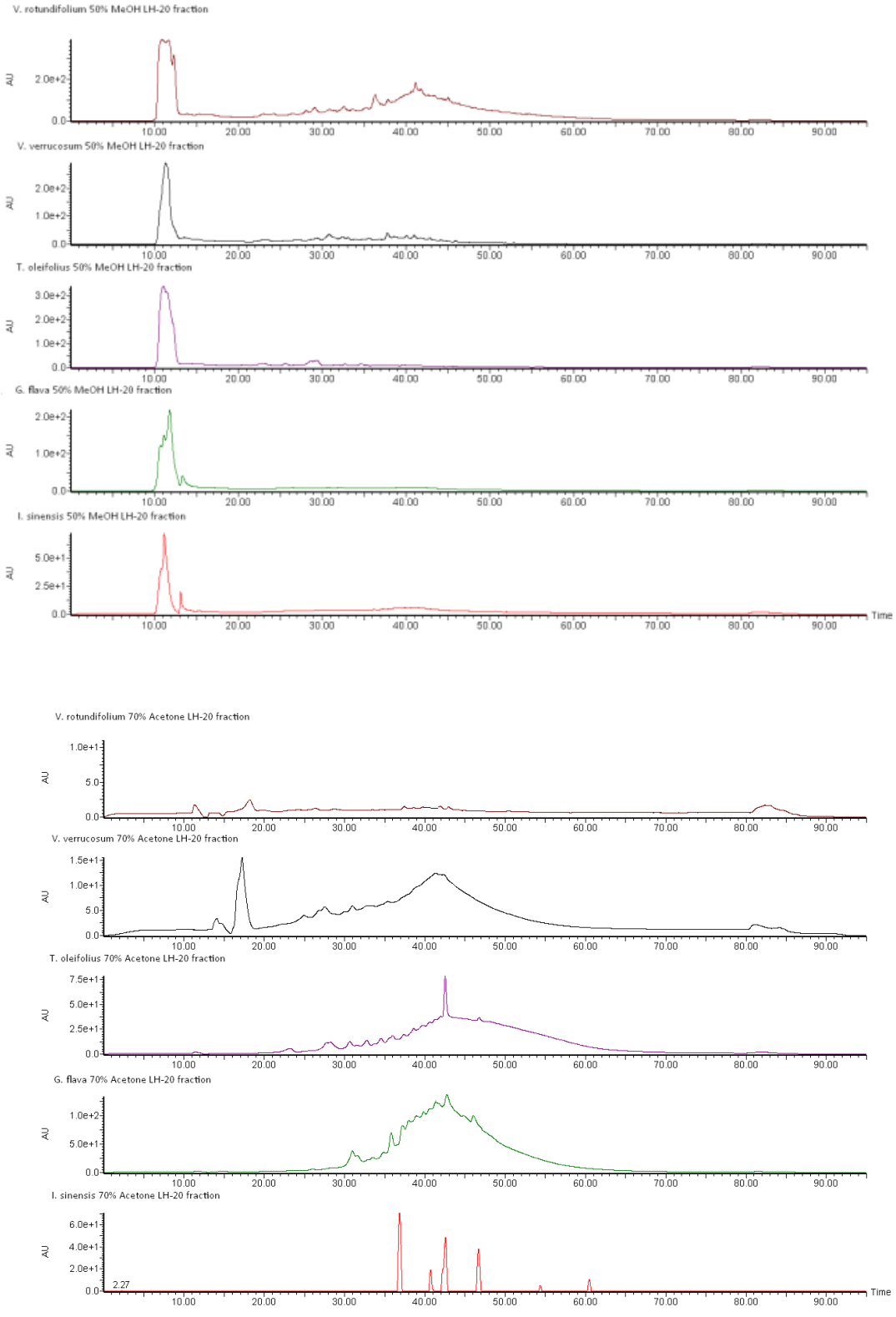

Figure 2–5 Representative HPLC chromatograms of MeOH:H_2O (1:1) and acetone:H_2O (7:3) for step LH-20 fractionation for fractions from plants for *V. verrucosum*, *V. rotundifolium*, *T. oleifolius* and *G. flava* and *I. sinensis* from the step LH-20 method

A broad and clean hump was eluted and retained for fraction OTC02101 from *V. rotundifolium* (**Appendix 2-9**), and this showed that Sephadex LH-20 gel was operating in a size-exclusion manner. If the molecules are eluted in a size-exclusion manner, the order of elution may include high molecular weight CT, followed by medium molecular weight CT and low molecular weight CT. The methanolic fractions were gummy and sticky, which may be suggestive of the presence of sugars.

Sharp peaks with a small hump were observed for fraction OTC02102. Another hump was found in fraction OTC02103-04, an indication that CT were distributed in most of the methanolic fractions. A broad hump with sharp peaks was obtained for fraction OTC02105-6, and this indicated that Sephadex LH-20 was operating in an adsorption chromatographic mode, in which the adsorbed brown band of CT was eluted with acetone-water (7:3). The results for the second batch of fractions (not shown) showed similar peaks.

In addition, similar chromatograms were obtained for the fractions isolated from plants collected in 2010. The HPLC-PDA chromatograms were observed for the LH-20 fractions from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava*, and *I. sinensis* (**Appendix 2-14 to 2-22**). The chromatograms for the methanol:water (1:1) fractions showed sharp peaks, while the chromatograms of the acetone:water (7:3) fractions had broad humps. However, the methanol:water (1:1) and acetone:water (7:3) fractions from *I. sinensis* did not exhibit any broad humps, and this may indicate the absence of CT. From the re-chromatographed fractions, purified CT was not obtained.

2.3.6. Phytochemical screening of linear gradient LH-20 fractions

A series of the gradient LH-20 fractions from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, and *G. flava* plants, which were collected in 2009, were screened for the presence of CT using RP-HPLC with PDA detection (**Appendix 2-23 to 2-26**). Sharp peaks were obtained for the water (OTD04801 and OTD04802) and 100% MeOH (OTD04807) fractions, while small and broad humps were found for MeOH:water (1:3, OTD04803), MeOH:water (1:1, OTD04804), 75% MeOH:water (3:1, OTD04805) and 100% MeOH (OTD04806) fractions from *V. rotundifolium*, suggesting the presence of CT. The signal intensity of the hump increased with the increasing concentration of the eluting solvent, suggesting an increase in the size of the CT polymer and distribution of CT in all the fractions. Aqueous acetone (3:7) fractions were not obtained as a brown band was not adsorbed onto the column. For *V. verrucosum*, sharp peaks were obtained

for the water and methanol fractions (OTD04201 to OTD04206), while broad humps were found in some methanol and aqueous acetone fractions (OTD04207 to OTD04210). Fractions OTD04205 and OTD04206 had few sharp peaks, suggesting that the compounds were well purified. However, the chemical characteristics of these fractions were not obtained as broad peaks were obtained in the ^{1}H and ^{13}C-NMR spectra, suggesting the presence of CT polymers. For *T. oleifolius*, sharp peaks were observed in fractions (OTD04401 to OTD04407), while broad peaks were observed in the methanolic (100% MeOH) fractions (OTD04408 and OTD04409) and the acetone:water (7:3) fraction (OTD04410). Fraction OTD04601 (water) from *G. flava* showed a broad hump, which suggested the presence of CT and Sephadex LH-20 matrix operating in a size exclusion manner. The sharp peaks were observed in the ensuing fractions (OTD04602 to OTD04608), while broad peaks were obtained for the last eluted methanol (100%, OTD04609) and acetone:water (7:3, OTD04610) fractions. From the re-chromatographed fractions, fraction OTE11605 isolated from OTD04601 of *G. flava* was well purified and hence was further characterised by ^{1}H and ^{13}C-NMR spectroscopy.

2.3.7. Thin layer chromatography

CT and low molecular weight phenolic compounds were visualised in the chromatograms, which were sprayed with freshly prepared vanillin (4%, w/v) in methanol: concentrated hydrochloric acid (4:1). Vanillin-HCl reagent binds to flavonoids containing a phloroglucinol ring (**Figure 1-6 in Chapter 1**). Red and immobile spots from the baseline were observed in fractions containing high molecular weight compounds confirming the presence of CT oligomers and polymers (**Figure not shown**). Yellow mobile spots were also observed.

2.3.8. Analysis of CT containing step LH-20 fractions by ^{1}H and ^{13}C-NMR

The CT fractions were isolated from *V. verrucosum*, *T. oleifolius* and *G. flava* and analysed by ^{13}C-NMR to provide information on the oxidation pattern of the B-ring extender units (procyanidin to prodelphinidin ratio) of CT polymers. Assignment of signals was made in comparison with published data in the literature (Fournié and Bonneville, 1996, Haslam, 1989, Porter et al., 1982, Sivakumaran et al., 2004b, Zhang and Lin, 2008). Initially, the fractions were analysed by the 500 MHz NMR, but the

spectra could not be interpreted due to low intensity signal intensity (**Appendix 2-27 to 2-30**). However, better spectra with high signal to noise ratio were obtained when the samples were analysed by the 700 MHz NMR. All the analysed step LH-20 fractions containing CT showed broad peaks in the ^{1}H NMR spectra and could not be interpreted. The broad ^{1}H signals could be attributed to atropisomerisation which is due to steric interactions of the interflavanoid bond. However, useful qualitative information was obtained from the ^{13}C-NMR spectra, but it did not provide quantitative information on the mean degree of polymerisation. This information could not be obtained because the peaks were broad and some of them were superimposed. The ^{13}C-NMR spectrum of fraction OTC02906 (acetone:water, 7:3) from *V. verrucosum* is illustrated in **Figure 2-6**. The peaks of CT (δ) from *V. verrucosum* were observed at 28.1, 30.8, 37.6, 54.6, 69.3, 72.6, 76.6, 82.6, 96.0, 114.6, 116.4, 119.7, 130.8, 144.6, 145.0, 154.0, 155.8, 156.9 ppm.

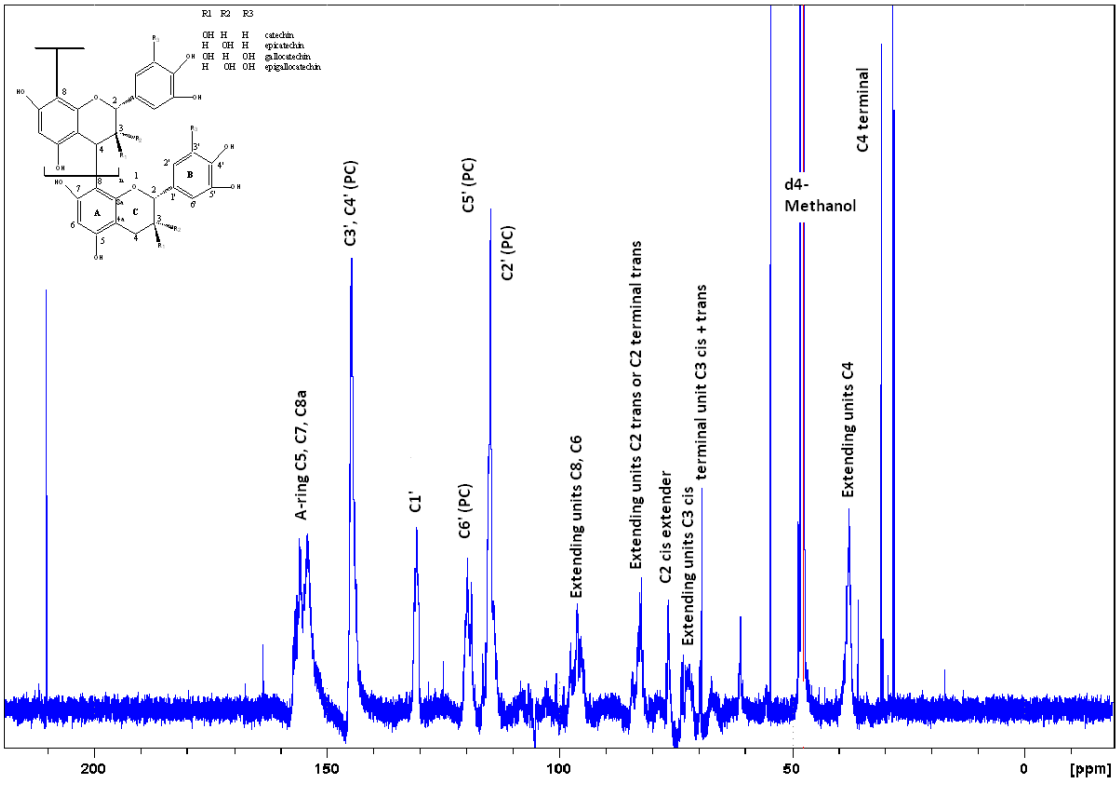

Figure 2–6 ^{13}C-NMR spectrum (d_4-methanol) of CT polymer of OTC02906 (acetone:water, 7:3) from *V. verrucosum* collected in 2009

The carbons in the extension unit (C4) signal resonated upfield at δ 37.6 ppm, whereas the terminal C4 signal was shown at δ 28.1 ppm. The region between 65 and 90 ppm is sensitive to the stereochemistry of the C-ring. The signals at 69.3 and 72.6 ppm could be attributed to C3 in the terminal units and the extender units, respectively. A peak at 76.6 ppm was assigned to C2 *cis* while at 82.6 ppm was attributed to C2 extender *trans* or C2 terminal. The ratio of the 2,3-*cis* to 2,3-*trans* isomers could be estimated through the distinct differences in their respective C2 chemical shifts, but the signal to noise ratio was too low to allow for this estimation. The signal at 96.0 ppm were assigned to C8 and C6 of the A-ring, while the signals normally at 106.4 and 108 ppm attributed to C6′ and C2′ of pyrogalloyl B-ring of prodelphinidin units (gallocatechin/epigallocatechin) were not observed. Furthermore, the peaks at 114.6, 116.4 and 119.7 ppm were assigned to the C2′, C5′ and C6′ of PC units in the B-ring. A peak for prodelphinidin was detected at 130.8 ppm for C4′ but could also be attributed to C1′. The ^{13}C-NMR spectra of two carbons in the B-ring bonded to the hydroxyl groups normally show a distinct signal at 145 ppm. Thus, the peaks at 144.6 and 145.0 ppm were assigned to C3′ and C4′ of procyanidin units (catechin/epicatechin). The resonance line of three carbons bonded to hydroxyl group in the B-ring at 146 ppm which usually represents prodelphinidin (gallocatechin/epigallocatechin) was not detected. Overall, this suggests that CT from *V. verrucosum* were purely procyanidins (i.e. 100% PC). The A-ring signals were observed downfield at 154.0 and 156.9 ppm for C5 and C7, respectively**.**

The ^{13}C-NMR spectrum of fraction OTC03406 isolated from *T. oleifolius* collected in 2009 is shown in **Figure 2-7.** The following signals (ppm) were obtained: 28.1, 30.7, 36.3, 54.6, 67, 71.6, 75.6, 80.7, 96.4, 101.0, 106.3, 113.8, 114.9, 117.6, 131.3, 144.1, 144.5, 153.6, 155.4, 155.9 ppm. There were additional peaks that could not be assigned in the spectrum, and this shows the diversity and complexity of the CT molecules.

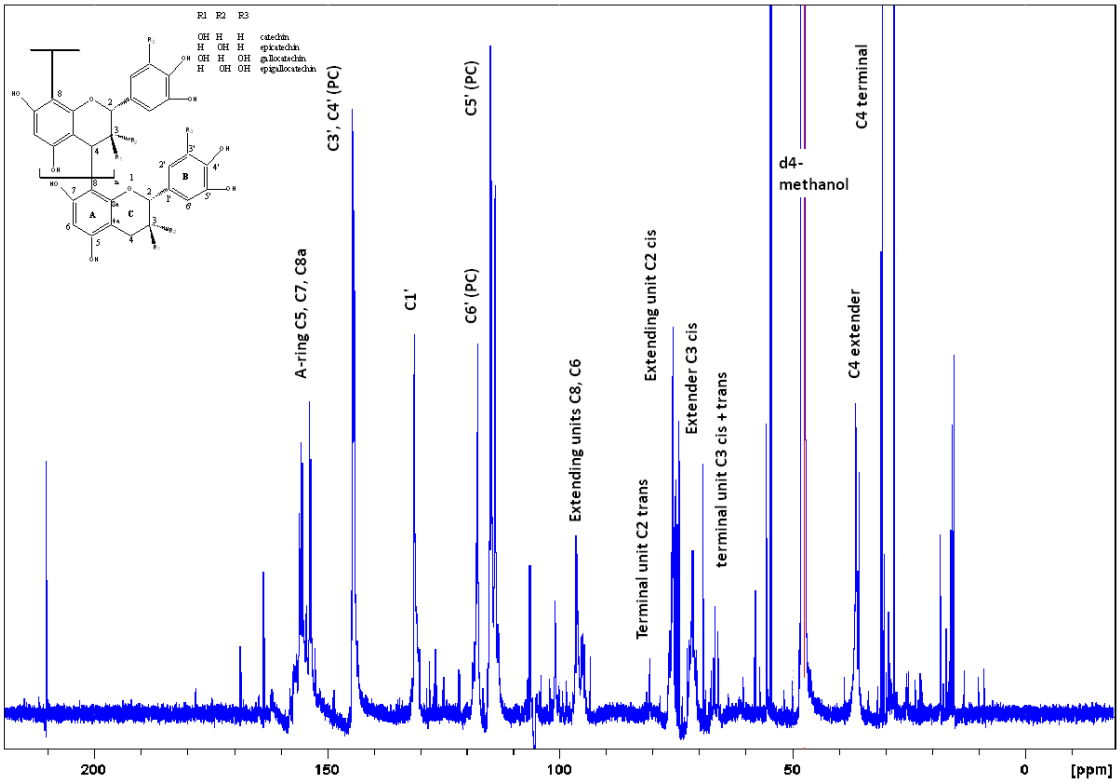

Figure 2–7 ^{13}C-NMR spectrum (d_4-methanol) of CT of OTC03406 (acetone:water 7:3) from *T. oleifolius* collected 2009

The C4 signal attributed to carbons in the extension units was shown at 36.3 ppm, whereas C4 signal for carbons in the terminal unit was shown at 28.1 ppm. The signals at 67 and 71.6 ppm could be attributed to C3 in the terminal units and the extender units, respectively. A peak at 75.7 ppm was assigned to C2 *cis*, while C2 *trans* signal was not observed. A peak at 80.7 ppm was assigned to C2 terminal. C3 *cis* extender resonated at 71.6 ppm. The ratio of the 2,3-*cis* to 2,3-*trans* isomers was estimated to be 100:0 since C2 *trans* signal at 84 ppm was not detected. The mean molecular weight can be integrated from the ratio of the areas of the two C3 signals for extension to terminal units. However, this information could not be obtained because of low signal intensity of C3 in the terminal at 67 ppm. The signals at 96.4 and 101.0 ppm were assigned to C8 and C6 in the A-ring, respectively, while the signals at 106.3 and 108 ppm were assigned to C6′ and C2′ of pyrogalloyl B-ring of prodelphinidin units. Furthermore, the peaks at 113.8, 114.9 and 117.6 ppm were assigned to the C2′, C5′ and C6′ of PC units in the B-ring. A peak for prodelphinidin was detected at 131.2 ppm for C4′. The peaks at 144.1 and 144.5 ppm were assigned to C3′ and C4′ of procyanidin

units (catechin/epicatechin). The ^{13}C-NMR spectra showed a distinct signal at 145 ppm, which is usually due to two carbons in the B-ring bonded to the hydroxyl groups. The resonance line at 146 ppm which is usually representing prodelphinidin (gallocatechin/epigallocatechin) and with three carbons bonded to hydroxyl group in the B-ring was not detected. Overall, this suggests that CT from *T. oleifolius* plant were homogeneously procyanidins. The A-ring signals were observed downfield at 155.4 and 155.9 ppm for C5 and C7, respectively**.** The spectrum of a CT containing fraction from *T. oleifolius* was similar to that of *V. verrucosum*. The ^{13}C-NMR spectrum of fraction OTC03706 (2009 plant samples) from *G. flava* is shown in **Figure 2-8**. The following peaks (δ) were observed for fraction OTC03706 from the 2009 plant samples (29.6, 32.2, 37.70, 56.0, 67.5, 72.8, 77.1, 79.8, 96.3, 97.9, 100.6, 102.5, 107.8, 115.3, 116.2, 119.2, 132.7, 145.5, 145.9, 155, 157 and 158). The *cis*:*trans* ratio of CT from *G. flava* was estimated to be 100:0 since the *trans* signal was not detected, and PC:PD ratio was also estimated to be 100:0 because the PD signal was not obtained. The mean molecular weight was not deduced due to low signal intensity, signal overlap and peak broadening.

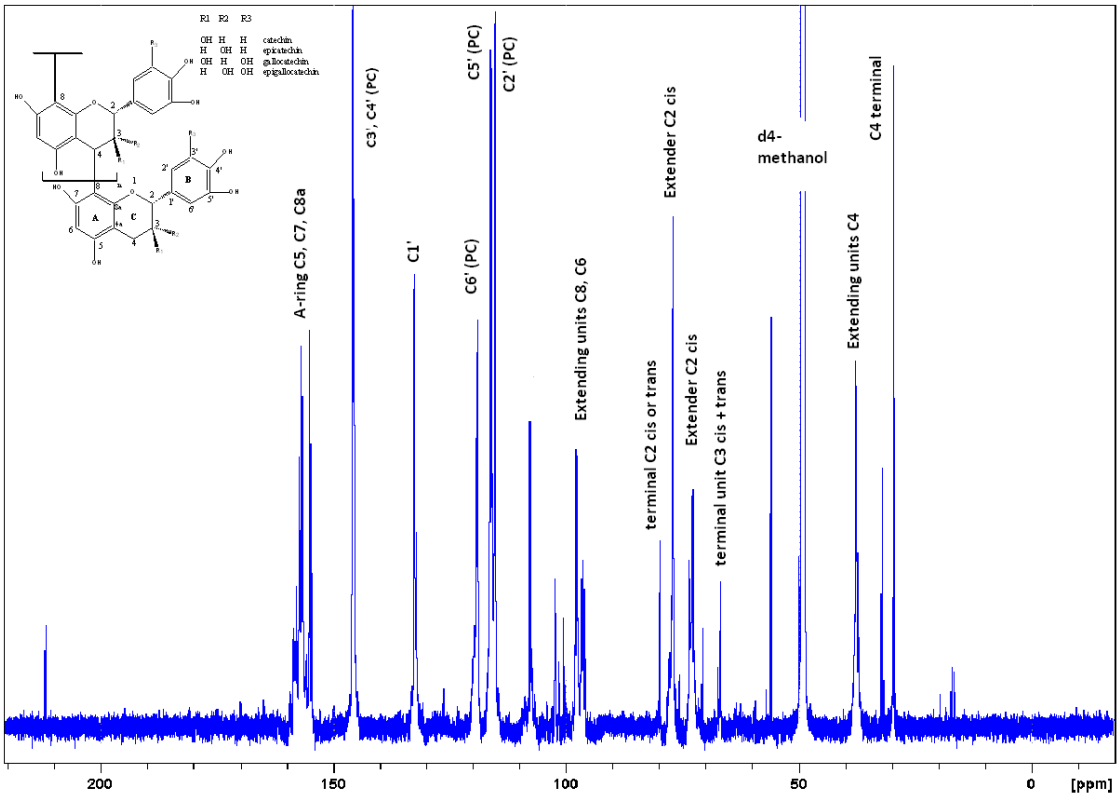

Figure 2–8 ^{13}C-NMR spectrum (d_4-methanol) of OTC03706 (acetone:water 7:3) from *G. flava* collected in 2009

Similar spectra were obtained for the CT fractions isolated from the same plants collected in 2010. The ^{13}C-NMR spectra were obtained for the following fractions: OTD03106 (acetone:water 7:3) from *V. verrucosum*, OTD03306 (acetone:water 7:3) from *T. oleifolius* and OTD03506 (acetone:water 7:3) from *G. flava* obtained from plants collected in 2010 (**Appendix 2-30**).

2.3.9. Analysis of CT containing linear gradient LH-20 fractions by ^{1}H and ^{13}C NMR

To improve the separation of CT oligomers from polymers, a linear gradient method was employed. The linear gradient fractions containing CT isolated from *V. verrucosum*, T. *oleifolius* and *G. flava* were run for NMR (OTD04210, OTD04410 and OTD04610 respectively). These fractions were isolated from plants collected in 2009. However, no signals were obtained in analysis with NMR. Good spectrum was obtained for OTD04805 and OTD04806 from *V. rotundifolium* and the re-chromatographed fraction OTE11605 from the same plant sample (**Appendix 2-30**). The following peaks in the ^{13}C-NMR spectrum of the fractions, which were analogous to peaks for the fractions above (**Section 2.3.8**), were observed: (δ) 28.1, 30.5, 36.4, 54.7, 65.8, 71.5, 75.9, 78.1, 96.5, 101.3, 106.5, 114, 115.5, 118, 131.9, 144.2, 144.5, 153.5, 155.2 and 155.8 ppm.

In summary, CT from *G. flava*, *T. oleifolius* and *V. verrucosum* were purely PCs. There was no evidence of galloylation and glycosylation of the CT polymers. The ^{13}C-NMR data of CT from *T. oleifolius*, *V. verrucosum* and *G. flava* were confirmed with published data from a variety of plant species such as birdsfoot trefoil (*Lotus corniculatus*) (Meagher et al., 2004) and mangrove species (*Kandelia candel* and *Rhizophora mangle*) (Zhang et al., 2010). None of the step and linear gradient LH-20 fractions containing CT produced a series of singly charged oligomeric ions nor multiply charged ions in ESI-MS and MALDI-TOF-MS. The ^{13}C-NMR CT data, although not quantitative, are commonly validated using thiolysis degradation reaction, which also provides information on the mean degree of polymerisation, PC:PD ratio and *cis*:*trans* ratio. Therefore, the results from the thiolysis experiment are discussed in **section 2.3.10** below.

2.3.10. Thiolysis

2.3.10.1. Analysis of thiolysis adducts by RP-HPLC

The chemical composition of CT of the plants collected in 2009 and 2010 (*T. oleifolius*, *V. verrucosum* and *G. flava*) was further characterised by strong acid catalysed degradation with benzyl mercaptan. The corresponding thioderivatives were analysed by a reverse phase-HPLC **(Figure 1-7 in Chapter 1, Literature Review)**. The degradation of these polymeric compounds with mild acids in the presence of phloroglucinol is well known despite incomplete degradation and low yields of the degraded products (Foo et al., 1997). Despite the unpleasant odour of benzyl mercaptan, it is the most ideal nucleophile in trapping all the carbocations and in providing good yields of cleavage products (Koupai Abyazani et al., 1993). However, studies with other nucleophiles should be carried out. In this study, complete degradation of the CT was observed in the chromatograms.

The HPLC-PDA chromatograms of the thiolysis adducts of CT from *T. oleifolius*, *V. verrucosum* and *G. flava* at 280 nm are shown in **Figure 2-9** and **Figure 2-10**. The chromatograms shown in both figures are for the thiolysis adducts from the following fractions: OTC02906 from *V. verrucosum*, OTC03406 from *T. oleifolius* and OTC03706 from *G. flava* from plants collected in 2009. The CT containing fractions from plants collected in 2010 were also used in the thiolysis experiment: OTD03106 from *V. verrucosum*, OTD03206 from *T. oleifolius* and OTD03406 from *G. flava*.

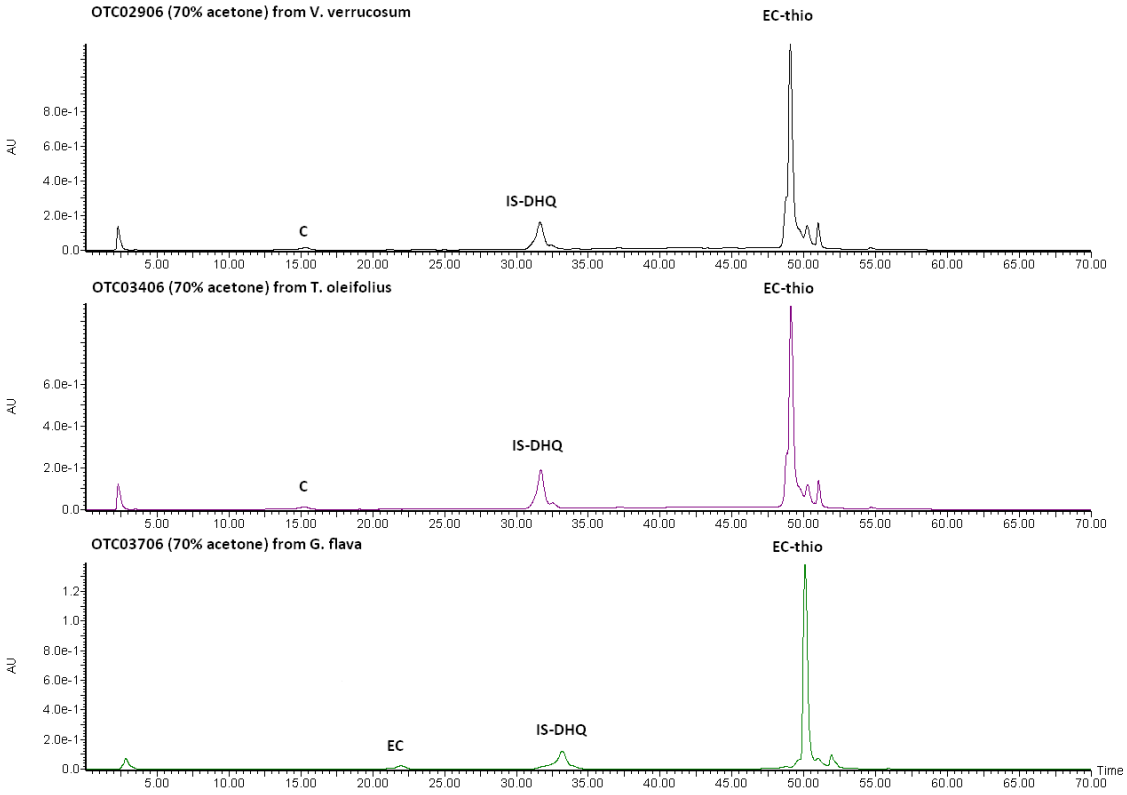

Figure 2–9 HPLC-PDA chromatograms of the thiolysis adducts of the CT from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 at 280 nm

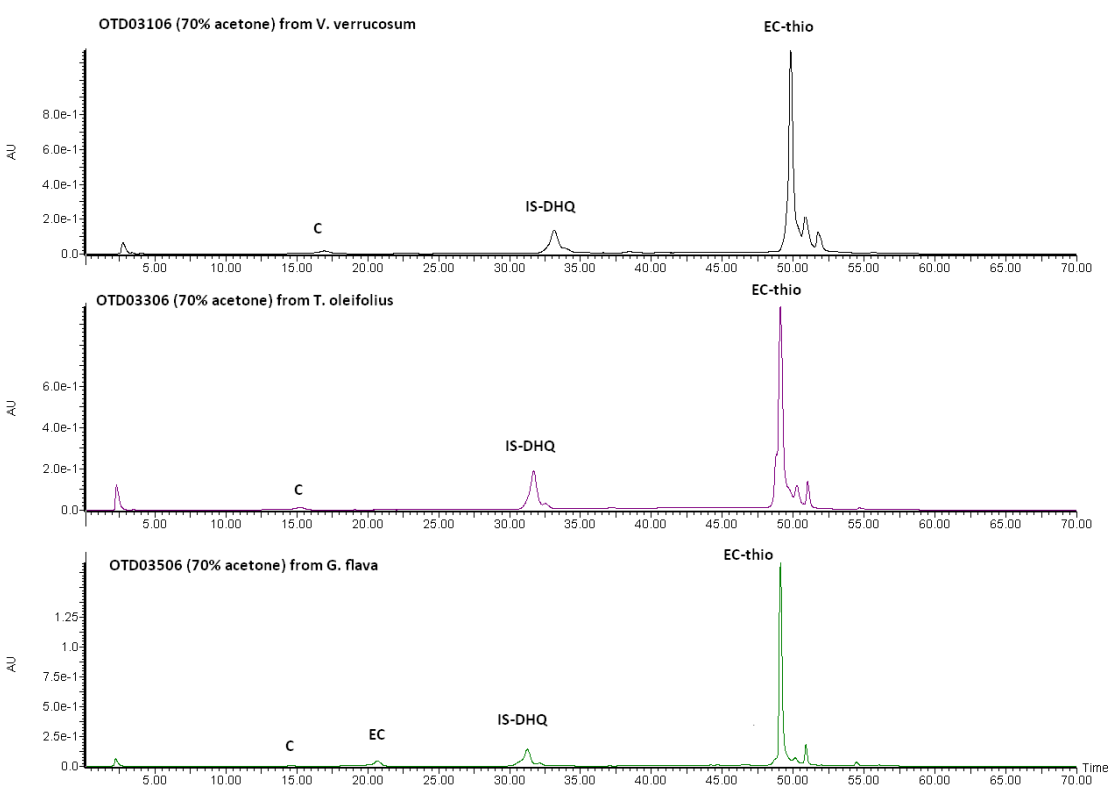

Figure 2–10 HPLC-PDA chromatograms of thiolysis adducts of CT from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2010 at 280 nm.

The sharp peaks were observed in the chromatograms after degradation of the CT polymer which were suggestive of the presence of the low molecular weight phenolics. The broad peaks before degradation were suggestive of the presence of CT polymers. After degradation, the CT polymers were converted into monomer units (free flavan-3-ols and flavan-3-ol thioether derivatives). The chromatogram of the undegraded CT polymer from the acetone:water (7:3) fractions compared to that of the degraded CT is illustrated in **Figure 2-11**.

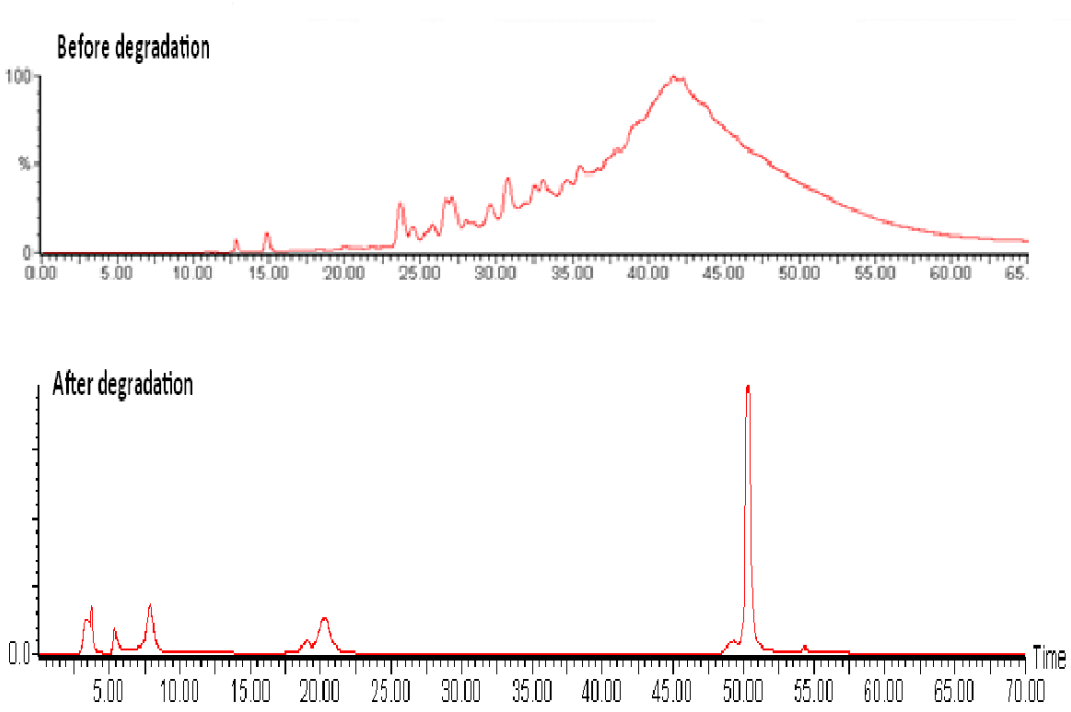

Figure 2–11 HPLC-PDA representative chromatogram of intact CT compared to that of degraded CT by the acid in the presence of benzyl mercaptan at 280 nm

The two main factors influencing the retention time are the stereochemistry and the overall polarity (Koupai-Abyazani et al., 1992). The substitution pattern of the B-ring is an important factor in the elution order of the thiolysis adducts. The retention time of the compounds with three hydroxyl groups on the B-ring (i.e. compounds with a pyrogalloyl group) are shorter than those of the corresponding compounds with two hydroxyl groups (i.e. compounds with a catechol group). In addition, the elution order of the compounds is influenced by the stereochemistry of the C3 position in the C-ring.

For example, (+)-catechin with 2R:3S configuration has a shorter retention time than (-)-epicatechin with 2R:3R configuration. The expected degraded products of the CT polymer were catechin, epicatechin, gallocatechin, epigallocatechin, catechin-thio, epicatechin-thio, gallocatechin-thio and epigallocatechin-thio. The retention times of the degraded products of CT from *V. verrucosum*, *T. oleifolius* and *G. flava* are collated in **Table 2-6**. The retention times of the degraded products of CT from all forage plants were similar, and the same compound from the forage plants eluting at 49-50 min was purified, isolated and characterised with ^{1}H and ^{13}C NMR.

Table 2-6 Retention times of the degraded products of CT from *V. verrucosum*, *T. oleifolius* and *G. flava* compared to those of the authentic standards.

Authentic standards (As)	Retention times of As	Retention times of *V. verrucosum* CT adducts	Retention times of *T. oleifolius* CT adducts	Retention times of *G. flava* CT adducts
Catechin	15.27	15.00	15.00	14.28
Epicatechin	21.20	nd	nd	19.67
Gallocatechin	nd	nd	nd	nd
Epigallocatechin	12.45	nd	nd	nd
Taxifolin	31.68	31.64	31.70	32.25
Catechin-thio or EC-thio	49.08	49.04	49.10	49.08
Epicatechin-thio	50.00	50.99	51.03	50.42
Gallocatechin-thio	45.22	nd	nd	nd
Epigallocatechin-thio	49.24	nd	nd	nd

Abbreviations: As-Authentic standards, nd- not detected

The flava-3-ols and the thiolysis adducts of CT extracted from *G. flava*, *T. oleifoilius* and *V. verrucosum* were identified by comparison to the retention times of authentic standards. However this task was not performed on CT from *V. rotundifolium*, as the CT containing fractions from this plant were not purified when these preliminary investigations were carried out. The degraded products were analysed by a reversed phase-HPLC, which demonstrated that CT extracted from these plants were predominantly procyanidin-type. From these qualitative results, catechin and epicatechin were present in the CT polymer from all forage plants (**Table 2-6**). Epicatechin was dominant in the extender units of CT from *T. oleifolius*, *V. verrucosum*

and *G. flava*. Catechin was present in the terminal units of *V. verrucosum* and *T. oleifolius* although with low signal intensity, while epicatechin was found in the terminal units of *G. flava*. The terminal units containing catechin and epicatechin eluted between 15.00 and 21.00 min, while the extender units containing catechin and epicatechin eluted between 49 and 50 min. The epicatechin benzylthioethers appeared as two epimers which could be for 3,4-*cis*-epicatechin benzylthioether (49.08 min, major product) and 3,4-*trans*-epicatechin benzylthioether (50.00 min, minor product). CT from all the forage species had similar chemical characteristics. There was no evidence of the presence of gallocatechin or epigallocatechin in the terminal and the extender units within the CT polymer from all forage plants. Furthermore, the individual units in the terminal and extender units showing galloylation were not observed. Thus, the terminal units in the CT polymer from *G. flava*, *T. oleifolius, V. verrucosum* contained catechin/epicatechin, while epicatechin was dominant in the extender units. This trend was also observed in the ^{13}C-NMR spectra of the CT of forages discussed in **section 2.3.8** above.

The relative amounts of various flavan-3-ols (from the terminal units) and their thioether derivatives (from the extension units) were measured by RP-HPLC to provide quantitative information on the mean degree of polymerisation (mDP), procyanidins:prodelphinidin (PC:PD) ratio and *cis*:*trans* ratio. The flavan-3-ol units in several leguminous forages consist of procyanidin (PC) monomers, catechin (C, 2,3-*trans*) and epicatechin (EC, 2,3-*cis*) and/or prodelphinidin (PD) monomers, gallocatechin (GC, 2,3-*trans*) and epigallocatechin (EGC, 2,3-*cis*) isomers. The thiolysis data for the structural composition and the mean degree of polymerisation of the fractions are presented in **Table 2-7**.

Table 2-7 Condensed tannin composition of step and linear gradient LH-20 fractions from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 and 2010 by thiolysis reaction products for the mean degree of polymerisation, % contributions of terminal and extender units of CT and ratio of prodelphinidin (PD) to procyanidin (PC) units.

Plant samples and fractions	Eluting solvent		Terminal (%)				Extender (%)					
		[a]mDP	[b]GC	[c]EGC	[d]C	[e]EC	GC	EGC	C	EC	[f]PC:[g]PD	*cis*:*trans*
Step LH-20 fractions												
V. verrucosum												
OTC02906 (2009)	Acetone:H_2O (7:3)	6.3	0	0	94	6	0	0	0	100	100:0	87:13
OTD03106 (2010)	Acetone:H_2O (7:3)	4.9	0	0	93	7	0	0	0	100	100:0	84:16
T. oleifolius												
OTC03406 (2009)	Acetone:H_2O (7:3)	6.1	0	0	95	5	0	0	0	100	100:0	87:13
OTD03306 (2010)	Acetone:H_2O (7:3)	6.1	0	0	95	5	0	0	0	100	100:0	87:13
G. flava												
OTC03706 (2009)	Acetone:H_2O (7:3)	4.7	0	0	8	92	0	0	0	100	100:0	99:1
OTD03506 (2010)	Acetone:H_2O (7:3)	3.0	0	0	0	100	0	0	0	100	100:0	100:0
Linear gradient LH-20 (2009)												
V. rotundifolium												
OTD04805	MeOH:H_2O (3:1)	18	0	0	44	56	0	0	0	100	100:0	98:2
OTD04806	100% MeOH	0	0	0	0	0	0	0	0	100	100:0	100:0
V. verrucosum												
OTD04208	100% MeOH	12.9	0	0	100	0	0	0	0	100	100:0	93:7
OTD04209	100% MeOH	4.8	0	0	96	4	0	0	0	100	100:0	83:17
OTD04210	Acetone:H_2O (7:3)	7.5	0	0	94	6	0	0	0	100	100:0	89:11
T. oleifolius												
OTD04410	100% MeOH	6.2	0	0	9	91	0	0	0	100	100:0	99:1
OTD04411	Acetone:H_2O (7:3)	6.5	0	0	100	0	0	0	0	100	100:0	87:14
G. flava												
OTC04609	100% MeOH	3.3	0	0	0	100	0	0	0	100	100:0	100:0
OTD04610	Acetone:H_2O (7:3)	2.7	0	0	25	75	0	0	0	100	100:0	100:0
OTE11605 (Re-chromatographed)	Acetone:H_2O (7:3)	5.5	0	0	47	53	0	0	0	100	100:0	93:7

Abbreviations: [a]mDP = mean degree of polymerisation. [b]GC = gallocatechin. [c]EGC = epigallocatechin. [d]C= catechin. [e]EC = (epi) catechin. [f]PC= procyanidin. [g]PD = prodelphinidin).

Catechin was the predominant substance in the terminal unit (94 and 93%, respectively) in CT from fractions OTC02906 and OTD03106 of *V. verrucosum*, while epicatechin was the dominant extender unit (100%) in both fractions. Fractions OTC03406 and OTD03306 from *T. oleifolius* showed a similar pattern. Thus, catechin was the predominant terminal unit (95%) while epicatechin was the dominant extender unit (100%) from both fractions. In contrast, epicatechin in CT from fractions OTC03706 and OTD03506 from *G. flava* was the predominant terminal unit (92 and 100%, respectively), while the extender unit consisted of epicatechin only (100%), with the overall characteristic of a procyanidin-type CT. Thiolysis provided information on the relative stereochemistry at C2 and C3 of CT. The *cis*:*trans* ratio of CT from the forage species ranged from 84:16 to 100:0. Gallocatechin and epigallocatechin were not detected in the terminal and extender units. Thus, CT from *V. verrucosum, T. oleifolius* and *G. flava* were PC and 2,3-*cis* stereochemistry dominant.

Similarly, the individual units within the CT polymers of the linear gradient fractions consisted of catechin and epicatechin (**Table 2-7**). The extender units from all fractions were purely epicatechin (100%), while the terminal units consisted of catechin and epicatechin. The PC:PD ratio (100:0) was similar for all fractions. The *cis*:*trans* ratio ranged from 83:17 to 100:0. The *cis*:*trans* ratio of fractions from *G. flava* was 100:0, which was similar to that of fractions from *V. rotundifolium*. Thiolysis of the re-chromatographed fraction, OTE011605, from the linear gradient fraction, OTD04601 (water), was shown to contain catechin (47%) and epicatechin (53%) in the terminal unit. The extender unit consisted of 100% epicatechin, and PC:PD ratio was 100:0, while the *cis:trans* ratio was 93:7. The similarity in PC content and *cis* stereochemistry for the extender and terminal units of all fractions could be regulated by their biosynthesis pathway.

The mean degree of polymerisation (mDP) can be estimated from the ratio of the terminal to extender units. In the step LH-20 fractions, the mean DP of CT from the forage species collected in 2009 and 2010 ranged between 3 and 6.3. The highest mean DP was observed in fractions from *V. verrucosum* and *T. oleifolius*, with a mDP of 6.3 to 6.1, respectively. However, the lowest mean DP of 3.0 was obtained in CT from OTD03506 from *G. flava*. Thus, there was no considerable difference in the chemical composition (*cis*:*trans* ratio, mDP and PC:PD ratio) in plants collected in 2009 and 2010. Thus, the step LH-20 fractions from these plants contained a low molecular weight CT (LMWCT).

For the linear gradient fractions, the mean DP of CT from the plant species ranged from 2.7 to 18. Fractions OTD04209 and OTD04210 from *V. verrucosum* consisted of low molecular weight CT (LMWCT), with a mDP of 4.8 and 7.5, respectively. Fractions from OTD04410 and OTD04411 from *T. oleifolius* had a mDP of 6.2 and 6.5, respectively. Furthermore, fractions OTD04609 and OTD04610 consisted of LMWCT with a mDP of 3.3 and 2.7, respectively. A medium molecular weight CT (MMWCT) was obtained from fraction OTD04806 (100% MeOH) from *V. rotundifolium*, with a mDP of 18. In addition, a MMWCT of 12.9 was isolated from OTD04208 from *V. verrucosum,* suggesting that Sephadex LH-20 could have been operating in a size exclusion manner. A high molecular weight CT (HMWCT) was not detected in any of the isolated fractions. Fraction OTE11605 had a LMWCT with a mDP of 5.5.

Overall, these results suggest that *V. verrucosum*, *T. oleifolius* and *G. flava* contain extractable CT with marked variations in chain lengths, which are procyanidin-type and dominated by the *cis* stereochemistry. The main constituent in the extender units were comprised predominantly of epicatechin, whereas catechin and epicatechin were the predominant terminal unit. These results were confirmed with the ^{13}C-NMR data of the intact CT (**Section 2.3.8**) and the purification of the degraded adducts (**Section 2.3.10.2 below**).

2.3.10.2. Identification and characterisation of the individual units within the CT polymer using open column chromatography

The degraded adducts of CT from *V. verrucosum*, *T. oleifolius* and *G. flava* were further purified by open column chromatography (silica gel, C-18 column). The purified isolates were screened with RP-HPLC-PDA and subsequently characterised with ESI-MS and ^{1}H and ^{13}C-NMR to study in more detail the structures that make up the CT polymer in both the terminal and extender units. Signal assignment was made in accordance with published data by Cai et al. (1991).

2.3.10.2.1. Characterisation of thiolysis adducts isolated from CT from *G. flava*

Trace amounts were obtained for the fractions (e.g. OTE18301-04) and hence analysis was not conducted. The characterisation of the thiolysis adducts of CT from *G. flava* revealed the presence of the following compound (OTE18305 (55% MeOH, 20 mg) epicatechin-4β-benzylthioether, an off white solid) as shown in **Figure 2-12**: ^{1}H-

NMR (d_4-methanol): Ph 7.20-7.45 (5H, m). 2′ 7.07 (1H, d, *J* = 2.0 Hz), 5′ 6.76-6.83 (1H, m), 6′ 6.76-6.83 (1H, m), 5.92 (1H, m, 6-H), 5.92 (1H, d, J = 2.3 Hz, 8-H), 2-H 5.26 (s), 3-H 4.07-3.86 (1H, m), 4-H 4.07-3.86 (1H, m), S-CH_2 4.09-4.00 (1H, m). ^{13}C-NMR δ 36.6 -SCH_2, 42.6 (C4), 70.2 (C3), 74.2 (C2), 94.4 (C8), 95.5 (C6), 98.8 (C4a), 113.9 (C2′), 114.6 (C5′), 117.9 (C6′), 126.8 (C4″), 127.9 (C3″ and C5″), 128.8 (C2″ and C6″), 130.7 (C1′), 139.0 (C1″), 144.4 (C3′), 144.6, (C4′), 155.9 (C5), 157.6 (C8a), 157.7 (C7).

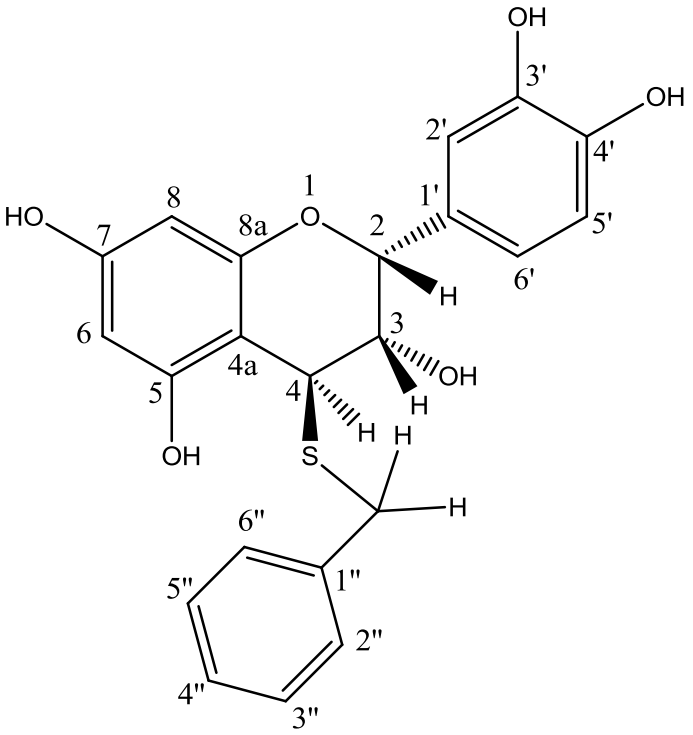

Figure 2–12 Chemical structure of epicatechin-4β-benzylthioether from *G. flava.*

OTE018306 (55% MeOH, 43 mg off white solid): epicatechin-4β-benzylthioether, an oily off white solid): ^{1}H-NMR (d_6-acetone): δ Ph 7.22-7.49 (5H, m), 2′ 7.07 (1H, d, 2.0 Hz), 5′ 6.79-6.84 (1H, m), 6′ 6.79-6.84 (1H, m), 6-H 5.94 (1H, d, 2.4 Hz), 8-H 6.04 (1H, d, 2.5 Hz), 2-H 5.30 (s), 3-H 4.01-4.12 (1H, m), 4-H 4.01-4.12 (1H, m), S-CH_2 4.01-4.12 (2H, m). ^{13}C-NMR (d_6-acetone) δ 36.3 SCH_2, 42.5 (C4), 70.3 (C3), 74.5 (C2), 94.9 (C8), 95.9 (C6), 99.0 (C4a), 114.5 (C5′), 114.7 (C2′), 118.4 (C6′), 126.8 (C4″), 128.2 (C3″ and C5″), 128.9 (C2″ and C6″), 131.1 (C1′), 139.1 (C1″), 144.5 (C3′), 144.6, (C4′), 156.3 (C5), 157.6 (8a), 158.0 (C7).

OTE018307 (55% MeOH, 60 mg): epicatechin-4β-benzylthioether, an off white solid): ^{1}H-NMR (d_6-acetone): δ Ph 7.23-7.49 (5H, m), 7.07 (1H, d, *J* = 1.4 Hz, 2′-H), 6.79-6.84 (1H, m, 5′-H), 6.79-6.84 (1H, m, 6′-H), 5.94 (1H, d, *J* = 2.2 Hz, 6-H), 6.05 (1H, d, *J* = 2.2 Hz, 8-H), 5.31 (s, 2-H), 3.95-4.12 (1H, m, 3-H), 3.95-4.12 (1H, m, 4-H), S-CH_2 3.95-4.12 (2H, m). ^{13}C-NMR (d_6-acetone) δ 36.3 SCH_2, 42.5 (C4), 70.3 (C3), 74.5 (C2), 94.9 (C8), 95.9 (C6), 99.0 (C4a), 114.5 (C5′), 114.7 (C2′), 118.4 (C6′), 126.8 (C4″), 128.2 (C3″ and C5″), 128.9 (C2″ and C6″), 131.1 (C1′), 139.1 (C1″), 144.5 (C3′), 144.6 (C4′), 156.3 (C5), 157.5 (8a), 158.0 (C7).

OTE018308 (55% MeOH): epicatechin-4β-benzylthioether, a brown solid): ^{1}H-NMR (d_6-acetone): δ Ph 7.23-7.49 (5H, m), 7.07 (1H, d, *J* = 1.4 Hz,2′-H), 6.79-6.84 (1H, m, 5′-H), 6.79-6.84 (1H, m, 6′-H), 5.94 (1H, d, *J* = 2.2 Hz, 6-H), 6.05 (1H, d, *J* = 2.2 Hz, 8-H), 2-H 5.31 (s), 3-H 3.95-4.12 (1H, m), 4-H 3.95-4.12 (1H, m), S-CH_2 3.95-4.12 (2H, m). ^{13}C-NMR (d_6-acetone) δ 36.3 SCH_2, 42.5 (C4), 70.3 (C3), 74.5 (C2), 94.9 (C8), 95.9 (C6), 99.0 (C4a), 114.5 (C5′), 114.7 (C2′), 118.4 (C6′), 126.8 (C4″), 128.2 (C3″ and C5″), 128.9 (C2″ and C6″), 131.1 (C1′), 139.1 (C1″), 144.5 (C3′), 144.6 (C4′), 156.3 (C5), 157.5 (8a), 158.0 (C7).

The HPLC-PDA chromatograms of the purified adducts are shown in **Appendix 2-31**. The ^{1}H-NMR and ^{13}C-NMR spectra of fractions OTE18305-08 (**spectra shown in Appendix 2-34 to 2-35)** were similar, which showed that the isolated compound (epicatechin) was prevalent in these fractions.

Thus, the results obtained demonstrate that epicatechin (2,3-*cis* stereochemistry) was the main extender unit in the CT polymer of *G. flava*, which suggested that CT from this plant consisted of epicatechin as their building block. In addition, they confirm the thiolysis and ^{13}C-NMR data of the intact CT which showed epicatechin to be prevalent in the polymer. Further efforts to isolate and characterise catechin proved unsuccessful, but this could be attributed to its low signal intensity.

2.3.10.2.2. Characterisation of thiolysis adducts isolated from CT from *T. oleifolius*

Trace amounts were obtained for the other fractions (e.g. OTF02001-05) and hence no analysis was conducted. Characterisation of the thiolysis adducts of CT from *T. oleifolius* showed the presence of the following compound from OTF02006 (55% MeOH, 31 mg, Rt 49.10, epicatechin-4β-benzylthioether, an off white solid) as shown

in **Figure 2-13**: ^{1}H-NMR (d_6-acetone): Ph 7.20-7.49 (5H, m), 7.07 (1H, d, J = 2.3 Hz, 2′-H), 6.74-6.84 (1H, m, 5′-H), 5.93 (1H, d, *J* = 2.3 Hz, 8-H), 6.05 (1H, d, *J* = 2.3 Hz, 6-H), 5.31 (s, 2-H), 3.95-4.09 (1H, m, 3-H), 3.95-4.09 (1H, m, 4-H), S-CH_2 4.09-4.01 (1H, m). ^{13}C-NMR δ 36.3 SCH_2, 42.5 (C4), 70.3 (C3), 74.5 (C2), 94.4 (C8), 95.5 (C6), 98.8 (C4a), 114.6 (C5′), 114.7 (C2′), 118.4 (C6′), 126.8 (C4″) 128.4 (C3″ and C5″), 128.9 (C2″ and C6″), 130.7 (C1′), 139.0 (C1″), 144.5 (C3′), 144.6 (C4′), 156.3 (C5), 157.6 (C7), 158.0 (C8a). The chemical structure of the isolated compound is shown below, and RP-HPLC-PDA chromatograms of the thioadducts and ^{1}H and ^{13}C-NMR spectra of this compound are shown in **Appendix 2-32 and Appendix 2-36 to 2-37**, respectively.

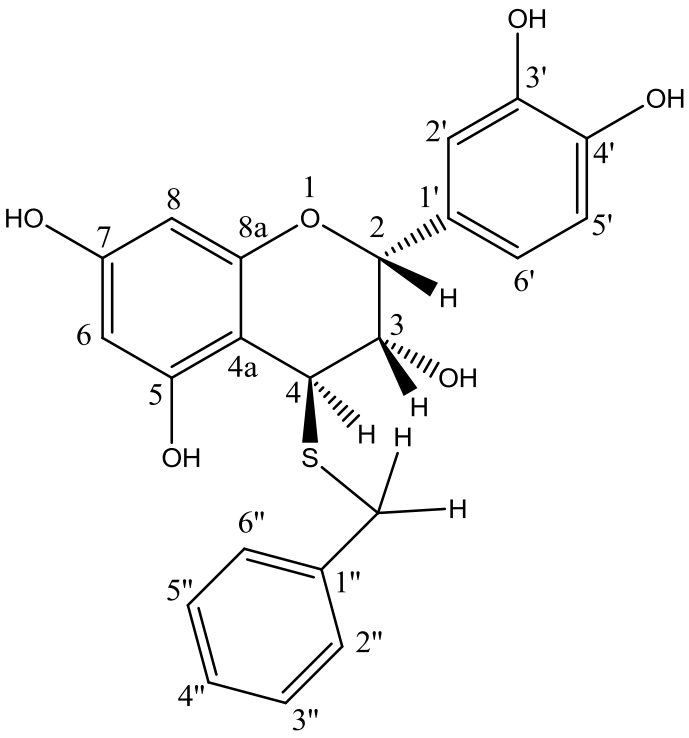

Figure 2–13 Chemical structure of epicatechin-4β-benzylthioether from *T. oleifolius.*

OTF02007 (55% MeOH, 51.6 mg off white solid Rt 49.10): epicatechin-4β-benzylthioether, an oily off white solid): ^{1}H-NMR (acetone-d_6): δ Ph 7.23-7.48 (5H, m), 7.07 (1H, d, *J* = 1.8 Hz, 2′-H), 6.74-6.84 (1H, m, 5′-H), 6.79-6.84 (1H, m, 6′-H), 5.93 (1H, d, *J* = 2.5 Hz, 6-H), 6.06 (1H, d, *J* = 2.4 Hz, 8-H), 5.30 (s, 2-H), 3-H 4.01-4.11 (1H, m), 4-H 4.01-4.11 (1H, m), S-CH_2 4.01-4.11 (2H, m). ^{13}C-NMR (d_6-acetone) δ 36.3 SCH_2, 42.5 (C4), 70.3 (C3), 74.5 (C2), 94.9 (C8), 95.9 (C6), 99.0 (C4a), 114.5

(C5′), 114.7 (C2′), 118.4 (C6′), 126.8 (C4″) 128.2 (C3″ and C5″), 129.0 (C2″ and C6″), 131.1 (C1′), 139.1 (C1″), 144.5 (C3′), 144.6, (C4′), 156.3 (C5), 157.6 (8a), 158.0 (C7). Epicatechin unit (2,3-*cis* stereochemistry) was prevalent in the thiolysis adducts analysed with HPLC. In addition, it was predominant in analysis of the CT polymers with ^{13}C-NMR.

2.3.10.2.3. Characterisation of thiolysis adducts from *V. verrucosum*

Trace amounts of the other fractions (i.e. OTF02701-05) were obtained, and hence no analysis was conducted. The characterisation of the thiolysis adducts of CT from *V. verrucosum* showed the presence of the following compound (OTF02706 (55% MeOH) and OTF02707 (60%MeOH) were pooled to make OTF02901 (10 mg, Rt 49.10) epicatechin-4β-benzylthioether, an off white solid) as shown in **Figure 2-14**: ^{1}H-NMR (d_6-acetone): Ph 7.23-7.49 (5H, m), 7.06 (1H, d, *J* = 1.9 Hz, 2′-H), 6.73-6.97 (1H, m, 5′-H), 5.84 (1H, d, *J* = 2.2 Hz, 6-H), 6.04 (1H, d, *J* = 2.2 Hz, 8-H), 5.30 (s, 2-H), 3.95-4.11 (1H, m, 3-H), 3.95-4.11 (1H, m, 4-H), S-CH_2 3.95-4.11 (1H, m). ^{13}C-NMR δ 36.3 SCH_2, 42.5 (C4), 71.2 (C3), 74.5 (C2), 94.5 (C8),), 95.7 (C6), 101.1 (C4a), 115.1 (C5′ and C2'), 118.4 (C6′), 126.8 (C4″), 128.4 (C3″ and C5″), 129.1 (C2″ and C6″), 131.1 (C1′), 139.1 (C1″), 144.7 (C4′), 145.1 (C3′), 155.6 (C5), 157.7 (C7), 158.4 (C8a). The chemical structure of the isolated compound is shown below, and RP-HPLC-PDA chromatograms of the thioadducts including the ^{1}H and ^{13}C spectra are shown in **Appendix 2-33 and Appendix 2-38 to 2-39**, respectively.

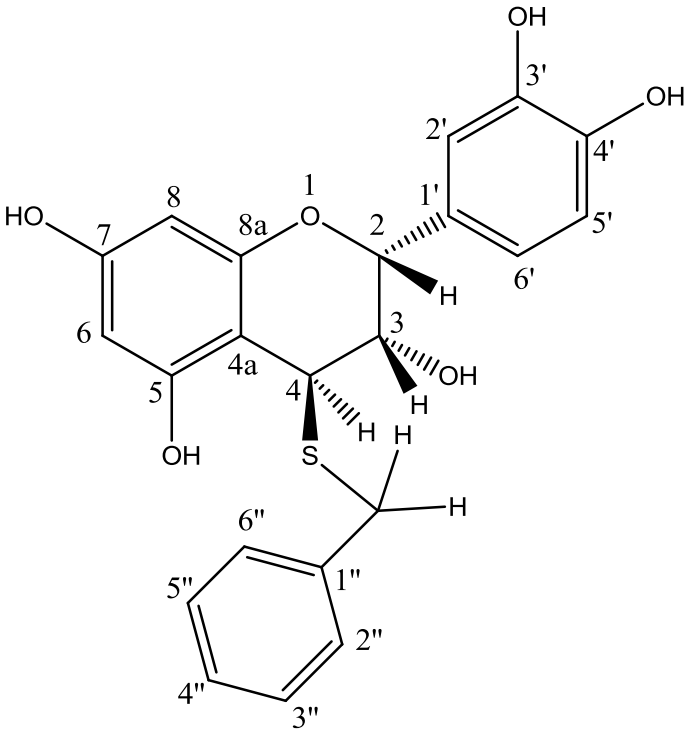

Figure 2–14 Chemical structure of epicatechin-4β-benzylthioether from *V. verrucosum.*

2.3.10.2.4. Characterisation of epicatechin-4β-benzylthioether by ^{1}H, ^{13}C, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC NMR

The ^{1}H and ^{13}C-NMR data of the isolated compound, epicatechin-4β-benzylthioether from *V. verrucosum*, *T. oleifolius* and *G. flava* were confirmed by the UV (**Figure 2.15**) and ^{1}H-^{1}H COSY and ^{1}H-^{13}C-HMQC experiments (**Table 2-8 and Figure 2-16 to 2-19**). In addition, the structure was confirmed by comparing the data with published data by Cai et al. (1991). These results showed that the plants did not exhibit considerable differences in the components determined because (-)-epicatechin was the predominant compound.

Table 2-8 ^{1}H and ^{13}C-NMR chemical shifts of the isolated epicatechin-4β-benzylthioether were confirmed by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC experiments (methanol-d_4).

Position C	δH	Multiplicity	Literature value δH (Cai et al. 1991)	δC	Flavan-3-ol rings
2	5.30	1H, s	5.30 (s)	74.9	C-ring
3	4.03	1H, m	4.03 (1H,d, *J* = 13.4Hz)	70.9	C-ring
4	4.10	1H, m	4.09 (1H,s)	42.6	C-ring
5				156.3	A-ring
6	6.06	1H, d, *J* = 2.3 Hz	6.04 (1H, d, *J* = 2.3Hz)	96.1	A-ring
7				158.0	A-ring
8	5.93	1H, d, *J* = 2.3 Hz	5.90 (1H, d, *J* = 2.3Hz)	94.7	A-ring
9 (8a)				157.5	A-ring
10 (4a)				99.0	A-ring
1'				139.1	B-ring
2'	7.06	1H, s	7.05 (1H,d, *J* = 2.0Hz)	114.7	B-ring
3'				144.5	B-ring
4'				144.6	B-ring
5'	6.82	1H, m	6.82 (1H, d, *J* = 8.1 Hz)	114.5	B-ring
6'	6.79	1H, m	6.79 (1H, dd, *J* = 2.0, 8.1 Hz)	118.6	B-ring
1"				131.1	Phthio
2"	7.47	1H, d, *J* = 6.5Hz)	7.21-7.50 (1H, m)	129.5	Phthio
3"	7.34	1H,t, *J* = 15Hz)	7.21-7.50 (1H, m)	128.7	Phthio
4"	7.24	1H,t, *J* = 14.9Hz)	7.21-7.50 (1H, m)	126.8	Phthio
5"	7.34		7.21-7.50 (1H, m)	128.7	Phthio
6"	7.47		7.21-7.50 (1H, m)	129.5	Phthio
1"'	4.05		4.02 (1H,d, *J* = 13.4Hz)	36.0	CH_2S

The signals attributed to the oxygenated and the non-oxygenated carbons in the A and B-ring of a flavan-3-ol molecule were observed. These assignments were supported by the observed small *meta* coupling constants of the A-ring (*J* = 2.3 Hz for H-6 and *J* = 2.3 Hz for H-8). Furthermore, the signals with their diagnostic coupling constants at δ 7.07 (1H, d, *J* = 2.0 Hz), 6.82 (1H, d, *J* = 8.1 Hz), 6.79 (1H, m) were assigned to hydrogens H2', H5' and H6' of the B-ring, respectively. H5′ appeared as a doublet (*J* = 8.1 Hz) due to *ortho* coupling with H6′, while H2′ appeared as a doublet due to *ortho* coupling with H6′. H6′ was both o*rtho* and *meta* coupled to H2′ and H5′. An aliphatic carbon signal at δ 36.0 ppm was assigned to the -SCH_2 of a benzylthiol and with their corresponding protons resonating upfield at δ 4.05 ppm. For the C-ring

signals, the determination of the absolute stereochemistry at C4 of the extender unit in the CT polymer is based on the 'γ effect' found in the ^{13}C-NMR spectra of the heterocyclic C-ring in the flava-3-ol system. An aromatic ring when it is attached at C4 position has a *trans* stereochemistry relative to the proton at C2 and has a minimal effect on the C2 signals. Thus, signals at δ 74.9 ppm were attributed to C2 carbons. For a *cis* orientation, a considerable downfield shift close to δ 79 ppm is usually obtained, suggesting the presence of *cis* orientation, but it was not observed. Further investigations should be conducted by removing the thiol moiety, if possible, and subsequently isolating and characterising the epicatechin compound.

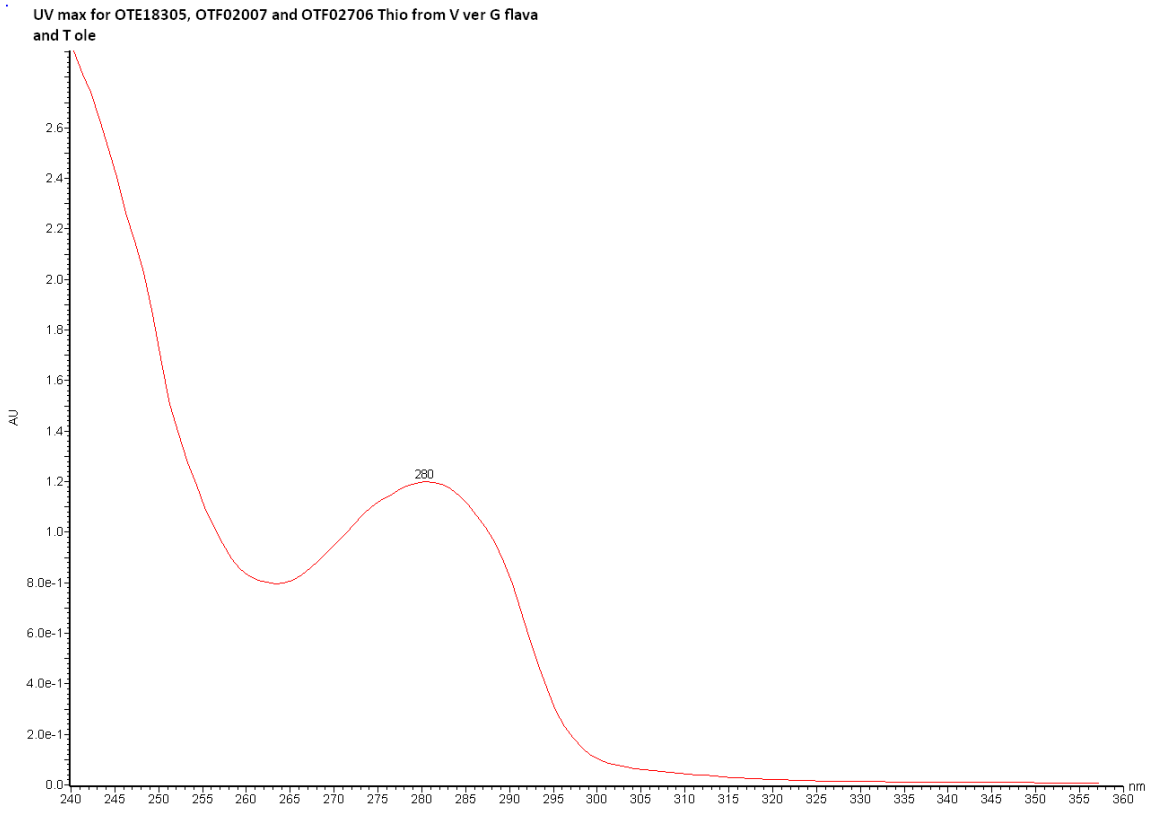

Figure 2–15 UV λ_{max} MeOH (nm) of (-)-epicatechin-4β-benzylthioether

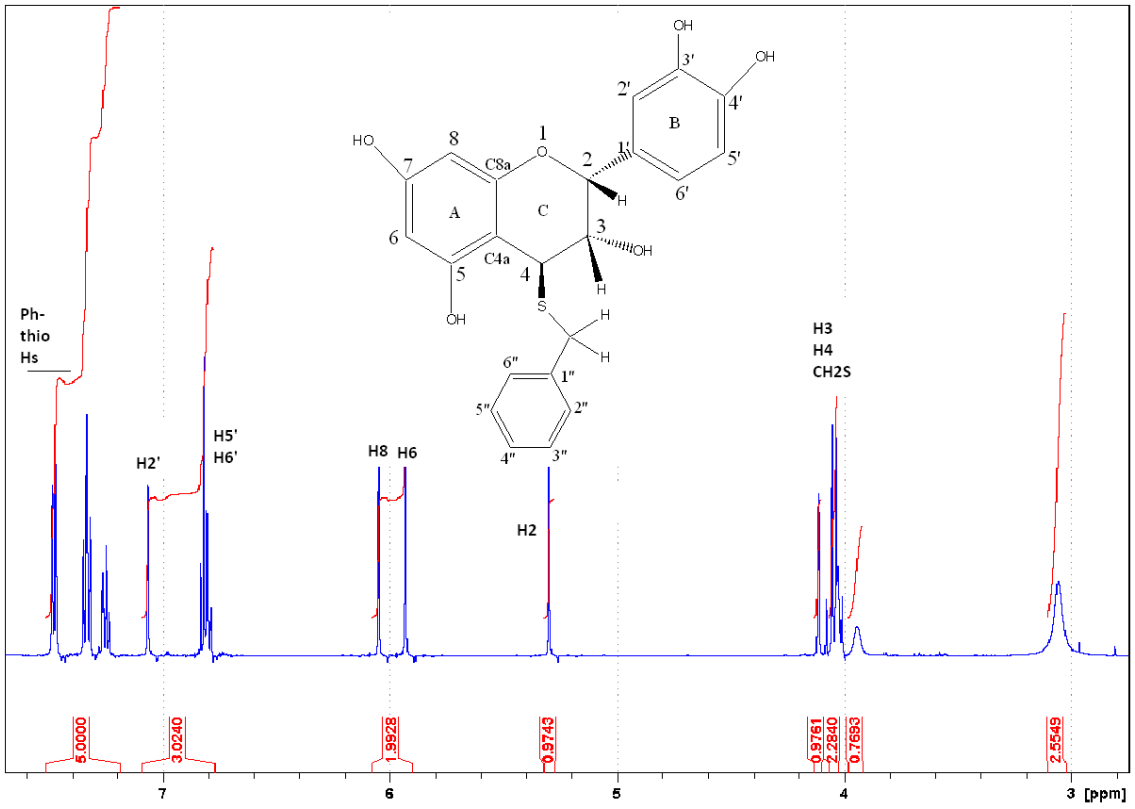

Figure 2–16 ^{1}H NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT

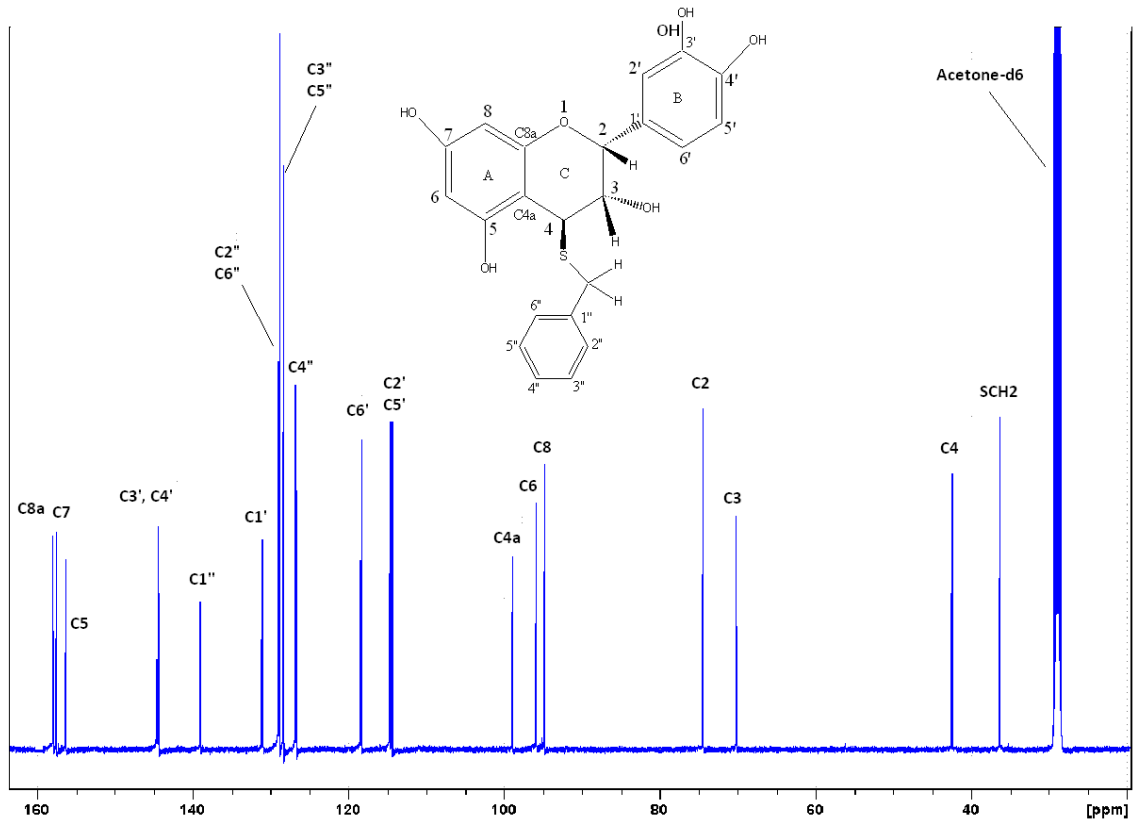

Figure 2–17 ^{13}C-NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT

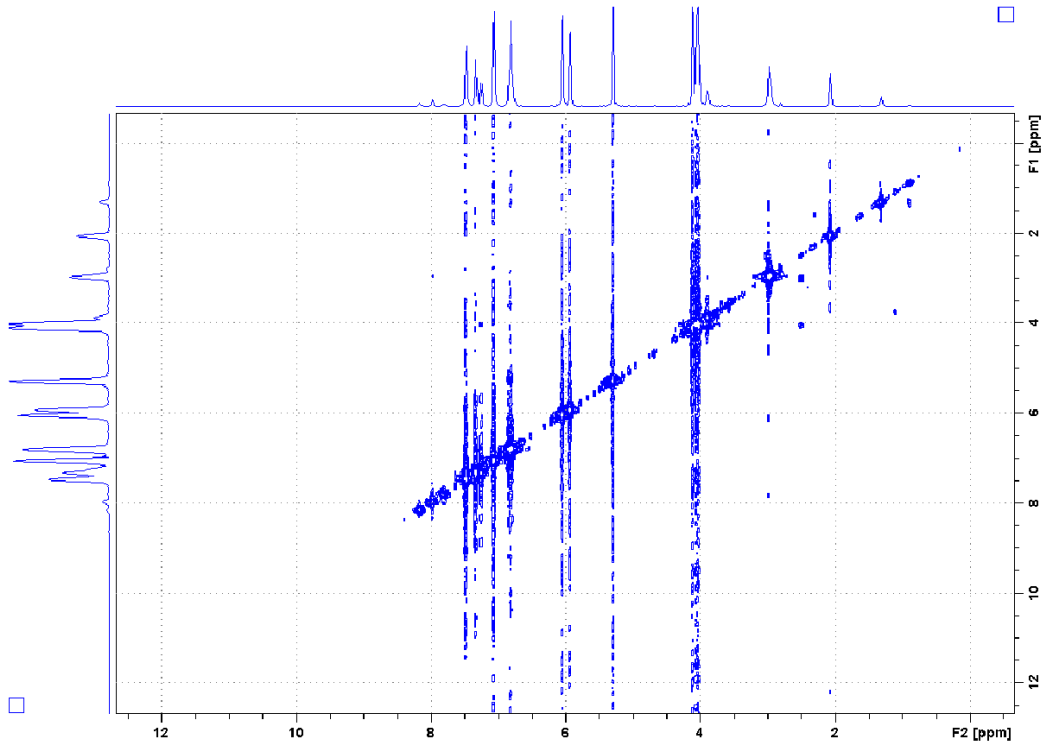

Figure 2–18 ^{1}H-^{1}H COSY spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT

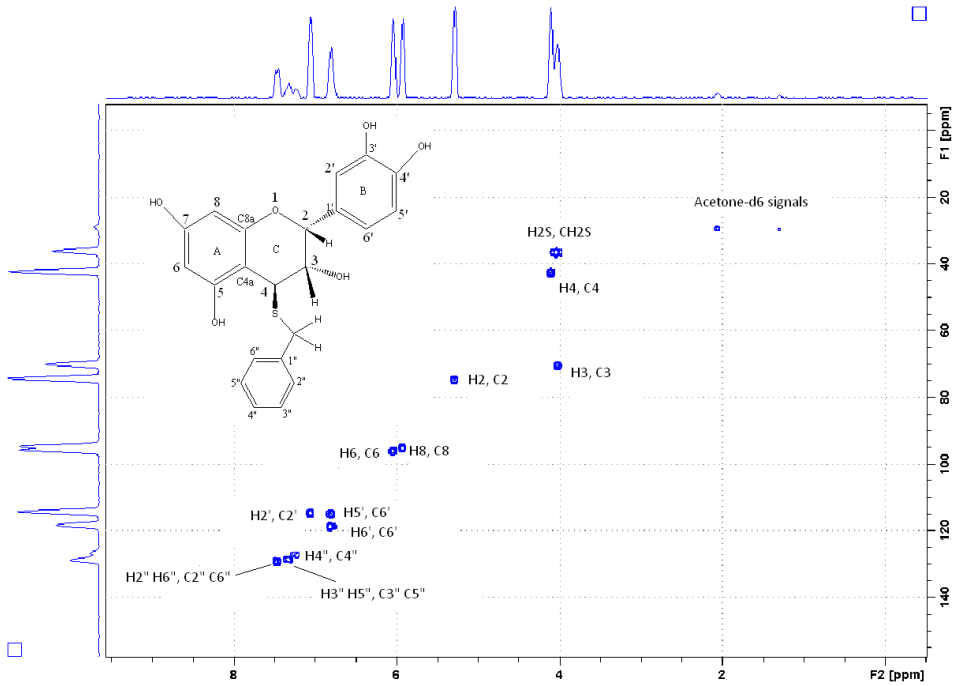

Figure 2–19 ^{1}H-^{13}C HMQC spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT

2.3.10.3. Analysis of thiolysis adducts by ESI-MS

The analysis of the thiolysis adducts by LC-MS to investigate the chemical composition of CT was acquired in negative ion mode. Initially, the samples were delivered to the ESI-MS probe via the HPLC. However, in delivery of samples through the HPLC, the sample could have been too dilute by when it reached the detector. Therefore, direct injection was done with the syringe pump and positive results were obtained. Fresh samples or adducts were prepared, and the samples were analysed immediately. The monomeric units attributed to the presence of catechin (R*t* = 15.00, $[M-H]^-$, *m/z* 289) and epicatechin or catechin benzyl thioether (R*t* = 50.00 $[M-H]^-$, *m/z* 411) from *V. verrucosum*, *T. oleifolius* and *G. flava* were detected.

2.3.10.4. Analysis of thioadducts by Fourier Transform Infrared (IR) Spectroscopy

The wavelength numbers (cm^{-1}) of epicatechin-4β-benzylthioether isolated from CT from *V. verrucosum*, *T. oleifolius* and *G. flava* are shown below: C-H 824, -C-O-alcohols, C-OH 1018, C-O-C 1038, C-OH 1147, Aromatic -OH 1284, , 1453 cm-1 alcohols C-O alcohols, 1518 C-H alkanes, cycloalkane 1468 C=C Aromatic 1603, 1494

and1453 cm-1, C-S-CH_2 1627, Aromatic C-H stretch and Aliphatic C-H stretch and O-H 3334 cm^{-1}. The IR spectrum of this molecule is shown in **Figure 2-20.**

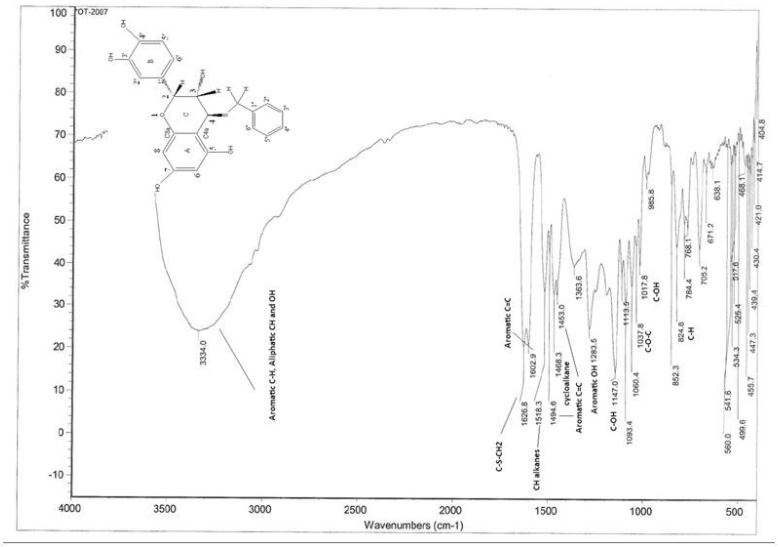

Figure 2–20 IR spectrum of epicatechin-4β-benzylthioether isolated from CT from *V. verrucosum, T. oleifolius* and *G. flava*

2.3.11. Condensed tannin composition and biological activity

The thiolysis degradation reaction demonstrated that CT from *V. verrucosum, V. rotundifolium, T. oleifolius* and *G. flava* and from Botswana were exclusively PC-type, with epicatechin as the predominant extender unit, and had variable chain lengths and *cis*:*trans* ratio.

Condensed tannins differed at many levels. Many CT polymers are mixtures of prodelphinidin and procyanidin units in differing ratios. Other CT polymers contain homogenous units, although homogeneous PDs are rare. In short, PC-type CT structure were isolated and characterised from *V. verrucosum, T. oleifolius* and *G. flava*. To the best of our knowledge, PC-type CT have never been reported in any non-forage and forage species in Botswana, so they are reported for the first time from these plants. Although quantitative information was not obtained in the ^{13}C-NMR spectroscopy, the spectral data showed that PC-type CT were present.

Procyanidin-type CT have been reported from a number of plant-derived food substances and forage species. A broad spectrum of biological activities have been discovered and some CT-based products are being marketed as nutraceuticals. For example, CT in grape seeds (Da Silva et al., 1991), apple (Guyot et al., 2001), cocoa (Adamson et al., 1999), lowbush blueberry and cranberry (Gu et al., 2002) are PC predominant. Furthermore, PC-type CT have been reported in *Aesculus* spp, *Vaccinium* sp, pear (*Cyonodia oblonga*), *Fragaria* sp. and apple (*Malus pumila* cv), and *Chaenomeles sinensis* (Foo and Porter, 1980). Procyandin-type CT are the most abundant in plants. CT in birdsfoot trefoil (*L. corniculatus*) are PC predominant, whereas CT from lotus major (*L. pedunculatus*) is PD predominant (Foo et al., 1997, Foo et al., 1996). CT polymers from *L. corniculatus* have PC-type extender subunits with epicatechin (0.67) dominating. On the other hand, *L. pedunculatus* has a PD-type extender subunits with epigallocatechin (0.64) dominating. The CT in sulla (*H. coronarium*) are PD predominant (Tibe et al., 2011), whereas the CT from white clover flowers (*Trifolium repens*) contain homogeneous PD (Foo et al., 2000). The CT in sainfoin (*O. viciifolia*) have variable compositions, which are attributed to the type of cultivar, age of the plant and seasonality (Foo et al., 2000, Koupai-Abyazani et al., 1993). The structural ratios of the *cis*:*trans* and PC:PD, and mean degree of polymerisation of CT from various plant sources are shown in **Table 2-9**.

Condensed tannins also varied in the 2,3-*cis*- or 2,3-*trans*- stereochemistry. The *cis* stereochemistry in the PC-type CT from *V. verrucosum*, *T. oleifolius* and *G. flava* was predominant. The biological activity of the CT from forages such as sulla and sainfoin is attributed to the predominance of the *cis* stereochemistry (Marais et al., 2000). Forages with *cis* predominant CT are always associated with higher activity. In addition, the additional hydroxyl groups in the CT structures may exhibit higher activity. For example, flavonoids with a 3′,4′–OH substitution system are reported to be more potent than those without this substitution pattern (Heim et al., 2002).

Thus, the CT in *G. flava*, *T. oleifolius* and *V. verrucosum* consisted dominatly of (-)-epicatechin extender units with a *cis* streochemistry. The role of the stereochemistry in the fourth position of the heterocyclic ring in the biological effects has not been investigated. Further research is required to investigate for biological activity in CT from other forage species in Botswana and on whether the activity is attributed to PC (catechin/epicatechin) or PD (gallocatechin/eipgallocatechin) predominant structures. PD-type CT were not observed in this study. However, PD units might have a higher activity due to the additional OH′s in the B-ring. It has also been reported that the high concentration of hydroxyl groups on the B-ring and functionalisation with esterified gallates may enhance the activity (Molan et al., 2003). However, in our study none of the CT were galloylated. Nevertheless, the results of our study are supported by the findings from Holderness et al. (2008) who found that PC predominant CT in apple peel were effective in inducing cell proliferation and stimulation of the innate immunity.

The biological effects of epicatechin-4β-benzylthioether are not known and should be invesigated. However, several *in vitro* studies have been conducted with epicatechin. For example, model epicatechin-containing dimers have been demonstrated to exhibit antiviral, antibacterial and superoxide radical-scavenging properties (De Bruyne et al., 1999). Motlhanka et al. (2008) reported that epicatechin showed good scavenging activity relative to 4'-O-methylepigallocatechin isolated from *Cassine transvaalensis*. Conversely, Molan et al. (2003) demonstrated that epicatechin had minimal antiparasitic activity relative to its galloylated derivatives. These results support the theory that functionalisation of the flavan-3-ols with gallate enhances the biological activity.

The effect of increasing molecular weight on the biological activity has not been investigated. The extent of oligomerisation may play an important role in influencing

biological activities of naturally occurring CT oligomers and polymers. However, CT oligomers (2-10 monomer units) were not detected with MALDI-TOF-MS or ESI-MS.

Other low molecular weight phenolics such as flavonoids could also be implicated in the biological activity. This was evidenced in the acetone-water extract of sainfoin consisting of a mixture of various substances including arbutin, kaempferol, quercetin, rutin, afzelechin, L-tryptophan, (+)-pinitol, sucrose and CT (Marais et al., 2000). Therefore, the biological activity from sainfoin may be attributed to CT acting independently or in synergy with other compounds. Also, it has been reported that CT from the same species do not provide the same biological activity. This was evidenced in *L. corniculatus* which is PC predominant but with increased amino acid absorption (Foo et al., 1996), while PD predominant *L. pedunculatus* protected proteins from degradation but inhibited amino acid absorption despite the higher number of hydroxyl groups (Foo et al., 1997).

Table 2-9 The structural ratios of *cis*:*trans* and procyanidin:prodelphinidin (PC:PD) producing units and molecular weights (M_W) of legume CT

Plant species	Common names	Tissue	CT (g/100 g DM)	*cis*:*trans*	PC:PD	Mw
L. corniculatus	Birdsfoot trefoil	Leaf	5	92:8	73:27	1800
L. pedunculatus	Lotus major	Leaf	8	75:25	30:70	3000
O. viciifolia	Sainfoin leaf	young	3	83:17	38:62	1620-2070
		old	-	48:52	7:93	-
H. coronarium	Sulla	Leaf	3.3-12.5	-	12:88-27:73	-
T. repens	White clover	Flowers	-	65:35	0:100	8500-9100

(Foo et al., 1982, Foo et al., 2000, Foo et al., 1996, Marais et al., 2000, Tibe et al., 2011)

2.4. Conclusions

The thiolysis degradation reaction and ^{13}C-NMR analysis have shown that CT from *G. flava*, *T. oleifolius* and *V. verrucosum* were predominantly *cis*, PC and had variable chain lengths. Epicatechin was found to be the main constituent in the extender units, and this compound was deduced to be responsible for the anti-parasitic and

immunostimulatory properties from the forage plants (Results in **Chapter 4** and **5**). The assays were carried out before the characterisation of the CT.

CHAPTER THREE:

ISOLATION AND CHARACTERISATION OF FLAVONOLS FROM *T. OLEIFOLIUS* AND *V. VERRUCOSUM*

3.1. Introduction

Plant phenolics such as flavonoids, phenolic acids and their derivatives can affect performance and productivity in ruminants. Flavonoids are a large group compounds that are further divided into different subclasses of flavonols (e.g. quercetin, kaempeferol), flavones (e.g apigenin), flavanones (naringenin), flavan-3-ols (condensed tannins), isoflavones (formononetein, genistein), neoflavones and anthocyanidins (cyanidin, delphinidin) (**Figure 3-1**). Some compounds may occur as -C or –O glycosides, in which one of the carbons is bound to a sugar moiety such as galactose, glucose and rhamnose. The sugar substitution pattern of flavonoids is favourable at the seventh and the third position on the A and C-rings, respectively. The basic core structure of flavonoids possesses the C_{15} flavone nucleus.

A range of flavonoids and phenolic acids of plant origin are commonly isolated and characterised by liquid chromatography (LC) coupled with a photodiode array (PDA) detector and mass spectrometry (MS) (Cuyckens and Claeys, 2004). Atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) are the widely used interfaces. These compounds have characteristic UV absorption spectra from which the aglycone moiety can be detected. However, the sugars do not have a strong UV absorption, and require hydrolysis and subsequent analysis of the individual components of the adducts with nuclear magnetic resonance (NMR) and comparison to the retention times of authentic standards. Low molecular weight phenolics such as kaempferol, rutin, quercetin, afzelin, (+)-pinitol, and flavanoid glycosides have been isolated and characterised from sainfoin (Marais et al., 2000). These, in addition to CT, may play an important role in the productivity and performance of ruminants. Foo et al. (2000) have also demonstrated that white clover flowers contain a plethora of phenolics in addition to CT. Thus, cinnamic acid derivatives, myricetin, quercetin and kaempferol derivatives were isolated and characterised from the white clover flowers. Flavonol glycosides have also been isolated and characterised from the forage legume, sulla, by APCI-MS (Tibe et al., 2011) A phenolic compound, formononetin, an isoflavone, is present in red clover and is known to be oestrogenic (Klejdus et al., 2001). This compound is not oestrogenic *per se*, but it is degraded to an equol in the rumen which

is oestrogenic. On the other hand, red clover products containing formononetin are available in the market and have beneficial effects of reduced menopause symptoms in women and reduced prostate enlargement in men and is used as a dietary supplement (Klejdus et al., 2001). This suggests that toxic plant metabolites could be transformed from undesirable agents to desirable health products. Iridoid glycosides (Aucubin) have been isolated from the herb plantain (*Plantago lanceolata*). These compounds are believed to possess anthelmintic properties (Stewart, 1996). Similar compounds have been isolated in plant-derived food substances and medicinal plants. For example, quercetin 3-glycosides were isolated from the leaves of *Solanum nigrum* (Nawwar et al., 1989). The sugars attached to the aglycone are rhamnose, galactose and glucose. In plant-derived food substances, flavonol glycosides have been isolated and characterised in tomato puree extract by using LC-ESI-MS (Mauri et al., 1999). NMR spectroscopy has also been used in the elucidation of phenolics from plant-derived food substances. For example, caffeoyl and their sugar derivatives have been isolated from *Echinacea pallida* and their structures characterised by this spectroscopic technique (Cheminat et al., 1988).

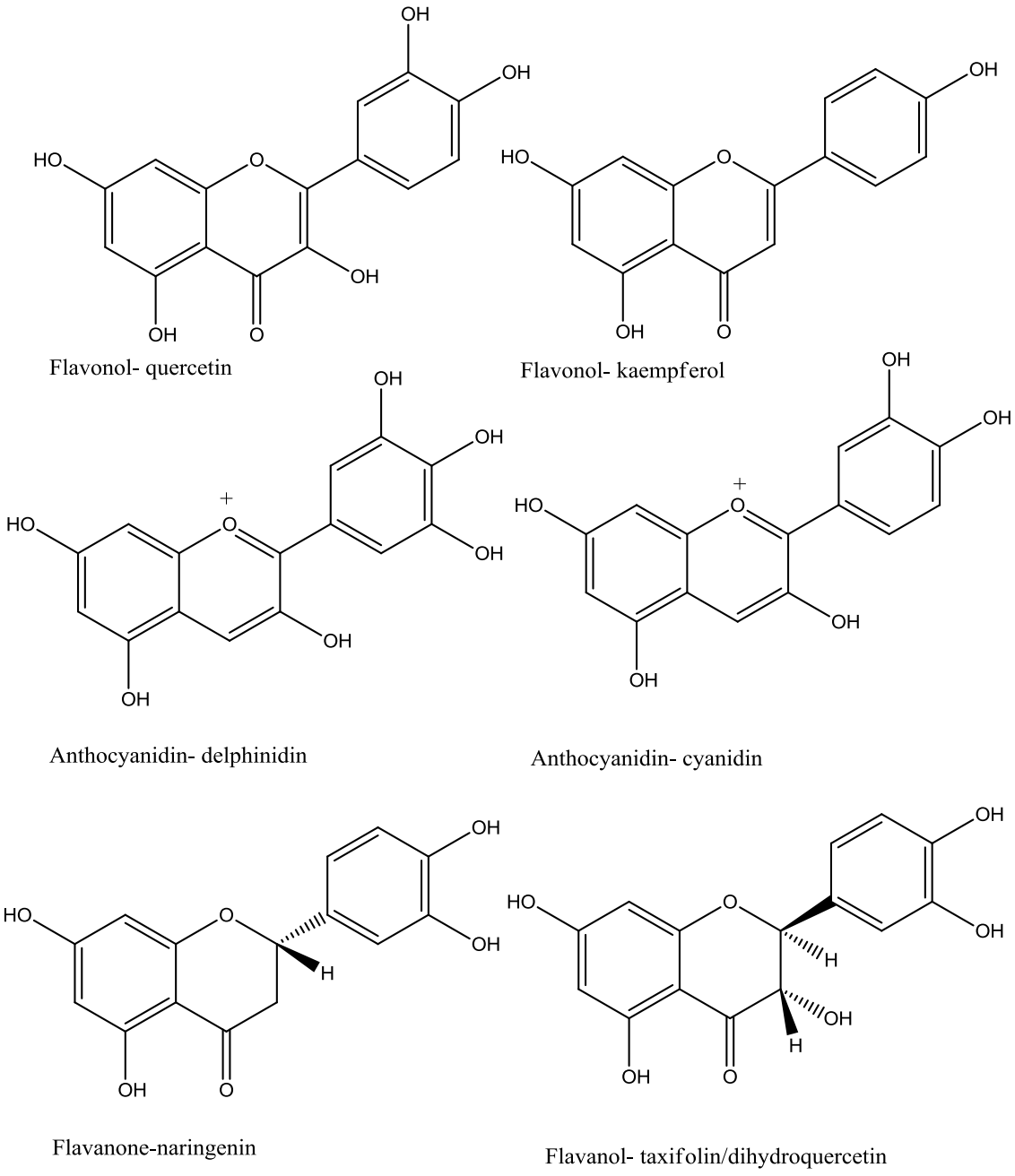

Figure 3–1 The main classes of flavonoids found in plants

Detailed investigation of the chemical composition of phenolics in forage plants in Botswana is unknown despite the purported nutritional and health properties of some of these plants. In other words, while *in vivo* and *in vitro* effects of CT from these forages in Botswana have been studied, their phenolic chemistry has not been studied in detail. In order to better understand the effects of these compounds upon ruminants, it is necessary to identify their chemical structures. The aim of the present study was to

characterise the chemical composition of flavonoids from Botswanan forage plants in order to develop a better understanding of their health benefits in ruminants. Smallholding farmers in Botswana have reported feeding plants such as *V. verrucosum*, *V. rotundifolium*, *T. oleifolius* and *G. flava* to cattle, sheep and goats temporarily as supplements, particularly during the dry season to meet their nutritional requirements and to improve their health (Madibela et al., 2000).

3.2. Materials and Methods

3.2.1. Characterisation of low molecular weight (LMW) phenolics from the step and linear LH-20 fractionation

The following fractions obtained from the step and linear gradient LH-20 fractionation (**Appendix 2-7, 2-8 and 2-9**) were characterized by ^{1}H and ^{13}C-NMR spectroscopy. The following step LH-20 fractions were semi-purified and thus were selected du were: OTD03403 (SF4-I, MeOH:H_2O 1:1) isolated from *T. oleifolius*, OTD03604 (SF4-II, MeOH:H_2O 1:1) fraction from *G. flava*, and OTD03804 (SF4-II, MeOH:H_2O 1:1) from *I. sinensis.* The selected linear gradient fractions that were characterised: OTD04204 (GF4, MeOH:H_2O 1:3), OTD04205 (GF5, MeOH:H_2O 1:1), OTD04206 (GF5, 100% MeOH) from *V. verrucosum*, OTD04405 (GF5, MeOH:H_2O 1:1), and OTD04406 (100% MeOH) from *T. oleifolius*. Only fraction OTD03403 (SF3-I, MeOH:H_2O 1:1) isolated from *T. oleifolius* had a better NMR spectra that could be interpreted.

3.2.2. Re-chromatography of the LH-20 fractions

3.2.2.1. Re-chromatography on Sephadex LH-20 of LMW phenolics from methanolic LH-20 fractions containingCT

Re-chromatography was performed on the following fractions: OTC02101 (SF1-I, MeOH:H_2O 1:1) and OTC02201 (SF1-II, MeOH:H_2O 1:1) from *V. rotundifolium.* A portion of *V. rotundifolium* CT extract was not adsorbed on Sephadex LH-20 but was eluted in the early aqueous methanol (Fr-1, 1:1) fraction. Thus, the following fractions containing CT were pooled and re-chromatographed: OTC02101 and OTC02201 from *V. rotundifolium* plant samples (2009) gave fraction OTE09201. Fractions OTD02901

and OTD03001 from *V. rotundifolium* (2010) plant samples were pooled to give OTE09202. These initial aqueous methanolic eluates from *V. rotundifolium*, which contained CT from RP-HPLC PDA, were subjected to re-chromatography on Sephadex LH-20 (XK 26/40) at 5 mL/min. An initial elution with water (100 mL) was followed by a wash with MeOH:H_2O (1:1, v/v; 200 mL) and lastly with acetone:water (7:3, v/v, 200 mL). The following fractions were obtained: OTE09501 (water, 696 mg, dark brown), OTE09502 (MeOH:H_2O 1:1, 289 mg, light brown) and OTE09503 (MeOH:H_2O 1:1, 30 mg, yellow) from the 2009 plant samples and OTE09701 (water, 999 mg, dark brown), OTE09702 (MeOH:H_2O 1:1, light brown, 280 mg), OTE09703 (MeOH:H_2O 1:1, 73 mg, yellow, OTE9704 (MeOH:H_2O 1:1, light brown, 16 mg) from the 2010 samples. Elution with acetone:water (7:3) could not be carried out since no brown band was adsorbed to the column after elution with water and methanol. All fractions were screened for the presence of CT and low Mw phenolics by using RP-HPLC PDA and TLC. No pure compounds with low molecular weight were obtained in this fractionation as evidenced from the HPLC chromatograms (not shown). The chromatographic conditions used in the original extracts were adopted (**Section 2.26).**

3.2.2.2. Re-chromatography on Sephadex LH-20 of linear gradient LH-20 fractions (water) containing CT and LMW phenolics

The gradient fractions from *T. oleifolius*, *V. verrucosum* and *G. flava* collected in 2009 were detected by RP-HPLC-PDA and showed the presence of condensed tannins (broad absorption peak at 280 nm) in their initial water fraction. The following fractions were re-chromatographed: OTD04201 (water) from *V. verrucosum*, OTD04401 (water) from *T. oleifolius* and OTD04601 (water) from *G. flava*. An initial elution with aqueous methanol (1:1, v/v 100 mL) was followed by a wash with water (200 mL) and lastly with aqueous acetone (3:7, v/v, 200 mL). Further purification of OTD04201 from *V. verrucosum* yielded OTE12201, OTE12202 and OTE12203, and the fractions were eluted with aqueous methanol (1:1, v/v). Further fractionation of OTD04401 isolated from *T. oleifolius* produced the following fractions: OTE11001 (water), OTE11002 (water) fractions. The fractionation of OTD04601 from *G. flava* yielded the following fractions: OTE11601 (water), OTE11602 (water). In addition, fraction OTE11605 (acetone:water 7:3) was purified from OTD04601 from *G. flava* and characterised. Fractions OTE12201, OTE11001 and OTE11601 were well purified, and

had the same HPLC-PDA profile and were characterised by ESI-MS and NMR. They were dissolved in d_4-methanol and run overnight by the 500 MHz Bruker NMR. But poor spectra with low signal to noise ratio were obtained and could not be interpreted. Fractions OTE11605 (acetone:water 7:3) were found to contain CT and were characterised in Chapter Two (**See section 2.5.2.3.1).** Chromatographic conditions used in the original extracts were adopted (**Section 2.26).**

3.2.2.3. Re-chromatography of methanolic fractions containing LMW by column chromatography (silica gel)

The following fractions were re-chromatographed and the purified isolates were characterised accordingly. The initial (first) methanolic fractions obtained from the step LH-20 fractionation were found to contain low molecular weight phenolics as evidenced from the HPLC chromatograms. Thus, the following fractions were purified by column chromatography (60 g of silica gel RP C-18): OTC02901 (SF1-I, MeOH:H_2O 1:1) from *V. verrucosum*, OTC03401 (SF1-I, MeOH:H_2O 1:1) from *T. oleifolius*, and OTC03601 (SF1-I, MeOH:H_2O 1:1) from *G. flava*. Each extract (1 g) was dissolved in MeOH:H_2O (10%) and loaded onto the column. Fractions were collected at a flow rate of 2.5 mL/min. The fractions (10-20 mL each) were collected in the test tubes and those with the same HPLC-PDA profile were pooled. Fraction 1 (test tube 1, 20 mL, yellow, 86.1 mg) was eluted with MeOH:H_2O (10%), and fraction 2 (test tube 2, 20 mL, trace) eluted with MeOH: H_2O (1:4), fraction 3 (pooled test tubes 3-18 eluted with 20% MeOH), fractions 4 (pooled test tubes 19-26, 30% MeOH:H_2O), Fraction 5 (test tubes 26-40, 50% MeOH). Fraction OTC02901 (MeOH:H_2O 1:1) from *V. verrucosum* was purified to yield the following: OTE13101, OTE13102, OTE13103, OTE13105, and OTE13105. The purification of fraction OTC03401 (MeOH:H_2O 1:1) from *T. oleifolius* yielded the following: OTE13801, OTE13802, OTE13803, OTE13804, and OTE13805. The purification of fraction OTC03701 (MeOH:H_2O 1:1) from *G. flava* yielded the following: OTE14601, OTE14602, OTE14603, OTE14604, and OTE13805. A pure fraction (OTE13801) from *T. oleifolius* was obtained by column chromatography and was characterised by HPLC, ESI-MS, NMR (^{1}H and ^{13}C-NMR). The fractions were dissolved in d_4-methanol and were run overnight by the 500 MHz Bruker NMR. But poor spectra with low signal to noise ratio were obtained and were difficult to interpret.

3.2.2.4. Re-chromatography of linear gradient LH-20 fractions from *V. verrucosum* using semi-preparative HPLC

Fraction OTD04205 (75% MeOH) and OTD04206 (100% MeOH) were re-suspended in methanol:water (1:3, v/v; 50 mg/mL) and loaded onto a preparative HPLC Phenomenex Jupiter RP C-18 column (250 x 10 mm i.d, 5 µm particle size) connected to a Waters HPLC system. The following mobile phase elution conditions were used: A= 0.1% formic acid in H_2O, B= 0.1% formic acid in methanol, gradient elution conditions: 0-10 min; 10% B, 10-60 min; 60% B, 60-65 min; 85% B, 65-70 min; 10% B, 70-75 min; 10% B. Detection was by UV absorbance at 255 and 355 nm. Flow rate of 2.5 mL/min was used. Fractions were combined from two HPLC runs and the methanol was removed by a rotary evaporator. The fractionation of OTD04205 (75% MeOH) from *V. verrucosum* yielded a pure fraction OTF05903, while the fractionation of OTD04206 (100% MeOH) from *V. verrucosum* gave a pure fraction OTF06003. Fractions were freeze-dried for removal of aqueous part of the fractions. The purified fractions were dissolved in methanol-d_4 or acetone-d_6 and run overnight by the 700 MHz Bruker NMR.

3.2.2.5. Phytochemical screening of the re-chromatographed fractions with HPLC-PDA and TLC

All re-chromatographed fractions were assessed for purity by a RP-HPLC-PDA. The chromatographic conditions used in the original extracts were adopted (**section 2.26).**

3.2.2.6. LC/ESI-MS/MS of Phenolics

The LC-MS/MS data were acquired on a Surveyor HPLC connected to a [-LTQ] linear ion trap mass spectrometer (Thermo Finnigan, San Jose, USA) equipped with an electrospray interface operated in positive ion mode. The negative ion mode was not used. The column used was a 150 mm x 2.1 mm, i.d., 5 µm, Phenomenex RP (C-18), injection volume of 10 µL, flow rate was 0.2 mL/min and the mobile phase and gradients were as above. The analysis was performed under the following settings: temperature of the heated capillary was set at 275 °C, spray voltage of +4.5 kV, and

normalised collision of 35%. The mass spectrometer was programmed to perform a full MS scan (*m/z* 100 to 20000) followed by three MS/MS scans on the most intense ions from the MS1 spectrum were run with the following dynamic exclusion settings: repeat count 2, repeat duration 0.5 min, exclusion duration 2.0 min. An aqueous acetonitrile solution (50:50, v/v) containing flavonoid fractions (100 µg/mL) was used in the analysis. ESI/MS/MS was conducted for the following samples: OTD03403 (SF1-II, MeOH:H_2O 1:1) from *T .oleifolius*, OTD04205 (GF5, MeOH:H_2O, 1:3) and OTD04206 (GF6, 100% MeOH) from *V. verrucosum*, OTD04405 (GF5, MeOH:H_2O, 1:3) and OTD04406 (GF6, 100% MeOH) from *T. oleifolius*, OTF05903 purified using semi-preparative HPLC from OTD04205, and OTF06003 purified from OTD04206 from *T. oleifolius.*

3.3. Results and Discussion

3.3.1. Characterisation of low molecular weight phenolics from LH-20 fractionation

Signal assignment and confirmation of the structure were made in accordance with published data (Foo et al., 2000, Markham, 1982, Nawwar et al., 1989). The compound was identified by ^{1}H NMR, ^{13}C-NMR and HPLC-ESI-MS and HPLC-ESI/MS/MS experiments. The isolated compound was found to be quercetin 3-O-glucosyl-(1→6)–glucoside.

The ^{1}H and ^{13}C-NMR spectral data are presented in **Table 3-1.** Quercetin 3-O-glucosyl-(1→6)–glucoside (also called quercetin-3-O-sophoroside) was characterised from fraction OTD03403 (MeOH:H_2O 1:1) from *T. oleifolius* obtained from a step LH-20 method. Freeze-dried powder (37 mg, yellow solid), UV λ_{max} MeOH nm 230, 250, 355 (**Figure 3-2**), HPLC R_t 31.97 min; ESI-MS/MS Ion trap: 626.99 $[M+H]^+$, 465 $[I+H]^+$, 303 $[A+H]^+$. ^{13}C-NMR (500 MHz, methanol-d_4) δ 17.0 (weak signal Rha), Glu 61.0 (C6‴), 73.6 (C5‴), 67.2 (C4‴), 76.1 (C3‴), 74.6 (C2‴), 103.6 (C1‴), Glu 68.3 (C6″), 71.8 (C4‴), 73.5 (C5″), 76.1 (C5″), 76.3 (C3″), 102.9 (C1″), Quercetin δ 93.5 (C8), 98.6 (C6), 104.1 (C10), 115.4 (C2′), 116.4 (C5′), 121.4 (C1′), 121.9 (C6′), 134.5 (C3), 144.4 (C3′), 148.1 (C4′), 157.0 (C9), 157.2 (C2), 161.5 (C5), 164.8 (C7), 178.0 (C4). ^{1}H NMR (500 MHz) 3.28-3.68 (m, sugars 7Hs), Glu 5.14 (d, *J* = 8.0 Hz H1″, 1H), 4.16 (d, *J* = 8.0 Hz H1‴, 1H), 6.21 (d, *J* = 2.0 Hz H6, 1H), 6.41 (d, *J* =2.0 Hz H8, 1H), 6.88 (1H, d, *J* = 8.5 Hz, H5′), 7.62 (d, *J* =2.2 Hz H2′, 1H), 7.65 (d, *J* = 8.5, 2.2 Hz, H6′,

1H), 7.93 (d, *J* = 2.2 Hz, H2′, 1H). The same structure was obtained from a linear gradient fraction OTD04404 (MeOH:H_2O 3:1) from *T. oleifolius*. These results were in complete agreement with those of Nawwar et al. (1989).

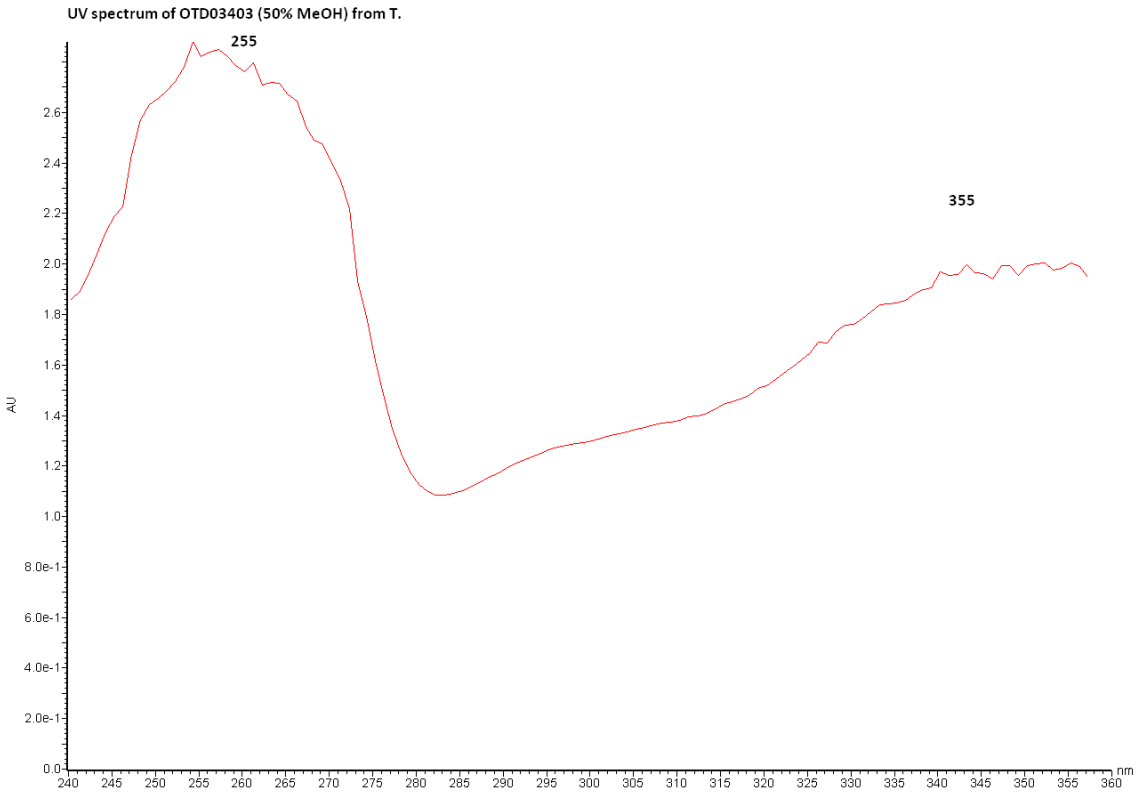

Figure 3–2 UV λ_{max} MeOH nm of quercetin 3-O-glucosyl-(1→6)–glucoside

The results showed the presence of quercetin with attached sugars at carbon 3. The spectral data indicated that the first pair of carbons had its protons resonating at δ H 6.21 (1H, d, *J* = 2.0 Hz) assigned to H8 protons coupled to C8 at δ 98.6, showing an AX coupling system. The other pair of protons was observed at δ 6.41 placed at H6 and assigned to δ 6.41 (d, *J* = 2.0 Hz) C6 (93.5). These pairs showed the aromatic proton spin system of a C5/C7 substituted A–ring of a flavonoid molecule with a *meta*–coupled protons pattern. The substitution pattern on A-rings was confirmed by the HMQC and COSY experiments. The proton and ^{13}C-NMR spectral data (ABX coupling system) indicated the presence of two sets of protons on B-rings in which the following signals were assigned: C2′ 7.93 (1H, d, *J* = 2.2 Hz, H2′) δC 116.4, C5′ 6.88 (1H, d, *J* = 8.5Hz, H5′) δC 115.4, δC6′ 7.62 (1H, dd, *J* = 8.5, 2.2 Hz, H6′) δC 121.9. In addition to the B-ring signals, a set of carbon signals for the oxygenated ring was 144.4 and 148.1 ppm for C3′and C4′. In the ^{13}C-NMR spectrum the shift at 134.5 ppm suggested an O-

glycosidic substitution at C-3, showing an upfield shift for a substituted quercetin. A downfield shift of about 10.2 ppm from 146.9 ppm of unsubstituted quercetin was obtained at C2 at 157.2. The carbon resonances confirmed the presence of two glucosyl units and one rhamnosyl unit. However, a weak proton ^{1}H-NMR spectrum of this compound did not give 3H doublet as expected for the presence of a rhamnose unit. The ^{13}C-NMR spectrum also showed a weak methyl signal at 17 ppm and was assigned to C6 of rhamnose unit. However, the rhamnose unit signals might have been for rutin, which was also present as evidenced in the ESI-MS/MS data. In a ^{1}H-^{13}C NMR HMQC spectrum, correlations of two anomeric sugar signals at 103.6 (δ H1 4.16) and 102.9 (δ H1 5.14) and their coupling constants (J_{H1-H2} = 8.0 Hz), were assigned to two C1 β-glucosyl moieties, respectively. In other words, chemical shifts, multiplicity and coupling constants helped in assigning of signals and linkages. A COSY ^{1}H-^{1}H spectrum of this compound showed a correlation between the glucose anomeric proton signal at δ 5.14 (H1) and a signal at δ 3.84 ppm (H2). Furthermore, the second glucose correlations of the anomeric proton signal at δ 4.16 (H1) and a signal at δ 3.06 ppm (H2) were observed. A carbonyl carbon signal was observed at 179 ppm and was assigned to the C4 carbon in the quercetin moiety. The ^{13}C-NMR spectrum of OTD03403 (MeOH:H_2O 1:1) was similar to a linear gradient fraction OTD04404 (MeOH:H_2O 1:1) isolated from *T. oleifolius*. The C6 signals at δ 61.0 and 68.3 ppm showed the presence of a terminal and extender glucose, respectively. Thus, it was concluded that the isolated compound was quercetin 3-O-glucosyl-(1->6)–glucoside (**Figure 3-3**) and it contained trace amounts of quercetin 3-O-rhamnosyl-(1->6)–glucoside (rutin) (**Figure 3-4**).

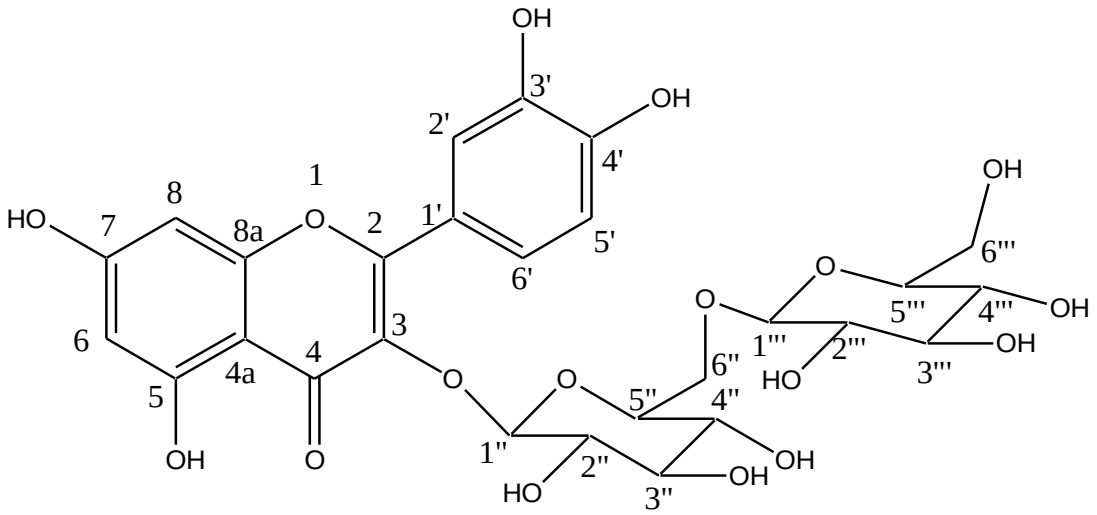

Figure 3–3 The structure of quercetin 3-O-glucosyl-(1→6)–glucoside

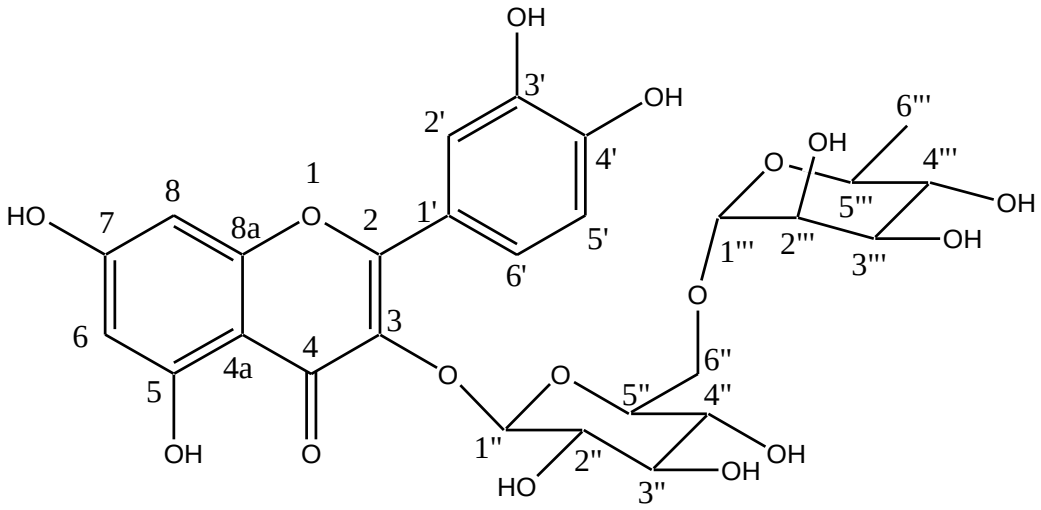

Figure 3–4 The structure of quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

This compound was confirmed by its *m/z* ratio using ESI/MS/MS at 627 (100%). This is the first report on the isolation and characterisation of these compounds from this plant. For further confirmation of the structure, the anomeric positions and interglycosidic linkages of the sugars to the quercetin moiety should be carried out by acid hydrolysis reaction and analysis of the products with HPLC and comparison made with authentic standards. The ^{1}H and ^{13}C-NMR spectra are shown in **Figure 3-5** and **Figure 3-6**, and these data were confirmed by the COSY and HMQC experiments (**Figure 3-7 and 3-8**).

Table 3-1 ^{1}H and ^{13}C-NMR (500 MHz) chemical shifts of quercetin-3-O-β-glucosyl-glucoside in methanol-d_4 and connectivity was made using COSY and HMQC experiments

Position C	δH	Multiplicity	δC	Literature values δC Olea et al. 1997 and Markham et al. 1992	δC Foo et al. 2000
2			157.2	156.1	156.2
3			134.5	133.3	133.5
4			178.0	177.4	177.5
5			161.5	161.2	161.3
6	6.21	1H, d, *J* = 2.0 Hz	98.6	97.7	98.8
7			164.8	163.9	164.6
8	6.41	1H, d, J = 2.0 Hz	93.5	94.0	93.6
9 (8a)			157.0	156.4	156.4
10 (4a)			104.1	103.0	103.8
1'			121.4	121.1	121.1
2'	7.93	1H, d, *J* = 2.2 Hz	115.4	115.3	115.2
3'			144.4	144.8	144.9
4'			148.1	148.5	148.6
5'	6.88	1H, d, *J* = 8.5Hz	116.4	116.2	116
6'	7.62	1H, dd, *J* = 8.5, 2.2 Hz	121.9	121.6	122
Glu1"	5.14	1H, d, *J* = 8.0 Hz	102.9	-	101.9
2"	3.06		74.6	-	71.3
3"	3.02		76.3	-	73.3
4"	3.91		71.8	-	68
5"	3.57		73.5	-	75.9
6"			68.3	-	60.2
Glu2"'	4.16	1H, d, *J* = 8.0 Hz	103.6	103.0	
2"'	3.06		74.6	74.9	
3"'	3.02		76.1	76.5	
4"'	3.91		67.2	69.8	
5"'			73.6	76.5	
6"'	3.81		61.0	60.8	

(Foo et al., 2000, Markham, 1982, Olea et al., 1997)

On acid hydrolysis with 2N HCl in MeOH (100%), quercetin-3-O-β-rhamnosyl-glucoside gave the compound, quercetin, by direct comparison with UV λ_{max} (255, 265sh, and 355 nm) and ^{1}H NMR spectra (500 MHz): 6.28 (d, *J* = 1.8 Hz, H6), 6.55 (d,

J = 1.6 Hz, H8), 7.00 (d, *J* = 8.5 Hz, H5′), 7.63 (d, *J* =2.2 Hz H2′, 1H), 7.70 (dd, *J*= 2.0 Hz, *J* = 10.0 Hz, H6″, 1H), 7.84 (d, *J* = 1.8 Hz, 1H, H2′). However, information on the chemical composition of the sugars could not be obtained. The presence of the aglycones can also be confirmed by acid hydrolysis and comparison to retention of authentic standards. Furthermore, the carbons involved in the linkage could be established by derivatisation (methylation and diazomethane) and with subsequent analysis of the adducts by NOE and HMBC and MS/MS experiments.

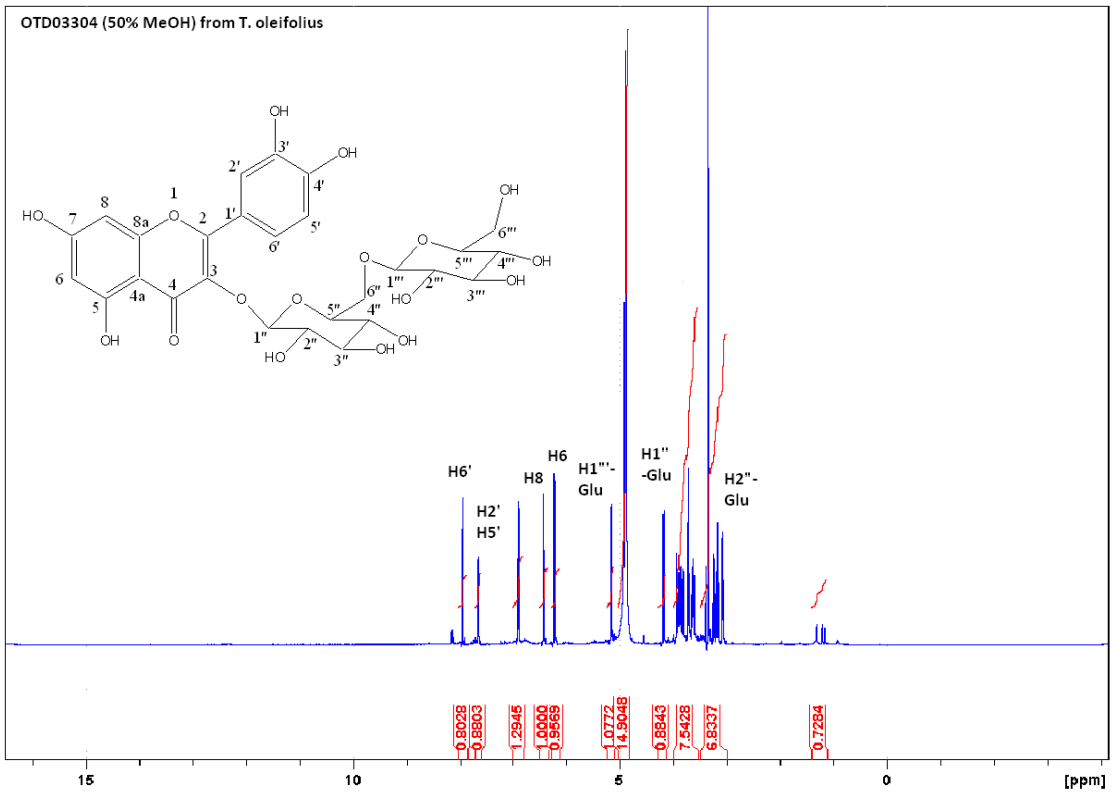

Figure 3–5 ^{1}H NMR spectrum of the isolated compound: quercetin 3-O-glucosyl-(1→6)–glucoside

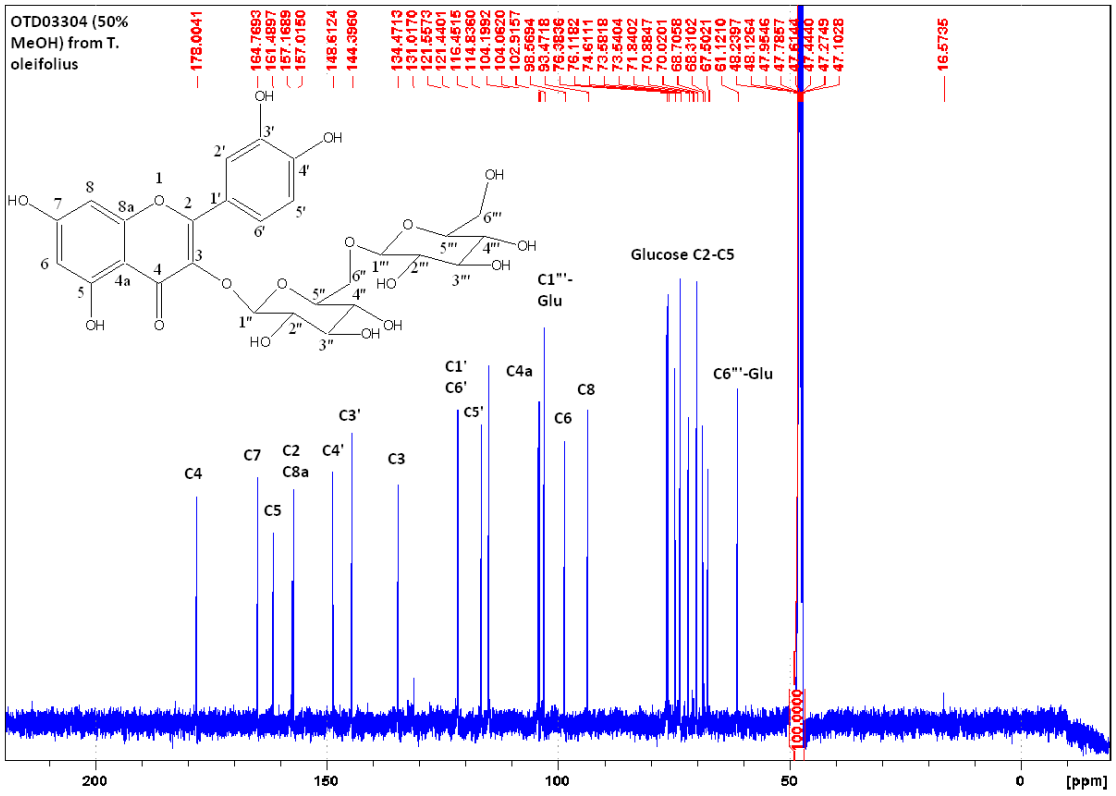

Figure 3–6 ^{13}C-NMR of OTD03403 (MeOH:H_2O 1:1) containing quercetin 3-O-glucosyl-(1→6)–glucoside

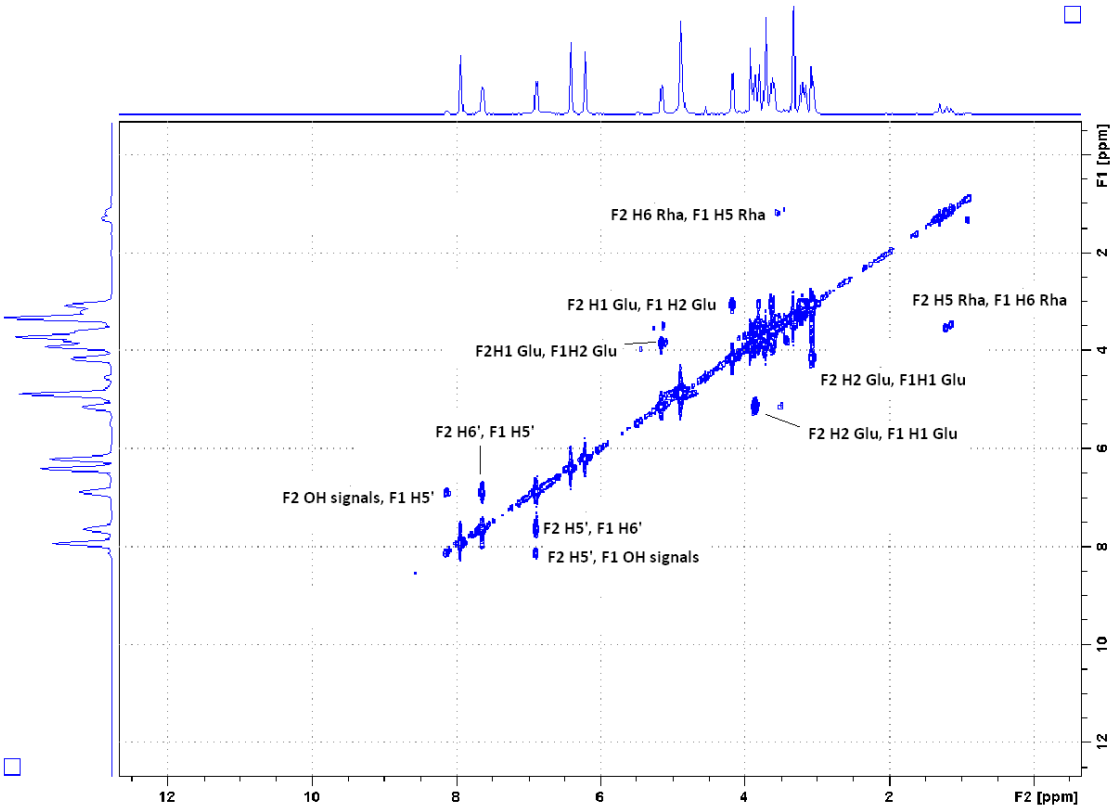

Figure 3–7 ^{1}H-^{1}H COSY of OTD03403 (MeOH:H_2O 1:1) with labelled cross peaks

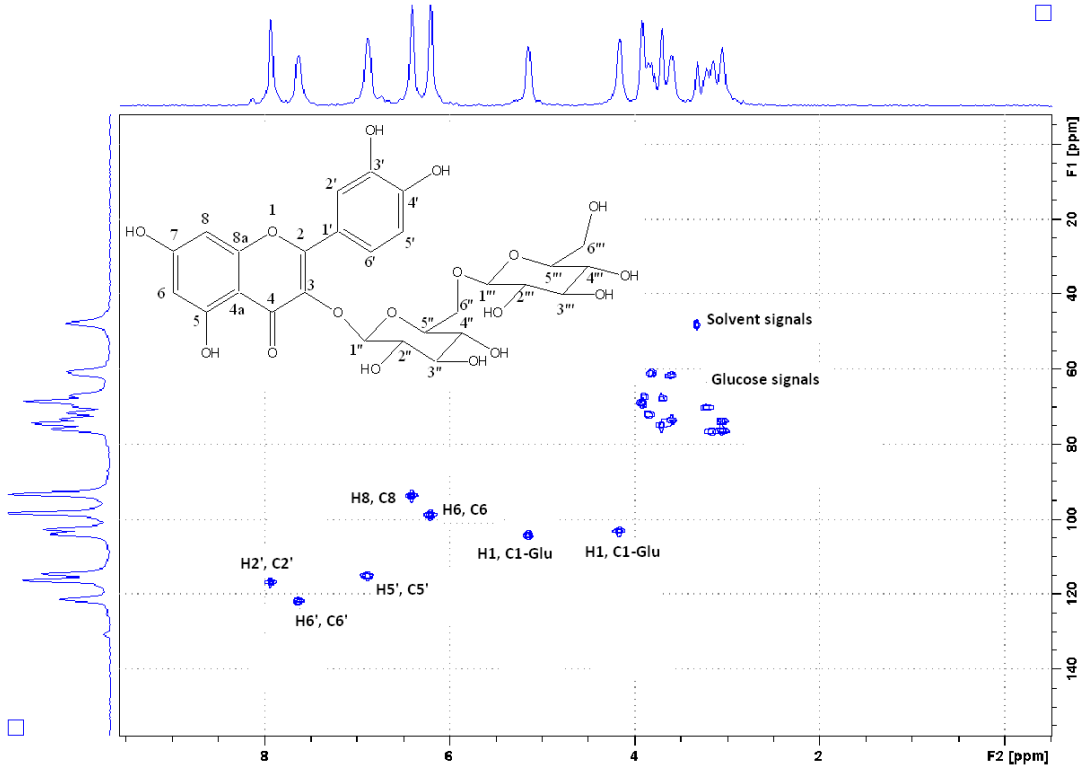

Figure 3–8 ^{1}H-^{13}C HMQC of OTD03403 (MeOH:H_2O 1:1) with labelled peaks

3.3.2. Re-chromatography of step LH-20 fractions containing LMW phenolics by column chromatography (silica gel)

The retention time of OTE13801 (MeOH:H_2O 1:9) was 12.30 min. This purified fraction was obtained from OTC03401 (SF1-I, MeOH:H_2O 1:1) from *T. oleifolius* by column chromatography. However, many signals were obtained and the spectrum could not be interpreted. ^{13}C-NMR chemical shift (δ) of fraction OTE13801 (MeOH:H_2O, 10%) and OTE15001 (MeOH:H_2O, 1:9) purified from OTC03401 (MeOH:H_2O 1:1) from *T. oleifolius*., chemical shift (δ): 177.9, 176.5, 158.7, 115.7, 99.4, 98.0, 84.7, 79.7, 77.9, 77.9, 76.2, 74.5, 74.4, 74.1, 73.8, 73.6, 73.2, 72.5, 72.4, 71.9, 71.7, 71.6, 71.2, 69.2, 69.2, 64.6, 64.3, 64.1, 63.1, 62.7, 60.9, 55.4, 49.7, 49.6, 49.5, 49.4, 49.4, 49.3, 49.2, 49.0, 48.9, 48.8, 47.2, 41.8, 40.3, 39.8, 39.3, 34.7, 30.5, 29.8, 29.3, 26.7, 25.7 and 25.2. The spectrum was predominant of sugar signals. The signals were observed between 60 and 100 ppm and these results were in agreement with the NMR data of a carbohydrate fraction from (Behrens et al., 2003). The signals at 177.8 and 176.5 ppm could be attributed to carbonyl groups. The signal at 158.7 ppm indicated the presence of an oxygenated A-ring of a flavan-3-ol molecule. Most of the fractions isolated

contained sugars, so they should be derivatised (methylation and acetylation) and purified by preparative TLC or HPLC to confirm the chemical structures. However, the yield of the fractions was not sufficient to carry out this task.

3.3.3. Re-chromatography of linear gradient LH-20 fractions using an open column chromatography

Fractions OTE12201, OTE11001 and OTE11601 were obtained and had the same HPLC-PDA profile and were characterised by ESI-MS and NMR. None of the fractions ionised with ESI-MS. The fractions were also dissolved in methanol-d_4 and were run overnight using 500 MHz Bruker NMR. But the spectra with weak signals were obtained. Fractions OTE11001 (water) from *T. oleifolius* and OTE11601 (water) from *G. flava* were isolated by column chromatography and characterised, but the spectra were complex and difficult to interpret. However, the signals between 60 and 90 ppm suggested the presence of sugars (**spectra not shown**).

3.3.4. Re-chromatography of a linear gradient LH-20 fraction (OTD04205) from *V. verrucosum* by semi-preparative HPLC

Fraction OTD04205 (75% MeOH) from *V. verrucosum* was purified to give OTF05903 which contained quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside. The ^{1}H NMR and ^{13}C-NMR spectral data are shown in **Table 3-2.** Freeze-dried light brown powder (20 mg), UV λ_{max} MeOH nm 230 (**Figure 3-9**), 255, 355; HPLC R_t 32.44 min; ESI-MS/MS m/z 742.96 [M+H]+, 610.99 [I+H]+, 464.97 [I+H]+ and 303.16 [A+H]+. The identity of the structure was confirmed by ESI/MS/MS at *m/z* 742, except that the apiose moiety was in the terminal unit rather than in the extender unit as revealed in the NMR data**.** The ^{13}C-NMR spectral characteristics were similar to those of rutin (Wu et al., 2007), except for the presence of the additional pentose signals.

Figure 3–9 UV λ_{max} MeOH (nm) of OTF05903 containing quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

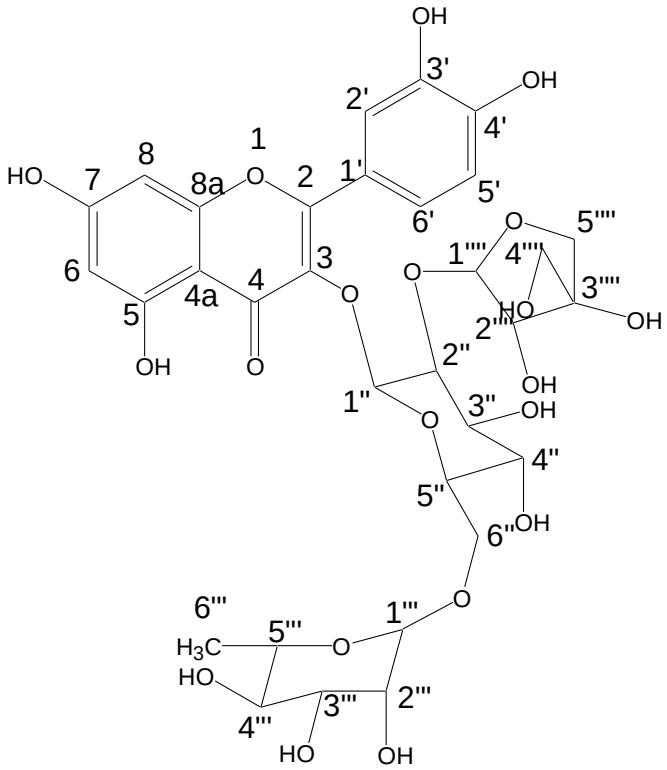

Figure 3–10 The structure of quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

The ^{1}H NMR spectrum of the aglycone-quercetin: 6.22 (d, *J* = 2.0 Hz H6, 1H), 6.45 (d, *J* = 2.0 Hz, H8), 6.93 (d, *J* = 8.5 Hz, H5′) 7.61 (dd, *J* = 2.2, 8.5 Hz, H6′, 1H), 7.64 (d, *J* = 1.9 Hz H2′, 1H). ^{1}H NMR of the sugars: glucose moiety 5.28 (d, *J* = 7.0 Hz 1-H), 5.41 (d, *J* = 7.5 Hz, 1-H apiose), 4.46 (broad s, 1-H rhamnosyl), 3.22-3.91 (m, sugar protons superimposed with hydroxyl protons). The data of the above structure (**Figure 3-10**) were confirmed by the correlated spectroscopy (COSY) and heteronuclear multiple quantum correlation (HMQC) experiments (**Figure 3-11 to 3-16**).

The results showed that the sugars were attached to quercetin at carbon 3. They are also in complete agreement with the signals for a quercetin and sugar moieties from the literature (Rastrelli et al., 1995, Wu et al., 2007). A set of carbon signals for the oxygenated ring resonated at 144.4 and 148.6 ppm for C3′and C4′. In the ^{13}C-NMR spectrum the peak at 134.5 ppm indicated an O-glycosidic substitution at C-3. In addition, a carbonyl signal C4 (δ 178.0 ppm) and C3 (δ157.6) were observed in the ^{13}C-NMR spectra, supporting an O-glycosidic substitution at C-3. The carbon signals confirmed the presence of a glucosyl, an apiose and a rhamnosyl unit. The ^{1}H NMR spectrum of this compound gave a 3H doublet (1.01, δ 3H, d, *J* = 6.0 Hz, Me-Rha H6) as expected for a rhamnose unit. The ^{13}C-NMR spectrum showed a methyl signal at 16.7 ppm, which was assigned to C6 of a rhamnose unit. The anomeric carbon of the rhamnose appeared as a broad singlet (δ 4.46, 1-H rhamnosyl overlapping with hydroxyl protons**)** in accordance with an alpha (α) configuration. A COSY ^{1}H-^{1}H spectrum of the isolated compound showed correlations between the anomeric proton signal at δ 3.41 (H5) and a signal at δ 1.01 (*J* = 6 Hz, H6). A COSY ^{1}H-^{1}H and ^{1}H-^{13}C-NMR HMQC and heteronuclear single quantum correlation (HSQC) spectrum showed correlations of the following carbons and hydrogens of an apiose sugar unit at position 1 (δ C1 109.3 H1, 5.41, 1.7 Hz) and 2 (δ C2 76.5 H2, 4.08, 1.7 Hz). A carbon signal resonating at 64.3 was assigned to C5 of the apiose unit, and this peak correlated with H5 at δ 3.70. Glucose C1 signal resonated at 102.9 (H1 at 5.28 ppm) and had a coupling constant of 7 Hz, suggesting a β-glucosyl proton unit. Glucose C6 resonated at δ 68.4 ppm suggesting that it was an extender unit bonded to a rhamnose (1→6) and an apiose (1→2) unit, with C5 resonating at δ 64.3. On acid hydrolysis, quercetin 3-O-α-rhamnosyl (1→2)-apioside-β-glucoside yielded the compound, quercetin, by direct comparison with UV and ^{1}H NMR spectra. Total correlated spectroscopy (TOCSY) experiments confirmed the presence of glucose and apiose sugars. The presence of CH,

CH_2 and CH_3 in the quercetin and sugar moieties was detected by a distortionless enhanced polarisation transfer (DEPT) experiment using a 135° pulse (**Figure 3-15)**. The DEPT spectrum indicated signals at δ 74.1 and 64.6 and the absence of signal at δ 80.1 with values for C-4, C-5, and C-3 of a β-apiosyl unit. For future studies, the interglycosidic linkages need to be confirmed by the nuclear Overhauser effect (NOE) and HMBC experiments, although these were confirmed in the literature. This compound is also reported in tomatoes (Slimestad et al., 2008), and we are not aware of any isolation of this compound from a forage plant.

Table 3-2 ^{1}H and ^{13}C-NMR (700 MHz) data for quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside in methanol d_4. The data were confirmed with HSQC, HMQC, H2bc, TOCSY and DEPT experiments and published work by Rastrelli et al. 1995.

Position Carbon	δH NMR	HSQC δH	Multiplicity from COSY and TOCSY	δ Carbon	DEPT (700 MHz)	Literature value δC Rastrelli et al. 1995
2				157.6[a]		158.9
3				134.5		132.2
4				178.0		179.3
5				161.1		163.1
6	6.22	6.21	1H, d, *J* = 2.0 Hz	99.9	CH (99.8[a])	99.8
7				164.0		165.7
8	6.45	6.48	1H, d, *J* = 2.0 Hz	94.0	CH (94.3)	94.8
9 (8a)				157.0[a]		158.4
10 (4a)				104.5		105.8
1'				121.4[b]		123.1
2'	7.64	7.66	1H, d, *J* = 1.9 Hz	116.4[c]	CH (115.6)	116.2
3'				144.4		145.8
4'				148.6		149.5
5'	6.93	6.95	1H, d, *J* = 8.5Hz	115.4[c]	CH (115.6)	116.8
6'	7.61	7.63	1H, d, *J* = 2.0, 8.5 Hz	121.9[b]	CH (122.6)	123.5
1"	5.28	5.29	1H, d, *J* = 7.0 Hz, Glu	102.9	CH (99.9[a])	102.2
2"	3.60		1H, d, *J* = 10 Hz	75.2	CH(75.2[b])	75.5
3"	3.57		1H, m	79.6	CH (78.2[e])	78.6
4"	3.34		1H, m	72.1	CH (76.4[b])	71.8
5"	3.29		1H,m	78.2	CH (78.2[e])	78.1
6"	3.34		1H, m	68.4	CH_2 (67.0[c])	68.3
1"'	4.46	4.51	Broad s Rhamnose	101.2	CH (100.9)	100.9
2"'				70.4	CH (70.4[d])	72.2
3"'	3.60			70.6	CH (70.7[d])	72.3
4"'	3.47			72.1	CH (72.1[d])	73.9
5"'	3.35		m	69.8	CH (69.8[c])	69.7
6"'	1.01		3H,d, *J* = 6 Hz	16.7	CH_3 (17.1)	17.9
1””	5.41	5.42	1H, d, *J* = 2.0 Hz, Apiose	109.3	CH (109.3)	110.5
2””	4.07	4.08	1H, d, *J* = 1.7 Hz	76.5	CH (76.7[b])	77.0
3””				79.9	CH (78.2[e])	80.9
4””	3.70	3.50	1H, d, *J* = 9.4 Hz	74.0	CH_2 (74.1[b])	75.4
5””	3.70	3.50	1H, d, *J* = 9.4 Hz	64.3	CH_2 (64.6)	66.7

Assignments with the same letter may be interchanged in each column.

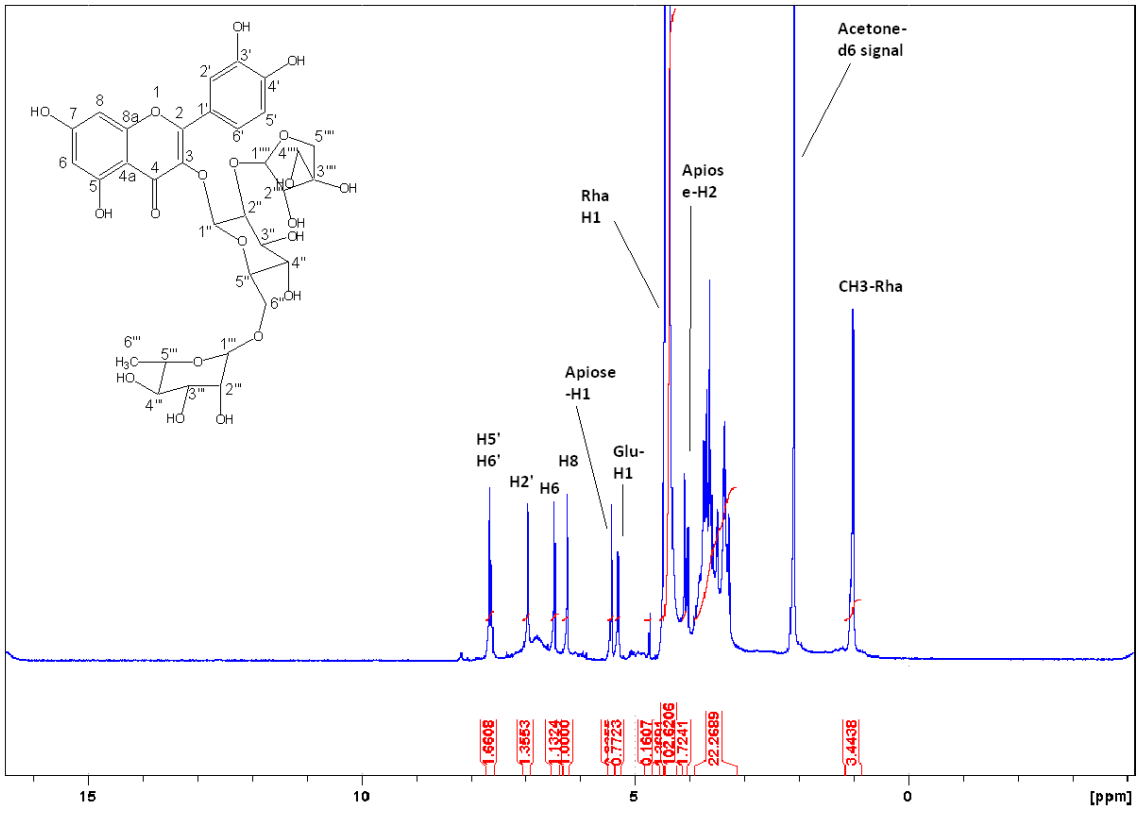

Figure 3–11 ^{1}H NMR spectrum of the isolated compound: quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

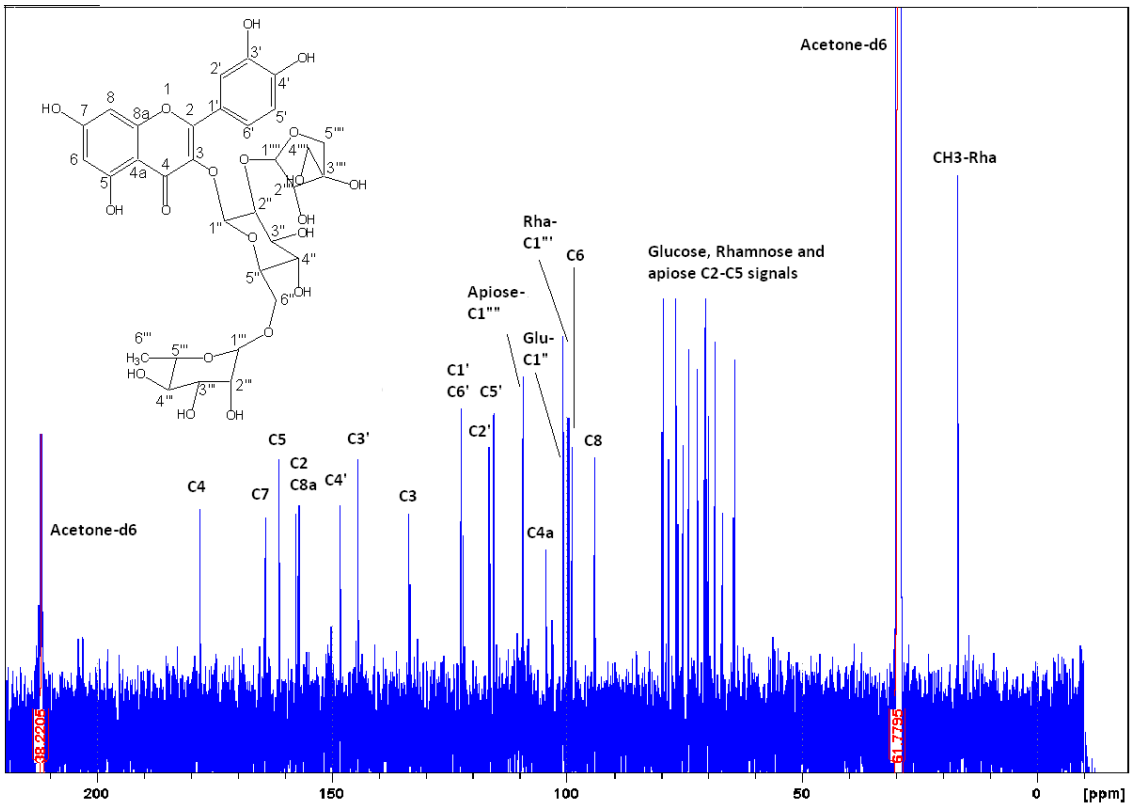

Figure 3–12 ^{13}C-NMR of OTD5903 (MeOH:H_2O 1:1) containing quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

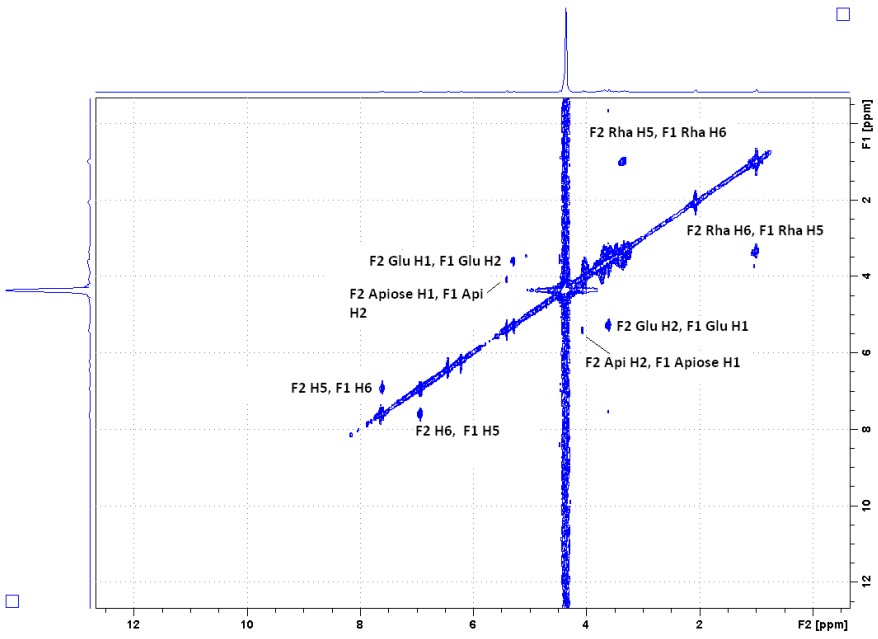

Figure 3–13 ^{1}H-^{1}H COSY of OTF05903 (MeOH:H_2O 1:1) with labelled cross peaks for quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

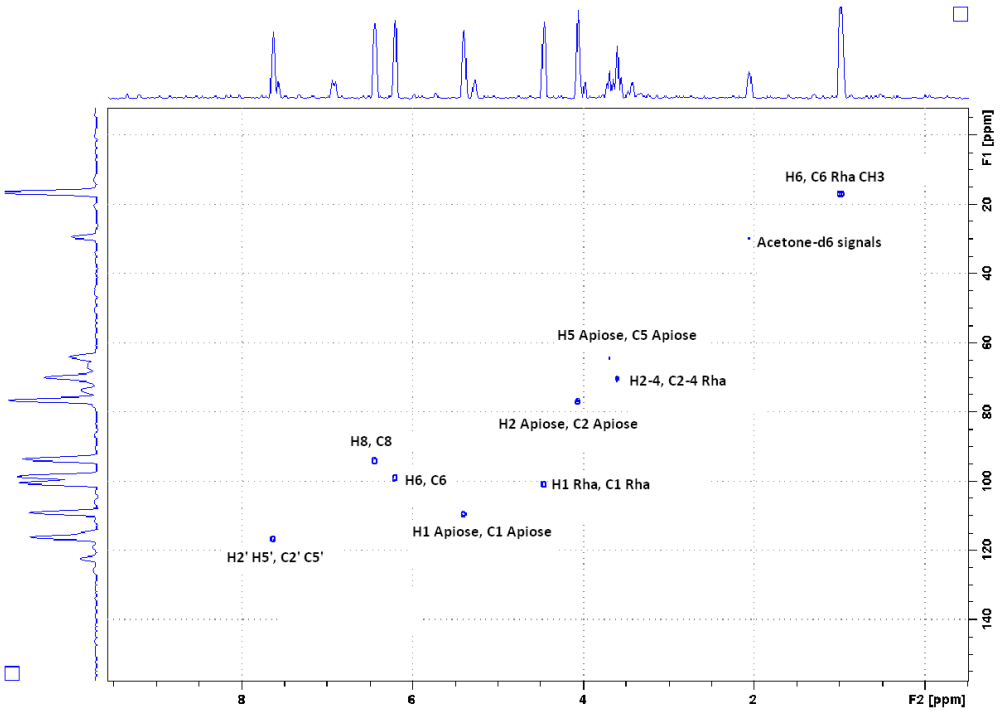

Figure 3–14 ^{1}H-^{13}C HMQC of OTF05903 (MeOH:H_2O 1:1) with labelled peaks for quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

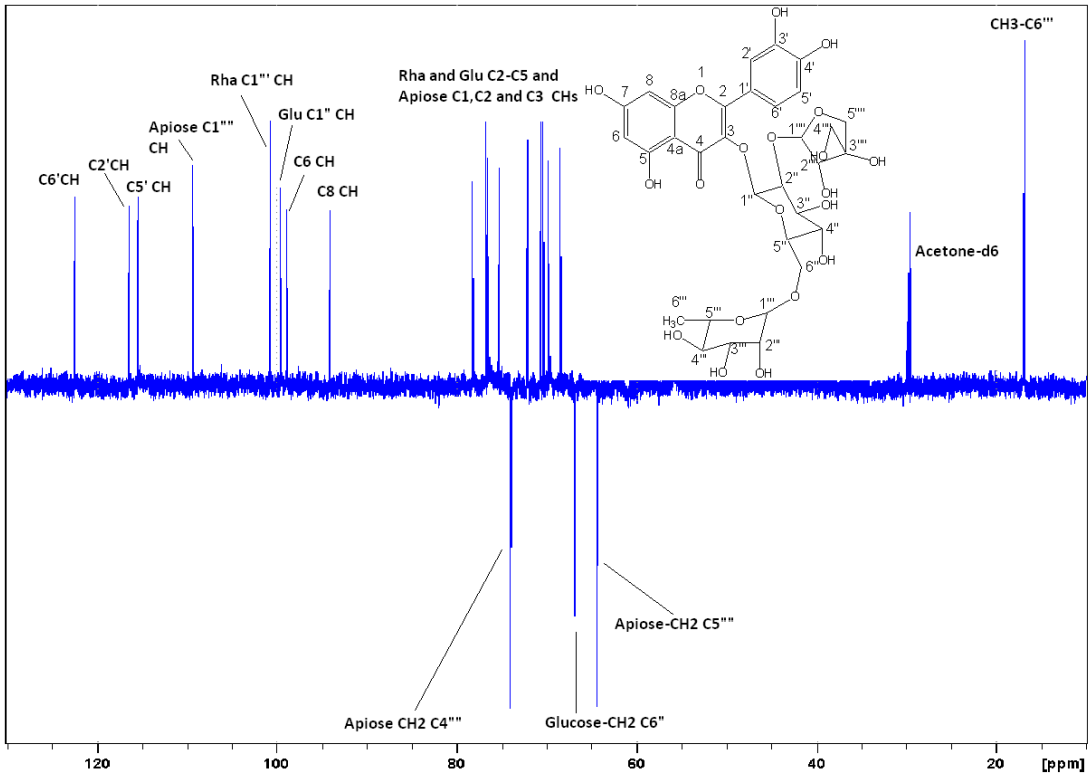

Figure 3–15 DEPT spectrum of quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

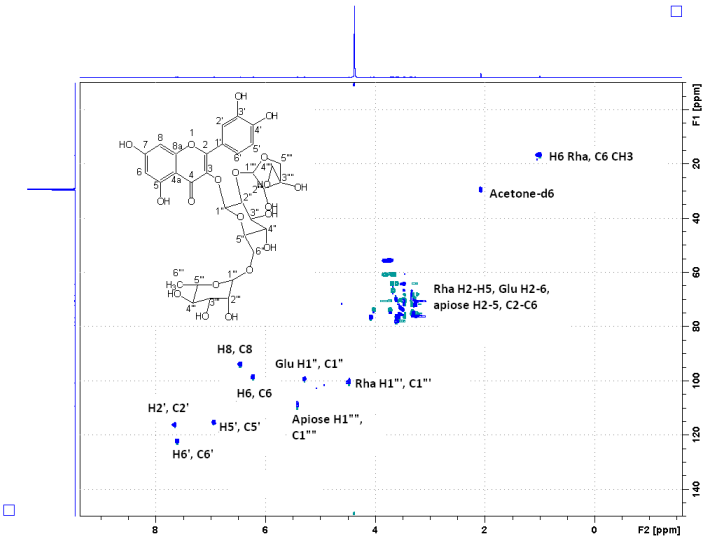

Figure 3–16 HSQC spectrum of quercetin 3-O-β-D-(1→2)-apiosyl-(1→6)-α-rhamnosyl-β-D-glucoside

3.3.5. Re-chromatography of a linear gradient LH-20 fraction (OTD04206) from *V. verrucosum* by semi-preparative HPLC

Quercetin-3-O-β-rhamnosyl-glucoside (rutin) was purified using a semi-preparative HPLC from fraction OTD04206 (75% MeOH) from *V. verrucosum* to give a semi-purified fraction OTF06003 (**Figure 3-17**). This fraction was found to be a mixture of a quercetin derivative and dihydroquercetin (**Table 3-3**). The NMR spectral data showed the presence of a quercetin linked at position 3 to a β-rhamnosyl (1-6) and a β-glucosyl unit (**Table 3-4**). The data were confirmed with the ^{1}H-^{1}H COSY and ^{1}H-^{13}C-NMR HMQC, HSQC and DEPT experiments (**Figure 3-18 to 3-22**). UV data λ_{max} nm: 230, 255, 355 and HPLC R_t: 34.35. ^{1}H NMR spectrum of the aglycone-quercetin: 6.25 (d, *J* = 2.0 Hz, H6), 6.27 (d, *J* = 2.0 Hz H6), 6.47 (d, *J* =2.0 Hz H8, 1H), 6.52 (d, *J* = 2.0 Hz, H8), 6.96 (d, 8.5 Hz, H5′, 1H), 7.67 (dd, *J*=2.2, 10 Hz, H6′, 1H), 7.11 (d, *J* = 2.2 Hz H2′, 1H), 7.13 (d, *J* = 2.2 Hz, H2′, 1H). ^{1}H NMR of the sugars: β-glucose moiety 5.14 (d, *J* = 8.0 Hz 1-H), 4.90 (d, *J* = 7.5 Hz 1-H β-glucose), 4.55 (d, *J* = 1.7 Hz, 1-H α-rhamnosyl), and 3.22-3.91 (m, sugar protons superimposed with hydroxyl protons).

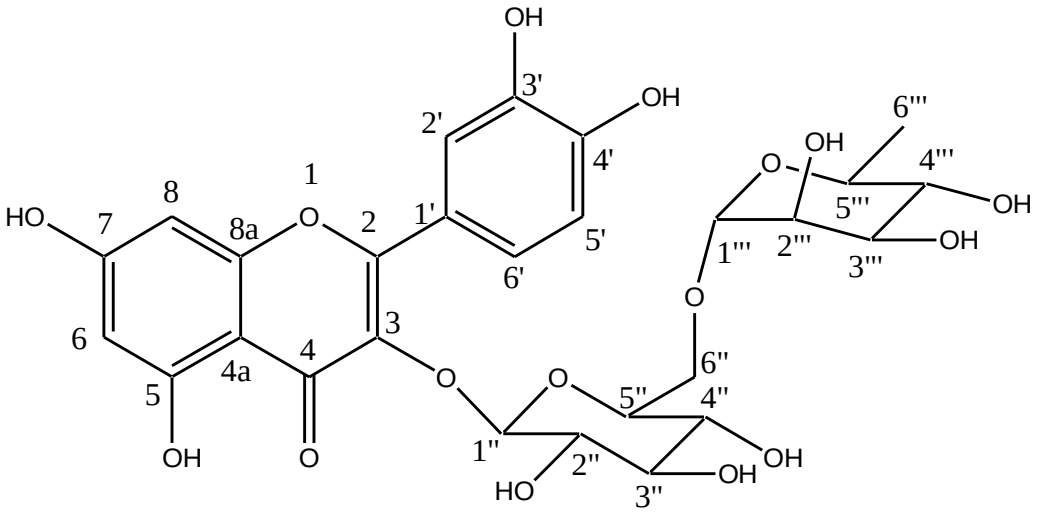

Figure 3–17 The structure of quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

The data were confirmed by the correlated spectroscopy (COSY) and heteronuclear multiple quantum correlation (HMQC), TOCSY, HSQC and DEPT experiments. The signals at δ 29.1 and 210 ppm were attributed to the acetone solvent. The peak at δ 48.7 ppm may be attributed to the methanol solvent, although it was not used in the experiment. The ^{1}H NMR spectrum indicated that OTF06003 was a triglycoside of quercetin on the basis of the chemical shift of theanomeric hydrogens in glucose and rhamnose (δ 5.14, 4.90 and 4.55). However, ESI/MS/MS data (*m/z* 611) showed that this fraction was a diglycoside. The third sugar, glucose, could be coming from a trace amount of quercetin-3-O-apiosyl glucoside, which was observed in analysis with ESI/MS/MS. The peaks at δ 5.14 and 4.90 were assigned to anomeric protons of two glucose units, with a coupling constant of ($J_{H1,H2}$ = 8.5 and 7.5 Hz) indicating a β configuration. The H-2 signals corresponding with H-1 signals were 3.74 and for two glucose moieties and for a rhamnose unit. The diequitorial-axial coupling constant $J_{H1,H2}$= 1.7 Hz suggested the anomeric H in rhamnose had an α configuration. These H signals correlated with the anomeric carbons of two glucose moieties and rhamnose (δ 101.0, 101.2 and 103.5), based on the HSQC spectral data. The presence of rhamnose was also confirmed by the signal that resonated at δ 1.10 and assigned to H6 (-CH_3), which correlated with δ 3.34 H5.

The ^{13}C-NMR confirmed the presence of glucose C6 resonating at δ 68.4 ppm suggesting that it was an extender unit bonded to a rhamnose (1→6) and a glucose (1→2) unit with C5 resonating at δ 61.0 suggesting that it was a terminal unit. These chemical shifts verified the presence of rutinosyl moiety [rhamnosyl-1,6–glucosyl].

Total correlated spectroscopy (TOCSY) experiments confirmed the presence of two glucose units and one rhamnose unit. The presence of CH, CH_2 and CH_3 in the quercetin and sugar moieties was detected by the distortionless enhanced polarisation transfer (DEPT) experiment using a 135° pulse. The DEPT spectrum indicated CH_2 (C6) signals at δ 67.1 and 61.1 for a glucose extender moiety and a terminal moiety. The CH_3 signal for H6 rhamnose was detected at δ 17.1.

The ^{1}H and ^{13}C-NMR data showed the presence of a dihyroquercetin compound (**Table 3-4 and Figure 3-23 to 3-26**). The C-2 signal at δ 83.1, C3 at δ 197 and C1' at δ 128.1, confirmed the presence of DHQ, with R_t 33.14, UV λ_{max} max 235, 285 and *m/z* 305.19. There was also an evidence of a signal at 5.06 (H1) and correlating with 4.67 (H2) for anomeric hydrogens in a COSY spectrum, which could be attributed to C2 and C3 of the dihydroquercetin.

Thus, it was concluded that the compounds identified from OTF06003 were quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin) and dihydroquercetin, and there was also a possibility of the presence of a dimer as evidenced in the pairs of signals in ^{1}H and ^{13}C-NMR, broad ^{1}H signals, as well as in ESI-MS/MS.

Table 3-3 ^{1}H and ^{13}C-NMR (700 MHz) data for compound quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

Position	TOCSY	1H NMR	δC	DEPT	Literature values	
Aglycone C	δH	multiplicity			δC Olea et al. 1997	Foo et al. 2000
2			157.9		156.6	156.2
			157.9			
3			134.0		133.0	133.5
			134.0			
4			178.0		177.2	177.5
			167.9			
5			161.5		161.4	161.3
			163.0			
6	6.25	d, *J* = 2.0 Hz	98.9	98.9CH	98.9	98.8
	6.27	d, *J* = 2.0 Hz	96.2	96.2CH		
7			163.7		164.7	164.6
			164.6			
8	6.47	d, *J* = 2.0 Hz	93.5	94.0CH	93.9	93.6
	6.52	d, *J* = 2.0 Hz	95.0	95.0CH		
9			157.1		156.9	156.4
			157.1			
10			104.3		104.1	103.8
			103.4			
1'			123.3		121.2	121.1
			122.4			
2'	7.11	d, *J* = 2.2 Hz	115.9	116.6CH	116.4	115.2
	7.13	d, *J* = 2.2 Hz	115.1	116.6CH		
3'			144.6		145.0	144.9
			145.1			
4'			148.5		148.8	148.6
			147.6			
5'	6.91	d, *J* = 8.5 Hz	115.1	115.1CH	115.5	116
	6.96	d, *J* = 8.5 Hz	115.9	115.1CH		
6'	7.67	dd, *J* = 2.2, 8.0 Hz	121.6	122.4CH	122.0	122
	7.74	dd, *J* = 2.2, 8.0 Hz	121.9	123.8CH		
βGlu1"	5.14	d, *J* = 8.0 Hz	101.0	101.0CH	98.9	101.9
2"	3.54	d, *J* = 11.0 Hz	74.6	76.8CH	74.2	71.3
3"			76.1	76.7CH	74.1	73.3
4"	3.85	m	73.6	76.1CH	70.8	68
5"			76.1	75.6CH	76.2	75.9
6"	3.71	s	68.3	67.0CH2	67.4	60.2
βGlu1"'	4.9	d, *J* = 7.5 Hz	101.2	102.3CH	101.1	
2"'	3.58	d, *J* = 2.4 Hz	71.8	74.3CH	70.8	
3"'	3.86	d, *J* = 2.4 Hz	73.6	73.4CH	70.6	
4"'	3.85		71.8	72.4CH	72.0	
5"'	3.92		68.7	71.9CH	68.6	
6"'			61.4	61.1CH2	68.0	
αRha 1"'	4.55	d, *J* = 1.7 Hz	103.9	103.5CH	101.1	
2"'	3.68		71.8	71.0CH	70.8	
3"'	3.58		71.8	69.9CH	70.6	
4"'	3.45		70.0	70.5CH	72.0	
5"'	3.34	d, *J* = 3.4 Hz	68.7	70.0CH	68.6	
6"'	1.07		17.0	17.5CH3	18.0	

Table 3-4 ^{1}H and ^{13}C-NMR (700 MHz) data of dihydroquercetin in OTF06003 in acetone-d_6

Position DHQ	δ H	1H NMR	δ C	DEPT	Literature data DHQ		
DHQ C		Multiplicity			13C and 1H NMR		
					Markham et al. 1982	Outrup et al. 1985 δ H	Outrup et al. 1985 δ 13C
2	5.06	1H, d, *J* = 11.4 Hz	83.4		83.1	5.00 (d, *J* = 11.3 Hz)	83.8
3	4.65	1H, d, *J* =11.5 Hz	71.9		71.7	4.61 (d, *J* = 11.3 Hz)	72.5
4			197.1		197.1		197.4
5			163.0		163.3		163.4
6	5.93	1H, d, *J* = 2.2 Hz	96.2	96.2CH	96.1	5.95 (d, *J* = 2.0 Hz)	96.4
7			164.6		166.8		164.3
8	5.97	1H, d, *J* = 2.2 Hz	95.0	95.0CH	95.1	5.99 (d, *J* = 2.0 Hz)	95.4
9 (8a)			167.9		162.5		167.2
10 (4a)			103.4		100.6		100.8
1'			128.6		128.1		129.0
2'	7.11	1H, d, *J* = 2.2 Hz	115.9	116.6CH	115.3	7.07 (d, *J* = 2.0 Hz)	115.2
3'			144.7		144.9		145.8
4'			145.8		145.7		145.1
5'	6.91	1H, d, *J* = 8.5 Hz	115.9	115.9CH	115.3	6.85 (d, *J* = 8.5 Hz)	115.2
6'	6.87	1H, d, *J* = 2.2, 10 Hz	121.9	122.4CH	119.2	6.90 (d, *J* = 8.5 Hz)	120.2

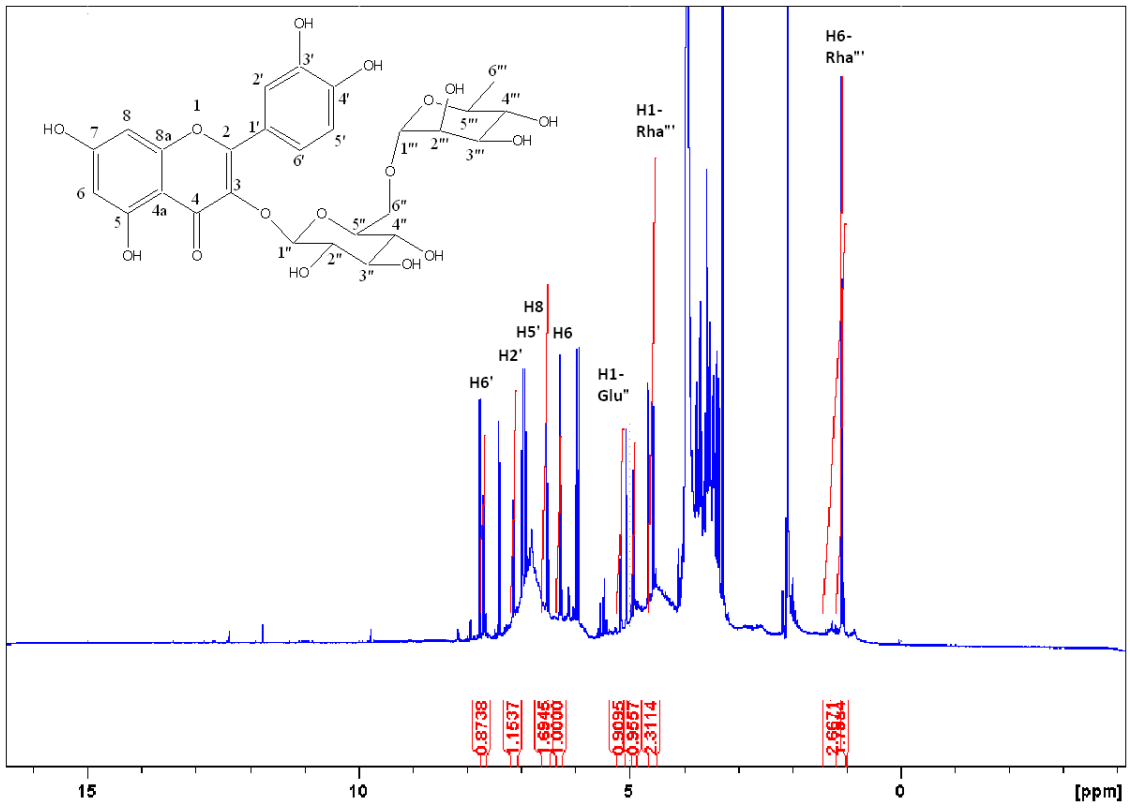

Figure 3–18 ^{1}H NMR spectrum of the isolated compound: quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

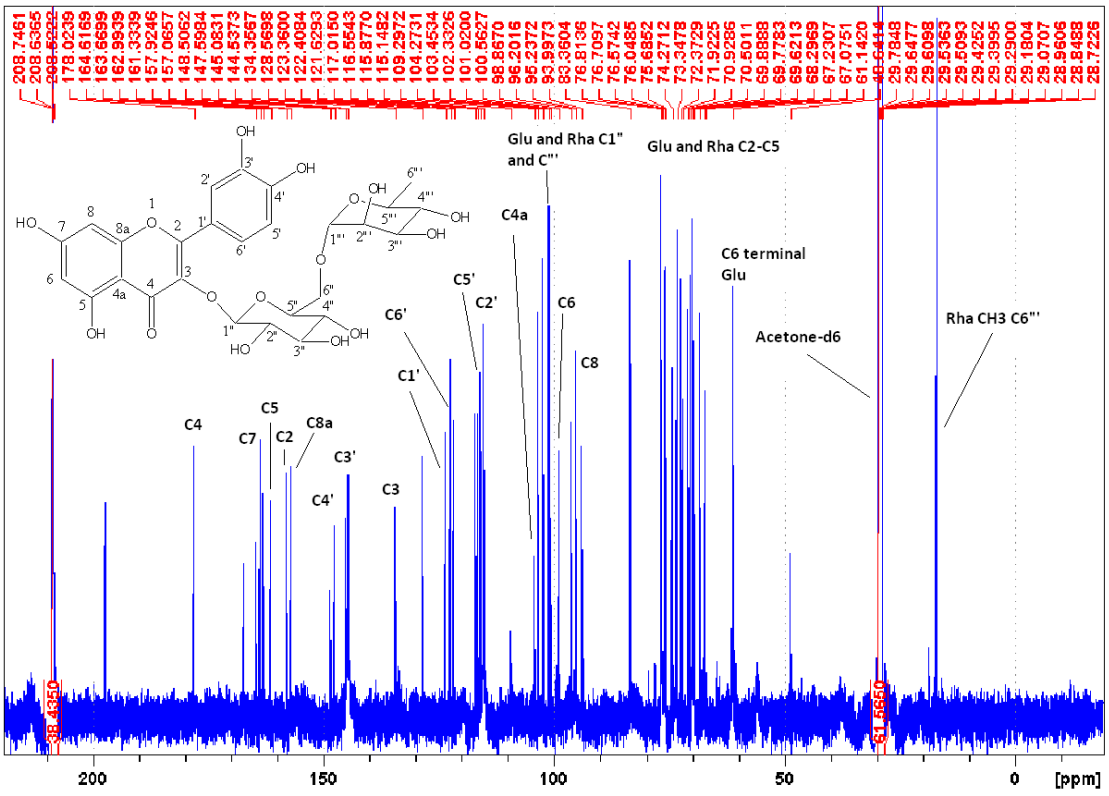

Figure 3–19 ^{13}C-NMR of OTD06003 (MeOH:H_2O 1:1) containing quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

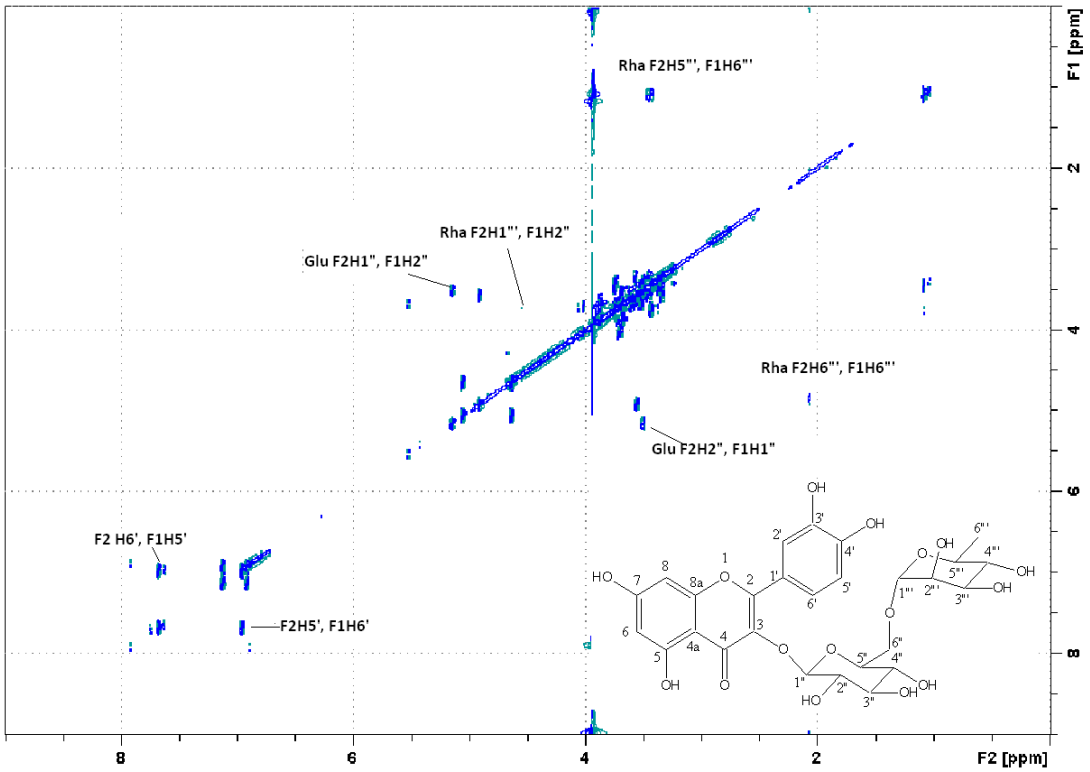

Figure 3–20 ^{1}H-^{1}H COSY of OTD06003 (MeOH:H_2O 1:1) containing quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

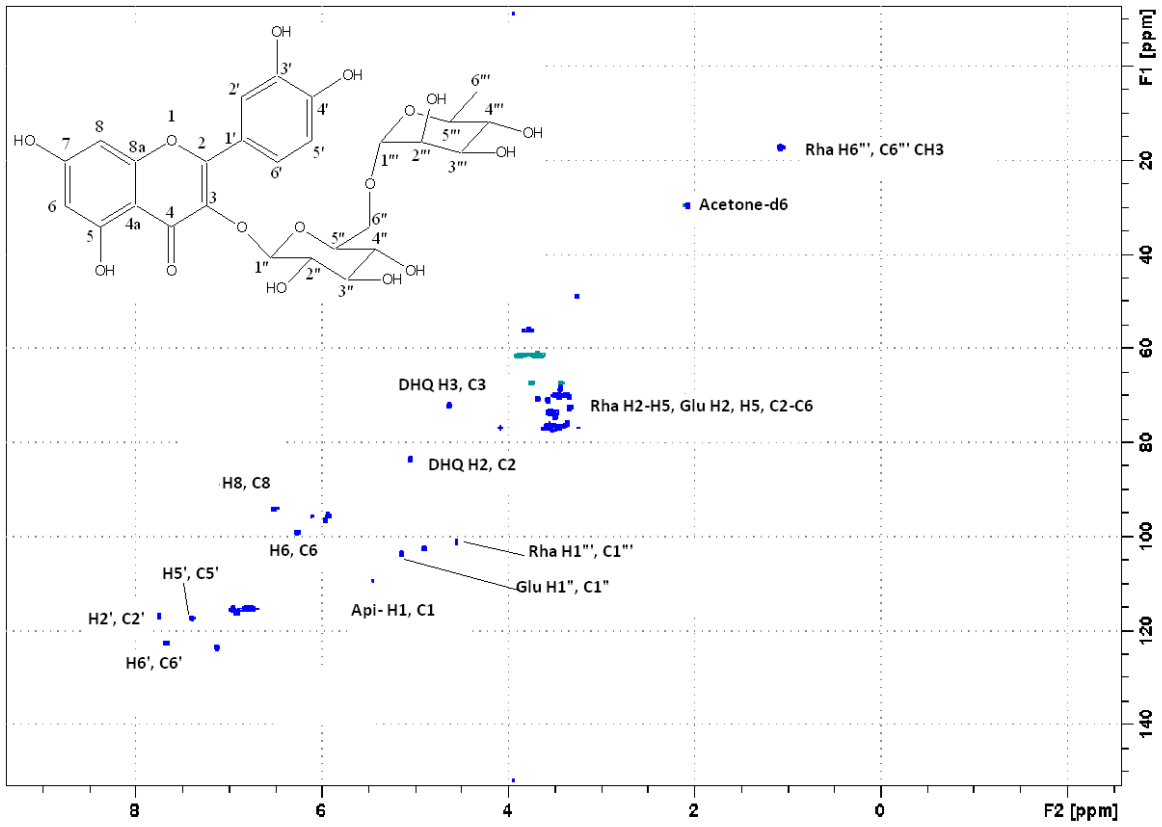

Figure 3–21 ^{1}H-^{13}C HSQC of OTD06003 (MeOH:H_2O 1:1) containing quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

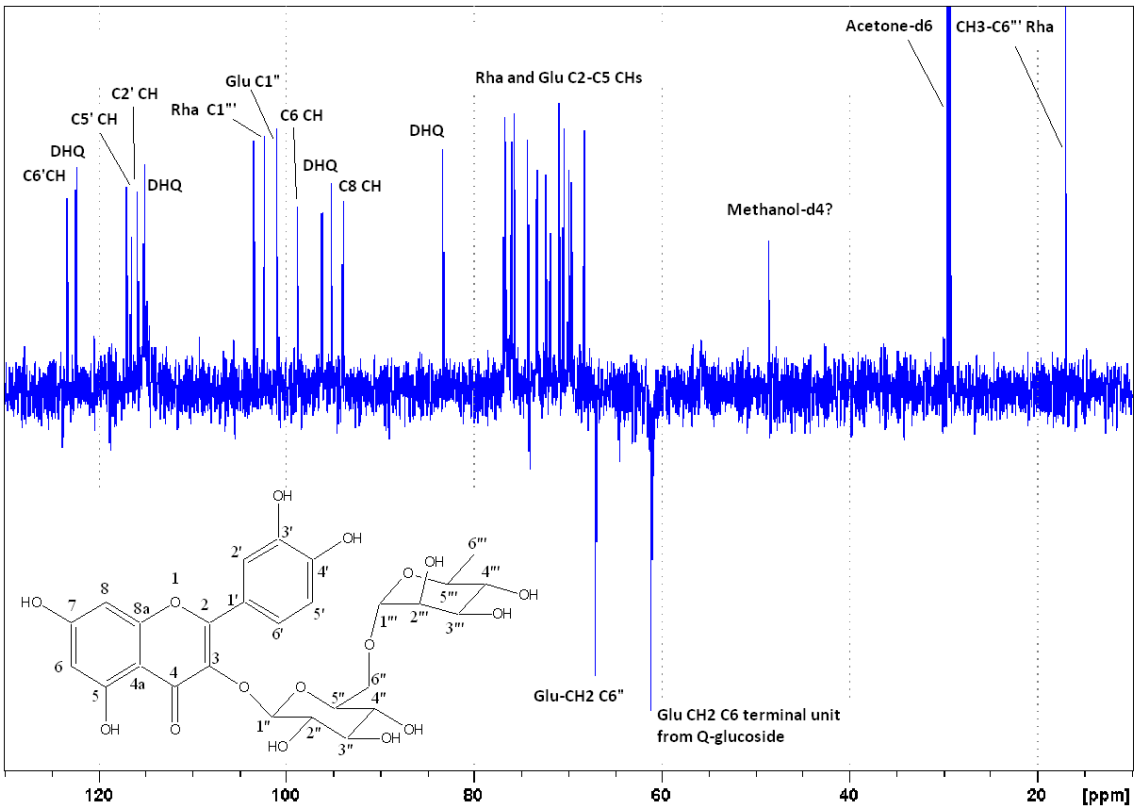

Figure 3–22 DEPT spectrum of of OTD6003 (MeOH:H_2O 1:1) containing quercetin 3-O-rhamnosyl-(1→6)–glucoside (rutin)

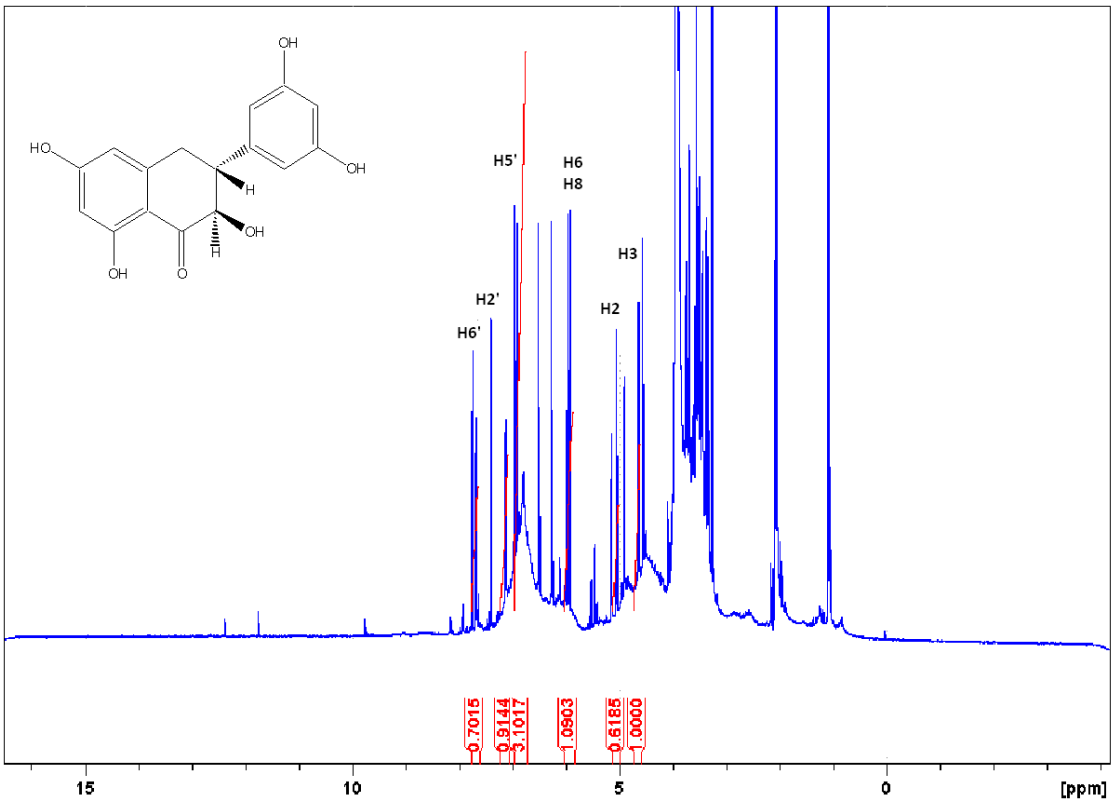

Figure 3–23 ^{1}H NMR spectrum of the isolated compound: 2,3-*trans* dihydroquercetin

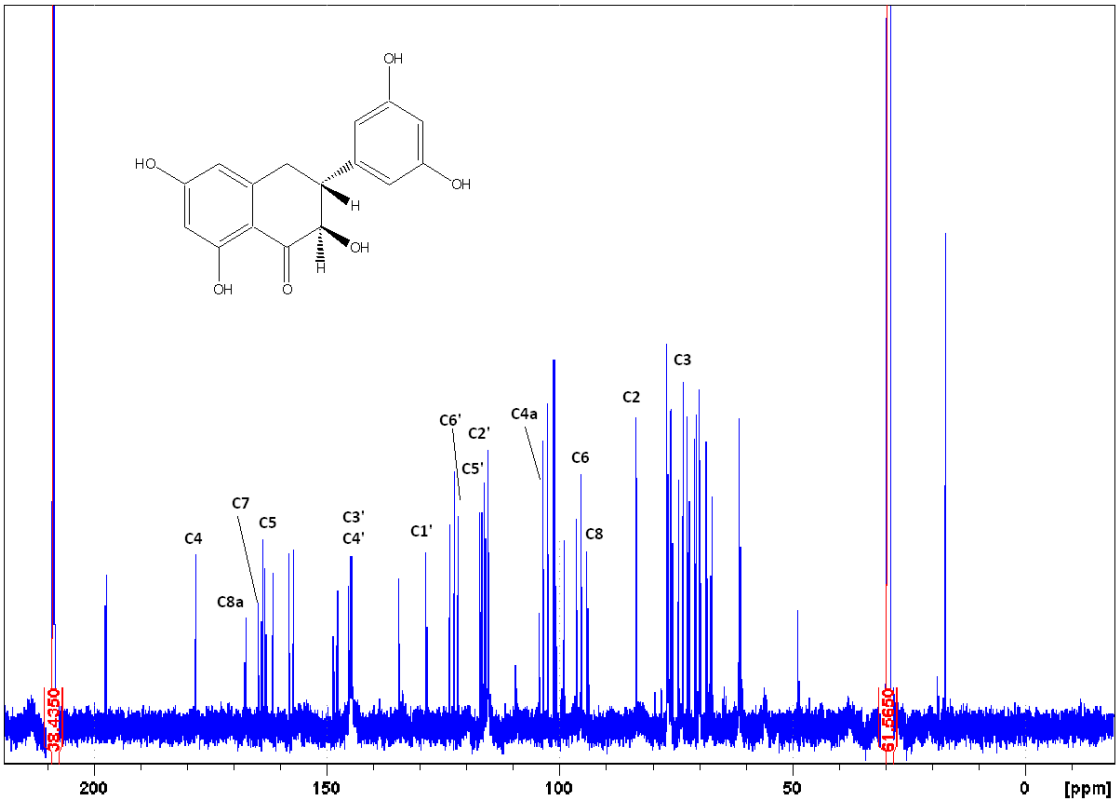

Figure 3–24 ^{13}C-NMR spectrum of the isolated compound: 2,3-*trans* dihydroquercetin

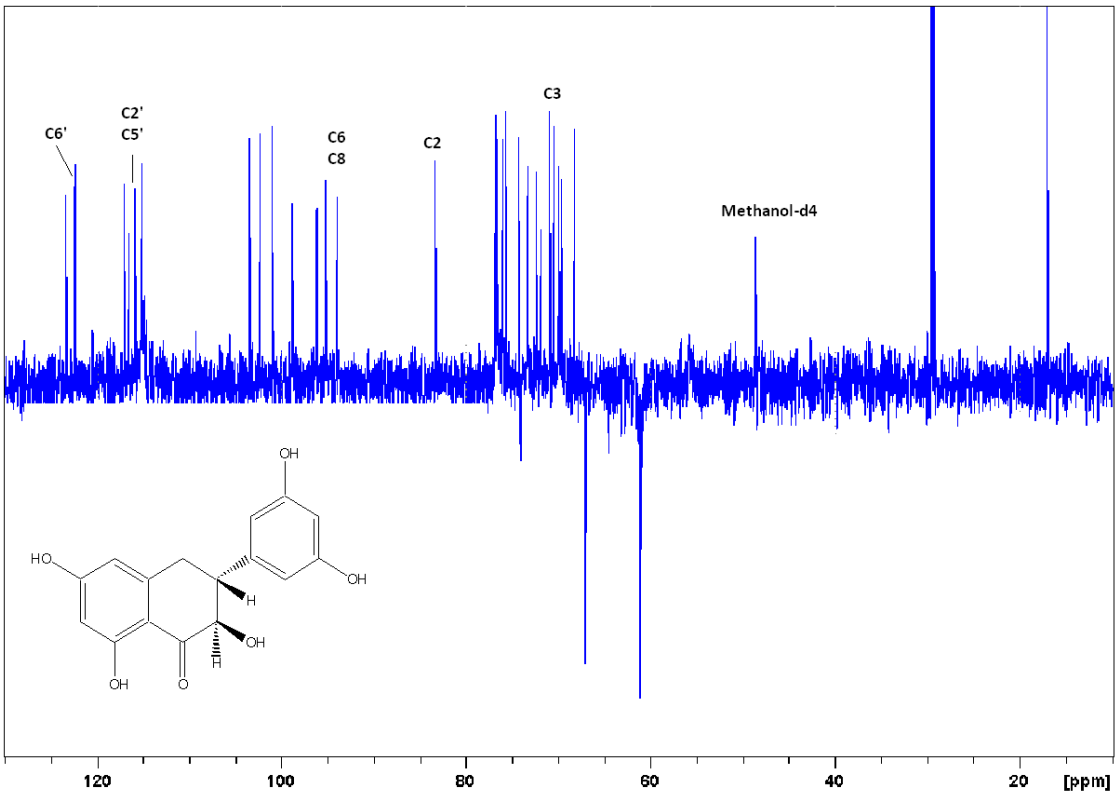

Figure 3–25 DEPT spectrum of 2,3-*trans* dihydroquercetin

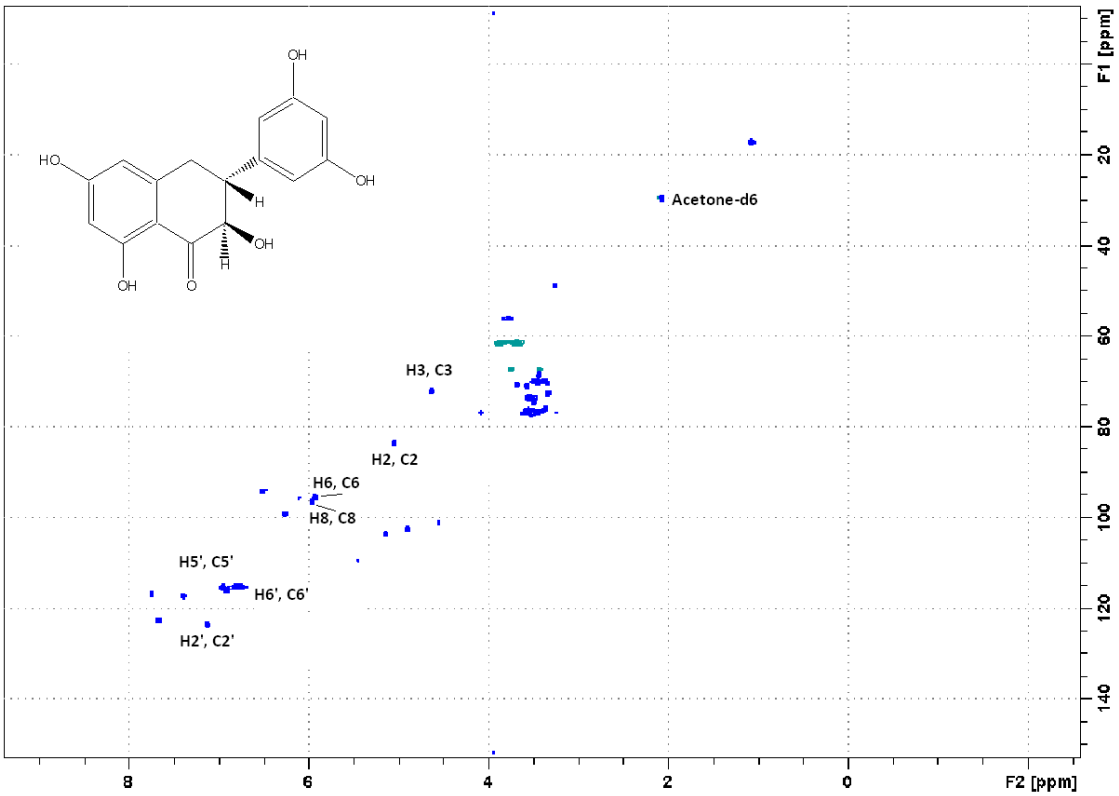

Figure 3–26 HSQC spectrum of 2,3-*trans* dihydroquercetin

3.3.6. LC/ESI-MS/MS of Flavonoids

The exact characteristics of the flavonoids and their sugar moieties were ascertained by NMR analysis (**Section 3.2**). The NMR data were confirmed by the LC/ESI/MS/MS, particularly for fractions OTD03403, OTD05903 and OTD06003. Twelve major flavonoids derivatives with attached sugars were separated by HPLC from the forage plants in fractions from *V. verrucosum* and *T. oleifolius*. Identification of the individual compounds was performed by comparison of PDA UV/Vis and ESI-MS spectrometric data with those of authentic standards and with those of published data. The published data by Grayer et al. (2004) and Vallejo et al. (2004) were used for identification of the compounds from these plants. LC-ESI-MS/MS analysis in the positive ion mode was used to identify the molecular weight (M^+) of the flavonoid glycosides, molecular weight information on the aglycone moiety and to ascertain whether there were sugar conjugates attached as evidenced by M^+-162 or M^+-146 or M^+-132 loss for glucose or galactose, rhamnose and a pentose (arabinose or apiose), respectively. The results obtained are summarised with the UV λ_{max} absorbance below and in **Table 3-5**. The ESI/MS/MS data showed the protonated aglycone $[A+H]^+$ ions at *m/z* 303, 287, 305 respectively, corresponding to quercetin, kaempferol and dihyroquercetin with the serial loss from the $[M+H]^+$ ion of rhamnose or glucose or apiose units to yield intermediate ions $[I+H]^+$ then further loss of a single hexose (glucoside) unit. This indicated that the hexose (glucose or rhamnose) was attached to the aglycone moiety, and the other sugars were attached on the hexose rather than to the aglycone.

Five flavonol glycosides were identified in this study. Full scan of fraction OTD03404 from *T. oleifolius* showed the presence of quercetin-3-*O*-diglucoside or sophoroside at *m/z* 626.99 (relative abundance (r.a) 100%, R_t 31.97, UV λ_{max} 230, 250, 355). This compound exhibited a neutral loss of 324 corresponding to sophoroside, a MS^2 fragment at *m/z* 465 $[M+H\text{-glucose}]^+$, a MS^3 fragment ion at *m/z* 303. The formed fragment at *m/z* 447.07 $[M+H\text{-}18]^+$ suggested that the sugars were attached at C3 of the aglycone moiety. The dimeric form $[2M+H]^+$ of this compound was shown at *m/z* 1252. The other fragments for MS^3 were shown at *m/z* 399, 369 and 345. The aglycone and sugar linkages were confirmed in the NMR data. Trace amounts of quercetin-3-*O*-rutinosides (rutin) at *m/z* 611 (r.a 100%, R_t 34.11 min, UV λ_{max} nm 230, 255, 355) was

also detected from this fraction. This compound showed a loss of 308 corresponding to glucose-rhamnose, and a MS^2 fragment at *m/z* 465 $[M+H\text{-glucose}]^+$, a MS^3 fragment at *m/z* 303. The other fragments for MS^3 were shown at m/z 369 and 345. The dimeric form $[2M+H]^+$ of this compound showed at *m/z* 1220.

The full scan spectrum $[M+H]^+$ of fraction OTD04405 from *T. oleifolius* exhibited a compound at m/z 758.99 (R_t 30.49, UV λ_{max} nm 265, 355), which was assigned to quercetin-glucosyl-apiosyl-glucoside. This compound exhibited a loss of 18 $[M+H-18]^+$ (m/z at 741), and a MS^2 fragment at *m/z* 627 $[M+H\text{-pentose}]^+$, 465 and 303, and a MS^3 fragment at *m/z* 597, 507, 477, 447 and 345. The dimeric form $[2M+H]^+$ of this compound showed at *m/z* 1516. Quercetin-diglucoside or sophoroside at *m/z* 626.99 (relative abundance (r.a) (R_t 33.65, UV λ_{max} nm 230, 255, 355) was also isolated. The dimeric form $[2M+H]^+$ of this compound showed at *m/z* 1252. Kaempferol-3-*O*-rutinoside at *m/z* 611.01 (R_t 33.47 min) was tentatively identified from *Tapinanthus oleifolius*. The–MS^2 $[M-H]^-$ fragmentation of 3-sophorosides is the typical for diglycosides at C3 position.

Five flavanol glycosides were isolated and identified from *V. verrucosum*. Fraction OTD04205 yielded quercetin-3-*O*-rhamnosyl-apiosyl-glucoside at *m/z* 742.99 (relative abundance (r.a) 100%, R_t 32.44, UV λ_{max} nm 230, 255, 355). This compound exhibited a loss of 18 $[M+H-18]^+$ (m/z at 725), and a MS^2 fragment at *m/z* 611 $[M+H\text{-pentose}]^+$, 465 and 303, and a MS^3 fragmentation for the precursor ions at *m/z* 285, 257, 229, 219, 165, 137 and 111. The dimeric form of this compound was detected at *m/z* 1484. Quercetin-3-*O*-dirhamnosyl-glucoside at *m/z* 756.90 (r.a) 100%, R_t 34.24, UV λ_{max} nm 230, 265sh, 355 was also detected. This compound showed a loss of COH_2 $[M+H-30]^+$ at *m/z* at 727 and 610 for the full scan, and a MS^2 fragment at *m/z* 593, 465, 447 and 303 $[M+H\text{-pentose}]^+$, and a MS^3 fragment at *m/z* 303, 285, 229, 183, 165, 153 and 93. The NMR data showed that the sugars were attached at position 3 of the aglycone moiety. The following compounds were isolated and identified from OTD04206: Quercetin-3-*O*-apiosyl-glucoside at *m/z* 596.95 (R_t 34.01, UV max nm 230, 255, 355) were tentatively identified from *Tapinanthus oleifolius*. Furthermore, this fraction produced a condensed tannin (CT) dimer such as epicatechin-4β→8-epicatechin at *m/z* 577.00 (R_t 16.62, UV λ_{max} 235, 280) and 2,3-*cis*-dihydroquercetin at *m/z* 305.16 at (R_t 33.14, UV λ_{max} 235, 285). Purified fraction (OTF05903) from OTD04205 yielded quercetin-3-O-rhamnosyl-glucosyl–apioside. Finally, purified fraction OTF06003 from OTD04206 confirmed the presence of epicatechin-4β→8-

epicatechin at *m/z* 577.00 (R_t 16.62, UV λ_{max} 235, 280) and dihydroquercetin at *m/z* 305.16 at (Rt 33.14, UV λ_{max} 235, 285). The anomeric position, linkages to the aglycone and interglycosidic linkages were confirmed by the NMR experiments (**Refer to Section 3.2.2).** These identified flavonoids may contribute to the beneficial properties for ruminants. A representative UV, HPLC-PDA chromatogram and mass spectrum is shown in **Figure 3-27**.

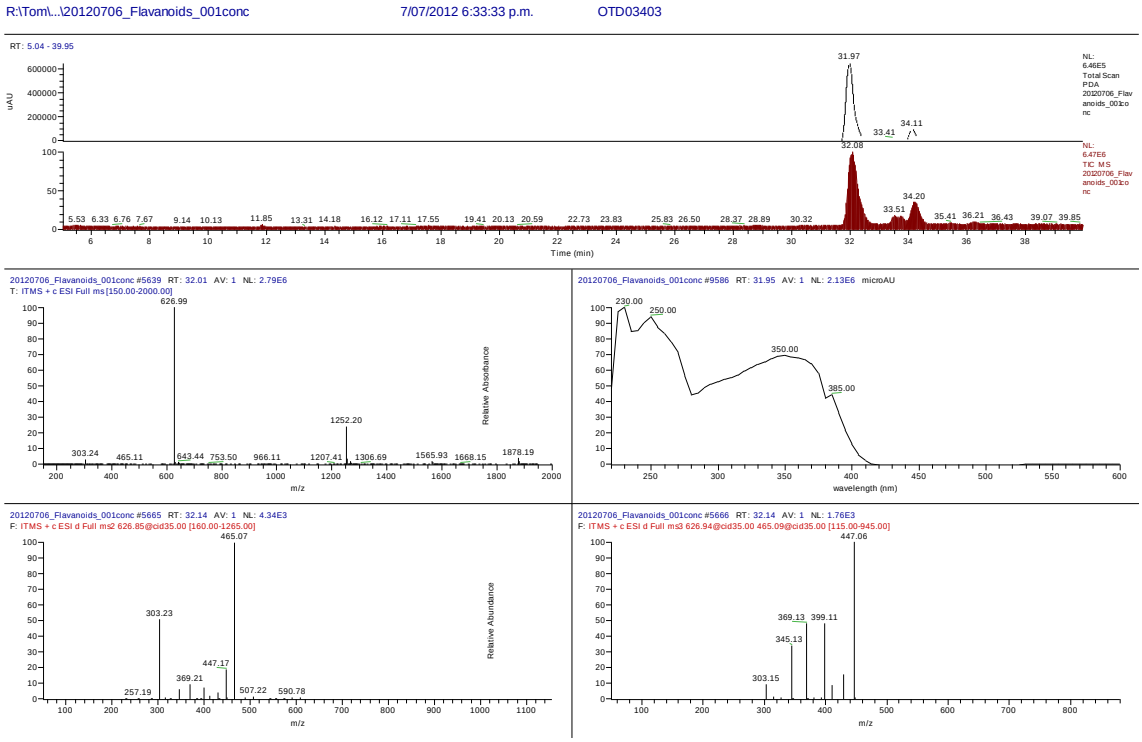

Figure 3-27 Representative HPLC-PDA chromatogram, UV and ESI-MS/MS spectrum of some of the LH-20 fractions

Table 3-5 Identified compounds, *m/z* of parent ions, *m/z* of intermediate ions, *m/z* of fragment ions, and retention times as determined by LC-DAD using ESI/MS/MS in positive ion mode for flavonoids from *T. oleifolius* and *V. verrucosum*

Plant name and fraction	Flavanoids	Rt (min)	UVλ max (nm)	[M+H]+ (m.u)	[I+H]+ (m.u)	Fragments [F+H]+ (m.u)
OTD03403 *T. olefolius*	Quercetin-3-O-diglucoside (sophoroside)	31.97	230, 250, 355	626.99	465.07	447.06, 399.11, 369.11, 345.13, 303.15
	Quercetin-3-O-rhamnosyl-glucoside (rutin)	34.11	230, 255, 355	611.01	465.11	447.14, 369.27, 345.14, 303.20
OTD04405 *T. oleifolius*	Quercetin-3-O-glucosyl-apiosyl-glucoside	30.49	265, 355	758.99	627.08	597.07, 507.13, 489.14, 465.17, 435.16,
						387.26, 345.16, 303.12
	Quercetin-3-O-diglucoside	32.02	230, 255, 355	627.00	465.07	447.06, 399.10, 369.20, 345.26, 303.25
	Kaempferol-3-O-diglucoside	33.47	225, 255, 355	611.01	449.04	431.09, 383.17, 353.11, 329.16, 299.26, 287
OTD04406 *T. oleifolius*	Quercetin-3-O-diglucoside	31.9	230, 255, 355	626.95	465.17	447.08, 399.12, 369.08, 345.17, 303.13
	Quercetin-3-O-apiosyl-glucoside	33.65	230, 255, 355	596.96	465.08	445.08, 345.18, 303.15
	Quercetin-3-O-diglucoside	34.16	230, 255, 355	610.99	465.05	303.1
OTD04205 *V. verrucosum*	Quercetin-3-O-glucosyl-apiosyl-glucoside	32.44	230, 256sh, 355	742.99	610.87	597.08, 579.36, 464.97, 345.06, 303.16
	Quercetin-3-O-dirhamnosyl-glucoside	34.24	230, 265sh, 355	756.90	610.98	465.19, 303.09
OTF05903 purified from OTD04205	Quercetin-3-O-glucosyl-apiosyl-glucoside	32.39	235, 280	742.96	576.08	477.09, 434.99, 345.22, 303.19
OTD04206 *V. verrucosum*	Epicatechin-epicatechin	16.62	235, 285	577.00	451.06	415.06, 409.13, 391.16, 289.12
	dihydroquercetin	33.41	235, 280	305.16	286.9	259.06, 195.04, 153.04
	Quercetin-3-O-rhamnosyl-glucoside (rutin)	34.55	230, 255, 355	610.97	465.12	447.09, 345.35, 303.10
OTF06003 purified from OTD04206	dihydroquercetin	33.14	235, 285	305.19	286.9	259.11, 195.06, 153.02
	Quercetin-3-O-apiosyl-glucoside	34.01	230, 265sh, 355	596.95	465.06	435.05, 345.13, 303.09
	Quercetin-3-O-rhamnosyl-glucoside (rutin)	34.35	230, 255, 355	611.02	465.02	447.19, 345.17, 303.13

Abbreviations: Rt-retention time, m-mass units, $[M+H]^+$ parent ion and fragment ions, $[I+H]^+$intermediate ion, $[A+H]^+$ aglycone ion

3.4. Conclusions

The aims of this study were to isolate and characterise flavonoids from *V. verrucosum* and *T. oleifolius*. The characterisation of flavonoids by NMR and LC-ESI-MS/MS has shown that quercetin was predominant in the purified fractions with attached sugars such as rhamnose, galactose, apiose and glucose.

CHAPTER FOUR:

THE EFFECT OF PURIFIED CONDENSED TANNINS FROM BOTSWANAN FORAGE PLANTS ON THE FREE-LIVING STAGES OF GASTROINTESTINAL NEMATODE PARASITES OF LIVESTOCK

4.1. Abstract

The effect of condensed tannins (CT) extracted from Botswanan forage plants on the free-living stages of a number of species of gastrointestinal nematode parasites derived from infected sheep were investigated using a number of *in vitro* assays. Fresh samples of five different plants (*Viscum rotundifolium, Viscum verrucosum, Tapinanthus oleifolius, Grewia flava and Ipomoea sinensis*) were collected in summer (February 2009 and 2010). Fractionation of each crude extract on Sephadex LH-20 column yielded low molecular weight phenolics and CT containing fractions. The potential impact of each purified CT fraction was evaluated for anti-parasitic effect using either an egg hatch, a larval development or a larval migration inhibition assay. Effects of three gastrointestinal nematode species derived from infected sheep (*Haemonchus contortus, Trichostrongylus colubriformis* and *Teladorsagia circumcincta*) were evaluated. Furthermore, effects of CT on adult worm motility were determined using the free-living nematode *Caenorhabditis elegans*. CT from *V. rotundifolium* and *I. sinensis* fractions from samples collected in 2009 and 2010 did not inhibit larval development. CT isolated from *V. verrucosum, T. oleifolius* and *G. flava* collected in 2009 completely inhibited the development of all parasite species. These CT fractions were more potent in inhibiting larval development of *H. contortus* than fractions from the same plant species collected in 2010. None of the CT fractions inhibited egg hatch or motility in *Caenorhabditis elegans*. However, a limited effect on larval migration was observed with some CT extracts. These results obtained suggest that CT extracts from Botswanan forage plants have anti-parasitic properties *in vitro,* and that further research is required to determine whether the findings are also observed in goats fed the plants in the field.

4.2. Introduction

Infection with gastrointestinal nematodes (GIN) significantly reduces protein utilisation and productivity in small ruminants, and animals can suffer from diarrhoea, inappetance, anaemia, loss of body weight and even death (Dalton, 2006). One of the widely employed control strategies of GIN is the use of anthelmintic drugs. However, the sustained use of these drugs usually results in the development of resistance in GIN (Sutherland and Leathwick, 2011). There are also problems with potential unavailability and unaffordability of the drugs in some regions, as well as improper and inappropriate use, particularly in developing countries (Sutherland and Scott, 2009). For these reasons, alternative control strategies, including the use of forage plants with putative anthelmintic activity, may have value in managing parasitism in grazing ruminants.

Numerous studies have reported either direct or indirect effects of condensed tannins (CT) from plants on GIN (Molan et al., 2002, Mupeyo et al., 2010b). Direct effects of CT may involve binding of CT to external and internal proteins of parasites and modifying either their parasitic and/or free living stages. Alternatively, CT may affect parasites indirectly by protecting dietary proteins from microbial degradation during passage through the rumen, enabling more proteins to dissociate in the small intestine, with the resulting increased absorption of amino acids leading to enhancement of the immune system (McNabb et al., 1998). Lambs grazing sulla (*Hedysarum coronarium*) containing CT (3.5 g/100g DM) had lower worm burdens and faecal egg counts than those grazing lucerne (CT 0.04 g/100g DM) (Niezen et al., 2002, Niezen et al., 1998). Similarly, sheep fed *Lotus pedunculatus* showed reduced worm numbers, egg excretion, and increased liveweight gain compared with sheep fed perennial ryegrass (Niezen et al., 1998). In these studies, the effects of CT from forages on GIN were hypothesised to be either direct or indirect. However, when extracts are administered to the animals as a drench, the antiparasitic effects have been reported as direct. For example, sheep infected with GIN and drenched with quebracho extracts had reduced faecal egg counts (Athanasiadou et al., 2001). The clearest indication of a direct effect of CT on GIN is observed in *in vitro* assays. According to Molan et al. (2002), purified CT extracts from seven herbages induced direct antiparasitic effects on *T. colubriformis* using a larval development assay, in which there was a correlation between the levels of CT present and the proportion of eggs able to develop to infective-stage larvae (L3).

While the effects of CT from some temperate and tropical forages on GIN have been investigated, the *in vitro* anti-parasitic effects of CT from Botswanan forage plants are unknown. In Botswana, smallholding farmers have reported feeding a range of parasitic plants such as mistletoes and browse plants as supplement to cattle, sheep and goats temporarily to meet their nutritional requirements and to putatively enhance their health and wellbeing (Madibela et al., 2000). In an *in vivo* study, goats fed *Viscum verrucosum* leaves and small stems had reduced faecal egg counts, which was attributed to ingestion of CT (Madibela and Jansen, 2003).

The aims of this study therefore were to investigate whether CT extracts from Botswanan forage plants could influence the ability of GIN to develop through to L3 *in vitro,* and for their effects on the free-living nematode *Caenorhabditis elegans*.

4.3. Materials and Methods

4.3.1. Plant collection

Fresh samples of five different plants (*Viscum rotundifolium, Viscum verrucosum, Tapinanthus oleifolius, Grewia flava* and *Ipomoea sinensis*) were collected from the Botswana College of Agriculture (BCA) farm in Gaborone, Botswana in summer (February 2009 and February 2010). The first three plants are mistletoes while the latter two are a shrub and a legume, respectively. The plant samples, consisting of leaves and small stems, were freeze-dried and couriered to New Zealand under Ministry of Agriculture and Forestry (MAF) approval. In addition, voucher specimens of the plants were deposited at the BCA herbarium.

4.3.2. Plant extraction

Freeze-dried and ground leaves and small stems (100 g) from each plant were extracted with acetone:water (7:3; v/v; 3L) containing ascorbic acid (1 g/L) and strained through cheesecloth to remove plant debris. The filtered extract was concentrated *in vacuo* at 40 $^{\circ}$C using a rotary evaporator to remove acetone, and the aqueous solution was subsequently defatted with dichloromethane. The aqueous layer was then concentrated *in vacuo* at 40 $^{\circ}$C using a rotary evaporator to remove residual dichloromethane, and freeze-dried to yield a brown condensed tannin crude extract.

4.3.3. Step fractionation (SF)

CT crude extracts were fractionated according to the method described by Meagher et al. (2004). Briefly, each CT extract (6 g) was dissolved in methanol:water (1:1, 30 mL). The extract was then loaded onto an XK 26/40 Sephadex LH-20 column (Pharmacia, Uppsala, Sweden), which was equilibrated with methanol:water (1:1). Four fractions (SF1-SF4; 150 mL each) were obtained after elution with methanol:water (1:1). Elution with acetone:water (7:3) yielded two fractions: SF5-I (150 mL) and SF6-I (350 mL), which were concentrated *in vacuo* and freeze-dried. The second batch (6 g CT extract/30mL aqueous methanol) was fractionated for each plant material to yield two CT fractions (SF5-II and SF6-II). In summary, fractionation yielded two purified CT-containing fractions (represented by SF6-I, SF6-II) from each plant: *V. verrucosum* (acetone:water; 7:3), *T. oleifolius* (aqueous acetone:water; 7:3) and *G. flava* (acetone:water; 7:3) fractions were used in the assays. Fraction 5 could not be used due to insufficient yield. For *V. rotundifolium*, CT were present in the first fraction (SF1, aqueous methanol; 1:1). The fractions were screened for the presence of phenolics using a reverse-phase high performance chromatography (RP-HPLC) coupled to a photodiode array (PDA) detector.

4.3.4. Gradient fractionation (GF)

Fractionation of the extracts on Sephadex LH-20 was carried out with a slight modification of the linear gradient method described by Meagher et al. (2004). This method was employed for the fractionation of the crude extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009. Each freeze-dried crude extract (17 g) was dissolved in 100 mL was loaded onto a Sephadex LH-20 column (XK 26/100, column volume: 400 mL) and coupled to a FPLC system. Elution was at a flow rate of 7 mL/min and was performed first with water (two fractions, 500 mL each, GF1 and GF2), then with methanol-water (1:1, v/v), followed by MeOH:H_2O (1:3, 250 mL, GF3), MeOH (1:1, 250 mL, GF4), MeOH (3:1, 250 mL, GF5), 100% MeOH (four fractions, 250 mL each, GF6, GF7, GF8 and GF9), and finally with acetone:H_2O (7:3, 2 fractions, 500 mL each, GF10, GF11). Small volumes of the fractions were retained and monitored by using RP-HPLC PDA.

4.3.5. Condensed tannin content

The extractable, protein-bound and fibre-bound CT concentrations were determined by the butanol-HCl colorimetric method described by Jackson et al. (1996). All CT contents were determined using CT extracted from *L. pedunculatus* as a reference standard.

The plant collection to condensed tannin content determination sections above and their results **in section 4.4** below were repeated and taken from **Chapter 2**.

4.3.6. Anti-parasitic assays

4.3.6.1. Parasites

Lambs were experimentally infected with monospecific *T. colubriformis*, *H. contortus*, and *T. circumcincta* species and were housed indoors, fed lucerne and given free access to water. Assays were carried out depending on positive patent infection and faecal egg counts (FEC) results.

4.3.6.2. Experimental design

All the experiments were conducted in triplicate. In the initial experiment, high-throughput anti-parasitic screening of all the crude extracts (aqueous acetone and ethyl acetate) and CT containing and low molecular weight phenolics fractions (125) from the plants collected in 2009 and 2010 was conducted (data shown in **Appendix 4-1, 4-2, 4-3, 4-4 and 4-5**). The anti-parasitic screening was conducted against *H. contortus* at 500 µg/mL. In the second experiment, the effective CT containing fractions (represented by SF6-I/II) from each plant obtained from the primary screening were confirmed for anti-parasitic effects against *H. contortus*, *T. colubriformis* and *T. circumcincta* at 100 and 500 µg/mL (data shown). In the last experiment, serial dilution (1:2) of the positive fractions was carried out using *H. contortus*, which had a high yield of eggs sufficient for the assays (data shown). Therefore, variations in anti-parasitic activity of CT in plants collected in different years were investigated.

4.3.6.3. Preparation of eggs

Faecal samples were collected from lambs housed indoors, experimentally infected with monospecific *Haemonchus contortus*, *T. colubriformis* and *T. (Ostertagia) circumcincta*. The faecal matter was mashed into a liquid slurry. The slurry was strained through a 250 µm and 100 µm sieves to remove debris and washings containing the eggs were retained on a 20 µm sieve. The eggs were obtained through floatation in saturated sodium chloride solution. They were centrifuged at 200 g (Centrifuge Eppendorf 5810R, Washington, USA) for two minutes to concentrate them. The concentrated eggs were diluted to ensure that 100 µL of the egg solution contained about 100 eggs. Detailed protocol for the extraction of eggs is shown **Appendix 4-6.**

4.3.6.4. Egg hatch assay (EHA)

Each crude extract, purified LH-20 fraction and distilled water (negative control) was incubated with the eggs of three different species of gastrointestinal nematodes (*H. contortus*, *T. colubrifomis* and *T. circumcincta*) at 25 ºC for 26 hrs at 500 µg/mL in triplicate. At the end of the incubation period, larval scoring was performed: 0-hatched larvae and 1-larvae completely unhatched. This scoring method was found suitable for high throughput screening.

4.3.6.5. Preparation of nutrient medium for larval development assay (LDA)

The nutrient medium was prepared from 1% yeast solution (Y-1000; Sigma) in 90 mL phosphate buffered saline (0.01 M; pH = 7.3) and 10 mL of Earle's balanced salt solution (E7510; Sigma), 0.015% *E. coli* in distilled water and amphotericin B solution (5 mg in 1 mL distilled water). The growth medium was prepared by mixing the yeast solution (45 mL), *E. coli* solution (45 mL) and 125 µL of the amphotericin B solution (antifungal).

4.3.6.6. Larval development assay (LDA)

The assay was carried out in 96-well microtitre plate according to the method described by Molan et al. (2002). Briefly, each CT fraction was cultured with the eggs of three different GIN (*H. contortus*, *T. colubriformis* and *T. circumcincta*) at 100 and 500 µg/mL in triplicate. The ingredients added in 96 well microtitre plates included the following: agar (2%, 100 µL), nutrient media (40 µL), CT extracts (2 µL for 100 µg/mL and 10 µL for 500 µg/mL), dimethyl sulfoxide (DMSO) as the negative control, and the eggs of different strains of GIN (60 µL). The culturing was carried out at 25 °C for seven days. At the end of the incubation period, larval scoring was performed; 0-active larvae and 1-100% dead larvae. Lastly, serial dilution (1:2) of the positive fractions was performed in the concentration ranging between 500 and 0.488 µg/mL using *H. contortus* eggs. Dose-titration was carried out only on *H. contortus* species of parasites since a high yield of eggs was recovered from the faecal samples. Low yield of eggs of *T. colubriformis* and *T. circumcincta* was obtained and the eggs were not adequate for serial dilution.

Detailed protocols for the larval development assay is shown in **Appendix 4-7**.

4.3.6.7. Larval migration inhibition (LMI) assay

The method involved preparations of CT test solutions and of L3 larvae which were combined and cultured in 48-well microtitre plates (Costar, Cambridge, MA). Each purified LH-20 fraction stock solution (1000 µg/mL) was prepared by dissolving 1 mg in 1 mL of phosphate buffered saline (PBS; 0.1 M, 0.05 M NaCl; pH 7.2). The L3 larvae were exsheathed in sodium hypochlorite (0.025% chlorine) and concentrated to 1500 larvae per mL of PBS. Each purified LH-20 fraction or phosphate buffered saline (negative control) was incubated with larvae (L3) of each exsheathed gastrointestinal nematode species (*H. contortus, T. colubriformis* and *T. circumcincta*) at 37 °C for 2 hours at concentrations of 500, 250, 125, and 62.5 µg/mL (n=8).

After the incubation, the solutions were transferred to sieves (7 mm ID with 20 µm mesh at the bottom) placed in a 48 culture plates and left overnight (18 hours) to enable the active larvae to migrate through the sieves. Larval counting was then performed using a microscope and LMI (%) was calculated.

For calculation of data and statistical analysis, the number of larvae which had migrated through the sieves was counted using 40x magnification and the LMI (%) was determined using the following equation;

$$\% \text{ inhibition} = (A-B)/A \times 100,$$

where A is the number of L3 larvae migrated through the sieves in the LMI assay in control incubations, and B is the number of L3 larvae migrated in incubations containing different concentrations of crude extracts and LH-20 fractions. Differences between treatments were analysed by general linear model (GLM; Minitab, version 15). The means from four pooled experiments were considered significantly different when $p < 0.05$. The model included fixed effects of concentration and plant extracts.

Detailed protocols for the larval migration inhibition assay, exsheathing of L3 and culturing of L3's are shown in **Appendix 4-8**, **4-9** and **4-10**, respectively

4.3.6.8. Adult worm motility test (AWMT)

This assay measures the ability of the test compounds to inhibit the worms from producing progenies. Each purified LH-20 fraction and PBS (negative control) were incubated with *Caenorhabditis elegans* worms (nematode model parasite) at 25 ºC for two days at a concentration of 100 and 500 µg/mL in triplicate. At the end of the incubation period, worm scoring was performed: 0- active worms and production of progenies and 1- inhibition of production of progenies by the worms.

4.4. Results

4.4.1. Phytochemical screening of the LH-20 fractions

Purified LH-20 fractions from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis* were screened for the presence of CT using RP-HPLC, which was coupled to a PDA detector. The methanol:water (1:1) fractions isolated from *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis* showed sharp peaks in contrast to the acetone:water (7:3) fractions from the same plants with broad unresolved humps (**Figure 4-1**). However, a small hump was obtained for the methanol fractions from *V. rotundifolium*, suggesting the presence of CT with the Sephadex LH-20 gel operating in

a size exclusion manner. The sharp peaks were indicative of the presence of the low molecular weight phenolics, while the broad humps were indicative of the presence of polymeric CT. The broad humps were found in the acetone:water fractions from *V. verrucosum*, *T. oleifolius* and *G. flava*.

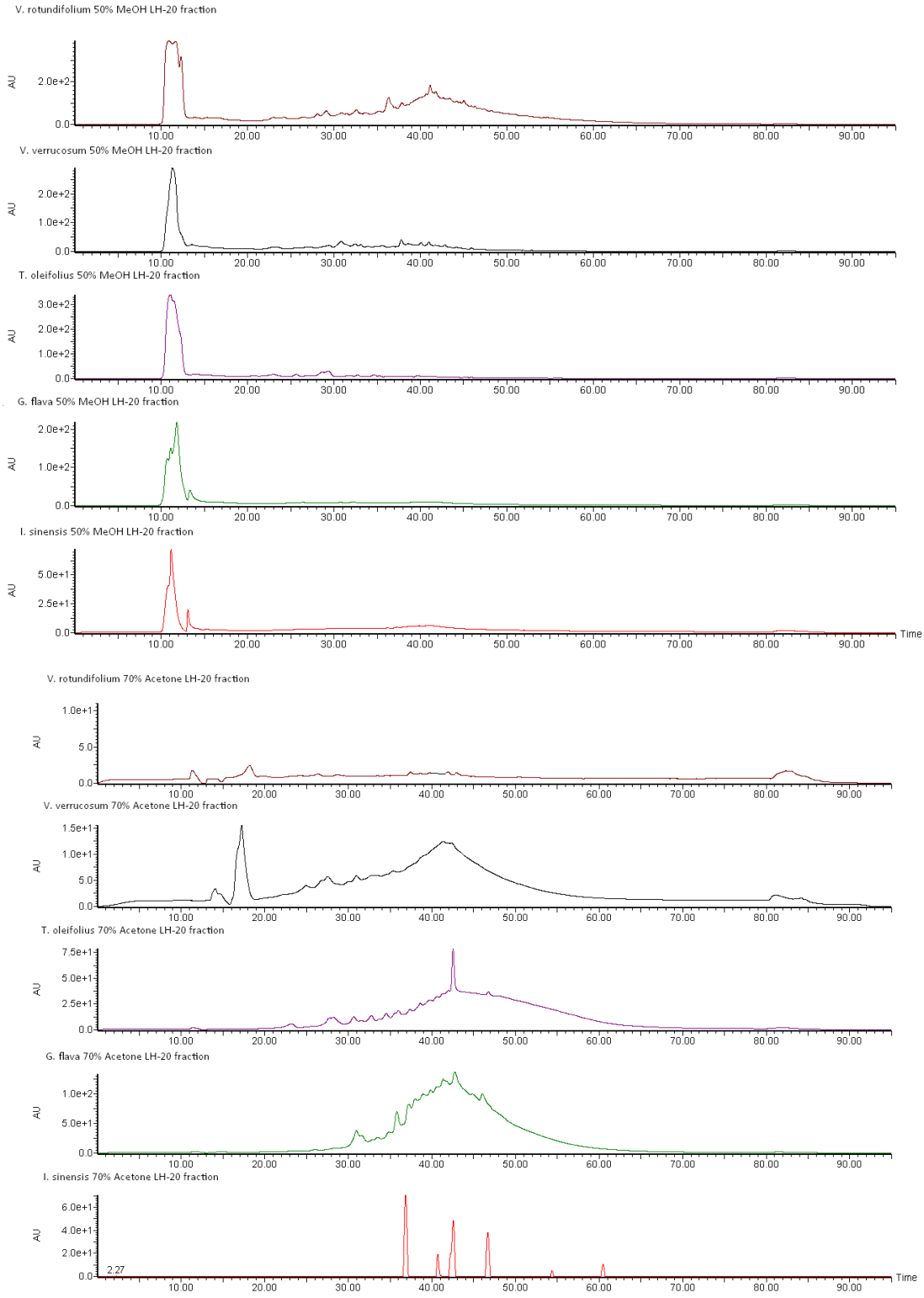

Figure 4–1 HPLC-PDA chromatograms of LH-20 methanol:water (1:1) fractions with sharp peaks indicating the presence of low molecular weight phenolics and aqueous acetone:water (7:3) fractions with a broad hump indicating the presence of condensed tannins in *V. rotundifolium*, *V. verrucosum*, *T. oleifolius*, *G. flava* and *I. sinensis.*

4.4.2. Condensed tannin concentration

CT content results from five forage plants are shown in **Table 4-1**. There were variations in the CT content between plant species. The total CT concentrations ranged from 0.2 to 12.7 (g/100g DM). *V. rotundifolium* and *I. sinensis* of plants collected in 2009 and 2010 contained trace amount of CT (<0.5 g/100g DM). *V. verrucosum* leaves and small stems collected in 2009 and 2010 contained 6.0% and 5.3% CT, respectively. *T. oleifolius* leaves and small stems collected in 2009 contained 5.1% CT while for those collected in 2010 contained 11.8% CT. *G. flava* leaves collected in 2009 had the highest CT concentration of 12.7% while *G. flava* collected in 2010 CT content was 9.7%. The butanol-HCl results indicated that significant amount of CT was found in *V. verrucosum, T. oleifolius* and *G. flava*, and these results were in agreement with the RP-HPLC-PDA results.

Table 4-1 Condensed tannin concentration (g/100g DM) by the butanol-HCl colorimetric assay

Plant name	Year	Free	Protein-bound	Fibre-bound	Total
V. rotundifolium	2009	0.1	0.4	<0.1	0.5
V. rotundifolium	2010	0.1	0.3	<0.1	0.4
V. verrucosum	2009	3.9	2.0	0.1	6.0
V. verrucosum	2010	3.2	2.0	0.2	5.3
T. oleifolius	2009	2.0	2.9	0.2	5.1
T. olefolius	2010	9.2	2.4	0.3	11.8
G. flava	2009	6.9	5.0	0.7	12.7
G. flava	2010	5.0	4.1	0.5	9.7
I. sinensis	2009	<0.1	0.1	0.1	0.2
I. sinensis	2010	<0.1	0.1	0.1	0.2

4.4.3. *In vitro* anti-parasitic assays

4.4.3.1. Larval development assay (LDA) of crude extracts

The results of the larval development assay of ten crude extracts from plants collected in 2009 and 2010 are shown in **Table 4-2** and **4-3**. Overall, the larval development assay results of the crude extracts isolated in 2009 and 2010 were similar because activity was not observed.

The ethyl acetate extracts did not exhibit any inhibition of larval development despite activity observed in OTC00302 fraction from *V. rotundifolium*.The ethyl acetate extract (OTC00302) isolated from *V. rotundifolium* plant showed anti-parasitic activity at 100 µg/mL against *H. contortus*. The defatted acetone-water (7:3) crude extracts (OTC01001) and ethyl acetate extract (OTC01002) isolated from *G. flava* leaves were effective in inhibiting larval development of *Teladorsagia circumcincta* at 100 µg/mL. However, anthelmintic effects were not exhibited at 500 µg/mL. The crude extracts from *V. verrucosum*, *V. rotundifolium* and *I. sinensis* did not exhibit antiparasitic activity at both these concentrations. The crude extracts and ethyl acetate extracts from plants collected in 2010 did not exhibit any inhibition of larval development. Overall, it was deduced that the crude extracts and ethyl acetate extracts from plants collected in both years did not exhibit activity against all the tested species of gastrointestinal nematodes, except the crude extract from *G. flava* (OTD01001).

Table 4-2 Effects of ten aqueous acetone crude extracts against four different species of gastrointestinal nematodes using a larval development assay *in vitro* at 100 µg/mL and 500 µg/mL in triplicate

Sample name	Date of collection	Aqueous crude extract	*H. contortus*		*T. circumcinta*		*T. colubriformis*	
			100	500	100	500	100	500
Viscum rotundifolium	27/02/2009	OTC00301	0	0	0	0	0	0
	10/02/2010	OTD01801	nd	0	0	0	0	0
Viscum verrucosum	27/02/2009	OTC00601	0	0	0	0	0	0
	12/02/2010	OTD01901	nd	0	0	0	0	0
Tapinanthus oleifolius	27/02/2009	OTC00801	0	0	0	0	0	0
	2/02/2010	OTD02001	nd	0	0	0	0	
Grewia flava	27/02/2009	OTC01001	0	0	**1**	0	0	0
	02/02/2010	OTD02101	nd	0	0	0	0	0
Ipomoea sinensis	03/03/2009	OTC01101	0	0	0	0	0	0
	02/02/2010	OTD02201	0	0	0	0	0	0

Abbreviations: larval scoring: 0 (not dead); 1 (100% inhibition or completely dead); nd-not determined

Table 4-3 Effects of ten ethyl acetate crude extracts against four different species of gastrointestinal nematodes using a larval development assay *in vitro* at 100 µg/mL and 500 µg/mL in triplicate

Sample name	Date of collection	Aqueous crude extract	*H. contortus*		*T. circumcincta*		*T. colubriformis*	
			100	500	100	500	100	500
Viscum rotundifolium	27/02/2009	OTC00302	**1**	0	0	0	0	0
	10/02/2010	OTD01802	nd	0	0	0	0	0
Viscum verrucosum	27/02/2009	OTC00602	0	0	0	0	0	0
	12/02/2010	OTD01902	nd	0	0	0	0	0
Tapinanthus oleifolius	27/02/2009	OTC00802	0	0	0	0	0	0
	02/02/2010	OTD02002	nd	0	0	0	0	0
Grewia flava	27/02/2009	OTC01002	0	0	1	0	0	0
	02/02/2010	OTD02102	nd	0	0	0	0	0
Ipomoea sinensis	03/03/2009	OTC01102	0	0	0	0	0	0
	02/02/2010	OTD02202	0	0	0	0	0	0

Abbreviations: larval scoring: 0 (not dead); 1 (100% inhibition or completely dead); nd-not determined

4.4.3.2. Larval development assay (LDA) of step LH-20 CT fractions

Fractions from *V. rotundifolium* and *I. sinensis* did not inhibit larval development at 100 and 500 µg/mL. Thus, these data were not shown due to lack of activity. The *V. verrucosum* fractions (SF6-I) derived from plants collected in 2009 completely inhibited (100% inhibition) larval development of *H. contortus*, *T. colubriformis* and *T. circumcincta* strains at both concentrations. However, *V. verrucosum* extracts of plants collected in 2010 (SF6-I and SF6-II) did not inhibit larval development. *T. oleifolius* fractions (SF6-I and SF6-II) completely inhibited development of all the tested GIN species at both concentrations. However, fractions from plants collected in 2010 did not inhibit larval development of all the tested GIN species. Therefore, increased CT content did not elicit any effects on the inhibition of larval development. Conversely, inhibition of larval development was found in *G. flava* with high CT content. The fractions of *G. flava* leaves (SF6-I and SF6-II) collected in 2009 were more potent and completely inhibited development of *H. contortus*, *T. colubriformis* and *T. circumcincta* strains to the L3 stage. However, minimal to no inhibition of larval development was attained in the fractions from plants collected in 2010. Overall, no pattern was observed in inhibition of larval development with the increasing CT content in the forage plants. However, forages containing trace amount of CT did not inhibit larval development. The results of inhibition of larval development of GIN species by the purified LH-20 fractions from plants collected in 2009 and 2010 are shown in **Table 4-4**.

Table 4-4 Effects of CT extracts from *V. verrucosum*, *T. oleifolius* and *G. flava* (70% acetone from first and second batch) on three different species of GIN using an egg hatch assay (EHA), larval development assay (LDA) and adult worm motility test (AWMT) *in vitro* at 100 and 500 µg/mL in triplicate.

Plant sample	Date of collection	Fraction Code	*H. contortus*			*T. circumcincta*			*T. colubriformis*			*C. elegans*
			EH	LD		EH	LD		EH	LD		AWMT
			500	100	500	500	100	500	500	100	500	500
Negative control			0	0	0	0	0	0	0	0	0	0
V. verrucosum	27/02/2009	SF6-I	0	**1**	**1**	0	**1**	**1**	0	**1**	**1**	0
		SF6-II	0	**1**	**1**	0	0	0	0	0	0	0
	2/02/2010	SF6-I	0	0	0	0	0	0	0	0	0	nd
		SF6-II	0	0	0	0	0	0	0	0	0	nd
T. oleifolius	27/02/2009	SF6-I	0	**1**	**1**	0	**1**	**1**	0	**1**	**1**	0
		SF6-II	0	0	**1**	0	**1**	**1**	0	0	**1**	0
	2/02/2010	SF6-I	0	0	0	0	0	0	0	0	0	nd
		SF6-II	0	0	0	0	0	0	0	0	0	nd
G. flava	27/02/2009	SF6-I	0	**1**	**1**	0	**1**	**1**	0	**1**	**1**	0
		SF6-II	0	**1**	**1**	0	**1**	**1**	0	**1**	**1**	0
	2/02/2010	SF6-I	0	0	**1**	0	0	0	0	0	**1**	nd
		SF6-II	0	0	0	0	0	0	0	0	0	nd

Abbreviations: larval scoring: 0 (not dead); 1 (100% inhibition or completely dead); SF6-I (step fraction 6-first batch); SF6-II (step fraction 6-batch 2); and AWMT (adult worm motility test), nd (not determined).

Furthermore, the fractions were investigated for the lowest toxic concentration using a dose-titration experiment against *H. contortus*. The results of this experiment are shown in **Table 4-5**. The lowest inhibitory concentration of larval development by the fractions from *G. flava* leaves collected in 2009 (SF6-I and SF6-II) was 62.5 µg/mL. In contrast, the lowest inhibitory concentration by the fractions from *G. flava* (2010) was 250 µg/L. This suggests that CT from *G. flava* (2010) were less potent than those from *G. flava* collected in 2009. The lowest inhibitory concentration of CT from *T. oleifolius* and *V. verrucosum* was 125 µg/mL. All the tested fractions did not inhibit egg hatch nor production of progenies by *C. elegans* worms *in vitro*.

Table 4-5 Lowest inhibitory concentration LDA of the positive CT extracts from *V. verrucosum, T. oleifolius* and *G. flava* against *H. contortus* using serial dilution ranging from 500 to 0.488 µg/mL(1:2)

Plant name	Date of collection	Sample code	Description	Two fold serial dilution (concentration in µg/mL)				
				500	250	125	62.5	31.25-0.488
V. verrucosum	27/02/2009	SF6-I	Acetone:water (7:3)	**1**	**1**	**1**	0	0
		SF6-II	Acetone:water (7:3)	0	0	0	0	0
T. oleifolius	27/02/2009	SF6-I	Acetone:water (7:3)	**1**	**1**	**1**	0	0
		SF6-II	Acetone:water (7:3)	**1**	**1**	**1**	0	0
G. flava	27/02/2009	SF6-I	Acetone:water (7:3)	**1**	**1**	**1**	**1**	0
		SF6-II	Acetone:water (7:3)	**1**	**1**	**1**	**1**	0
G. flava	2/02/2010	SF6-I	Acetone:water (7:3)	**1**	**1**	0	0	0
		SF6-II	Acetone:water (7:3)	**1**	**1**	0	0	0

Abbreviations: larval scoring: 0 (not dead); 1 (100% inhibition or completely dead); nd-not determined

4.4.3.3. Larval development assay (LDA) of linear gradient LH-20 CT fractions

Some linear gradient fractions showed anti-parasitic activity against *H. contortus* at 500 µg/mL (**Table 4-6**). From the primary screening of 41 fractions at 500 µg/mL, a total of three fractions were active in complete inhibition of larval development (**Appendix 4-2**). The active fractions with a score of 2 are: GF-4 and GF-6 from *V. rotundifolium*, and GF3, GF-6, GF-8, GF-10 from *V. verrucosum*, and GF-5, GF-7, GF-8, GF-9, GF-10 and GF-11 from *T. oleifolius*, and GF-9, GF-10, GF-11 from *G. flava*. This showed that activity was more prevalent in the oligomeric fractions than CT polymers despite the fact that no activity was observed in the CT containing fractions from the step LH-20 fractionation. This method was used to improve the separation of CT oligomers from polymers. The positive fractions were serially diluted (1:2) from a concentration of 500 µg/mL to 9.76 µg/mL with *H. contortus* strain. The lowest inhibitory concentration of 250 µg/mL was observed in the tested fractions (**Table 4-6**). The tested fractions were ascertained to contain condensed tannin oligomers and polymers. *G. flava* was more potent against certain species of parasites, particularly *H. contortus*.

Table 4-6 Lowest inhibitory concentration LDA of the positive LH-20 fractions from different plants against *H. contortus* different using serial dilution (1:2)

Plant name	Date of collection	Sample code	Description	Two fold serial dilution		
				500	250	125-0.488
V. verrucosum	27/02/2009	GF-10	Acetone:water (7:3)	**1**	0	0
T. oleifolius	27/02/2009	GF-8	100% methanol	0	0	0
		GF-9	100% methanol	**1**	0	0
		GF-10	Acetone:water (7:3)	**1**	**1**	0
		GF-11	Acetone:water (7:3)	**1**	**1**	0
G. flava	27/02/2009	GF-9	100% methanol	**1**	**1**	0
		GF-10	Acetone:water (7:3)	**1**	**1**	0
		GF-11	Acetone:water (7:3)	**1**	**1**	0

Abbreviations: larval scoring: 0 (not dead); 1 (100% inhibition or completely dead); nd-not determined

4.5. Larval migration inhibition assay

The aim of this study was to investigate whether CT isolated from Botswanan forage plants could inhibit the effects of parasitism using a larval migration inhibition assay *in vitro*. The primary screening of the LH-20 fractions was performed at 500 µg/mL (data not shown). From the screening, most of the fractions were not active and further investigations were then carried out with CT containing fractions. The results for the larval migration inhibition of *H. contortus* are shown in **Figure 4-2**. The tests were not conducted with other species of parasites due to insufficient recovery of the third stage L3 larvae from the faecal matter.

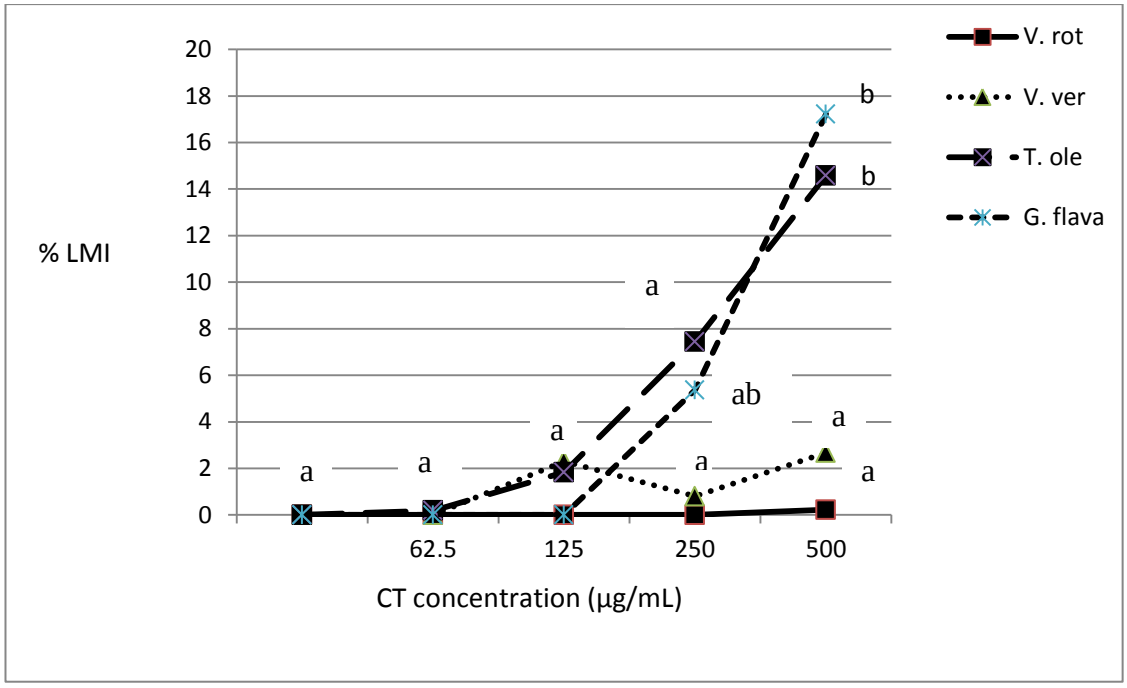

Figure 4–2 Larval migration inhibition of *H. contortus* by LH-20 fractions (SF6-I and SF-II) isolated from Botswanan forage plants in 2009 (*V. verrucosum*, *V. rotundifolium*, *T. oleifolius* and *G. flava*) at 62.5 to 500 µg/mL. Each point represents the mean of four experiments in duplicates (n=8). Standard error of the mean= 2.06. Means with the same letter (s) are not statistically significant.

Higher inhibition of larval migration was obtained in CT fractions from plants collected in 2009 (**Figure 4-2**). At 500 µg/mL, CT purified from *G. flava* and *T. oleifolius* (2009) markedly inhibited 17.2% and 14.6% ($p<0.05$) of the larvae (L3),

respectively from passing through the sieves compared to the negative control (PBS). The CT from *V. rotundifolium* and *V. verrucosum* did not inhibit the migration of the larvae at all concentrations ($p>0.05$).

CT extracted from *V. verrucosum* and *G. flava* (2010) inhibited 14.8% and 9.4% ($p<0.05$) of the larvae (L3) from passing through the sieves at 500 µg/mL relative to the negative control (PBS) (**Figure 4-3**).

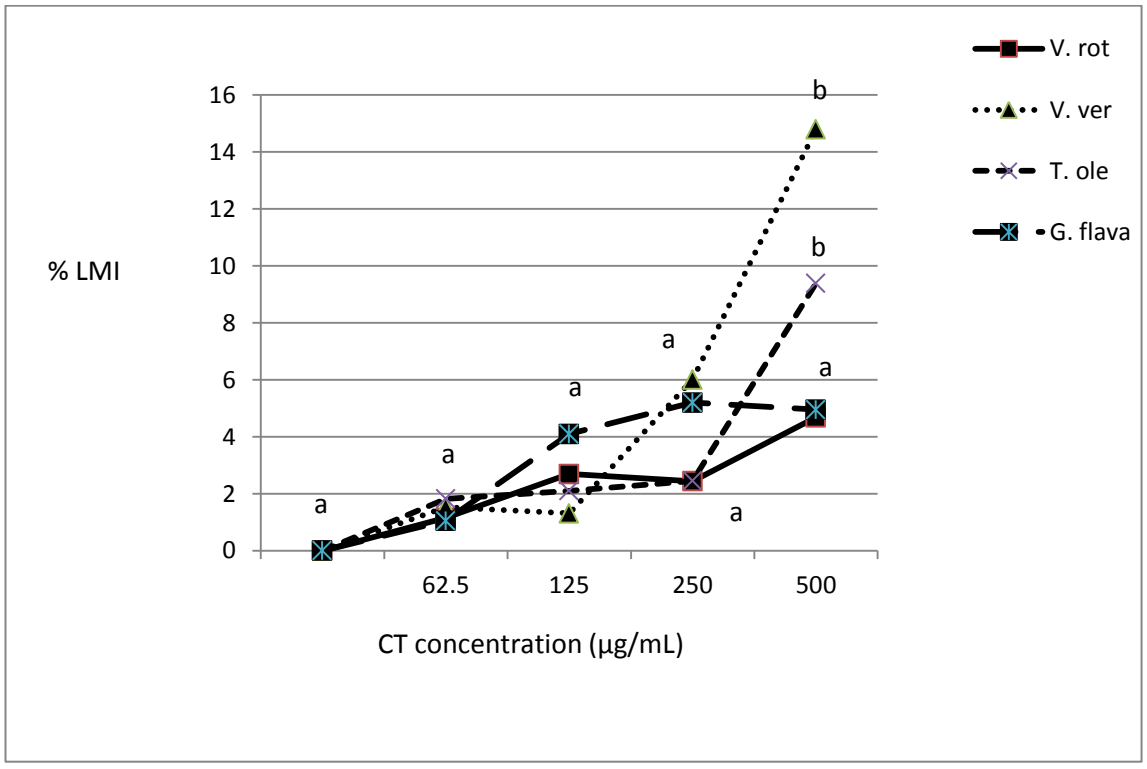

Figure 4–3 Larval migration inhibition of *H. contortus* by LH-20 fractions (SF6-I and ST6-II) isolated from Botswanan forage plants in 2010 (*V. verrucosum, V. rotundifolium, T. oleifolius* and *G. flava*) at 62.5 to 500 µg/mL. Each point represents the mean of four experiments in duplicate (n=8). Standard error of the mean=0.98. Means with the same letter (s) are not statistically significant.

However, the CT isolated from *V. verrucosum* (2009) at the same concentration did not inhibit larval migration. At 250 µg/mL, minimal inhibition of larval migration was observed. Also, the CT from *V. rotundifolium* did not inhibit the migration of the larvae at all concentrations. The inhibition of larval migration was dose-dependent. In

summary, the CT isolated from *G. flava* and *T. oleifolius* collected in 2009 were more inhibitory to larval migration than those from *V. rotundifolium* and *V. verrucosum* ($p<0.05$). In addition, the CT isolated from *V. verrucosum* and *T. oleifolius* collected in 2010 were more inhibitory to larval migration than those from *V. rotundifolium* and *G. flava* ($p<0.05$). These minimal effects may not be of physiological significance.

4.6. Discussion

The results obtained in this study demonstrate that CT extracted from a selected Botswanan forage plants can completely inhibit development of GIN species in an *in vitro* LDA. In the present study, CT extracted from *V. verrucosum, T. oleifolius* and *G. flava* completely inhibited larval development of GIN following isolation from sheep faeces. Previous studies have also demonstrated that the *in vitro* anti-parasitic effect of CT on GIN development. For example, CT extracted from *Hedysarum coronarium, Lotus pedunculatus, Lotus corniculatus, Onobrychis viciifolia, Dorycnium rectum* and *Dorycnium pentaphylum* each showed anti-parasitic effects against *T. colubriformis* (Molan et al., 2002, Molan et al., 1999). In addition, CT from *Lotus* species and sainfoin (*O. viciifolia*) also demonstrated anti-parasitic effects on cattle nematodes (Novobilsk et al., 2011). While few studies have been able to demonstrate corresponding *in vivo* anti-parasitic effects, it is interesting to note that goats fed *V. verrucosum*, one of the plants used in the current study, had lower faecal egg counts compared to relevant control animals (Madibela and Jansen, 2003).

The mechanisms responsible for the inhibition of larval development *in vitro* are not well understood. However, the high affinity of CT for proteins suggests that they may either bind directly to external proteins, while they may interact with internal proteins of the larvae following feeding. It is also possible that CT may bind to nutrients in the growth medium, thus impede their availability and inducing larval mortality. Putative external interactions involve the induction of toxicity from CT on the external surface of the larvae (Geary et al., 1993). Our results have indicated that CT were more potent in inhibiting larval development than egg hatching, larval migration and the production of eggs by *C. elegans*. A similar pattern was obtained with anti-parasitic effects of CT from forages grown under temperate conditions (Molan et al., 2002), in which CT extracted from *H. coronarium, L. pedunculatus, L. corniculatus* and *O. viciifolia* decreased larval development by an average of 91%, decreased egg hatch by

an average of 34% and inhibited larval migration by an average of 30%. This suggested that these CT may be ineffective in penetrating the cuticle of eggs, larvae and/or adult worms. Another study by Marie-Magdeleine et al. (2010) suggested that transcutilar diffusion was a common route for the uptake of anthelmintic drugs. However, the drugs were lipophilic, suggesting that they may more readily penetrate the external surface of helminths compared with hydrophilic CT. While there was a less marked effect of CT on larval migration in this and previous studies, what impact there was of CT may have been due to interference with the neurophysiology and neuromusculature of the larvae, resulting in paralysis, immobility and mortality (Molan et al., 2003, Molan et al., 2004).

CT derived from *G. flava* were more effective in inhibiting larval development than those from the other forages as evidenced from dose-titration. It is noted that the lowest active concentration of 62.5 µg/mL was significantly less than previous reports of CT activity, in which concentrations in excess of 200 µg/mL were required (Molan et al., 2002). The relatively high activity of *G. flava* CT suggests an investigation of potential *in vivo* efficacy should be undertaken.

In addition to the relatively high efficacy of *G. flava* CT in the current study, there were further, observable differences in the anti-parasitic activity between CT from different plant species. CT extracts from *G. flava* and *T. oleifolius* induced significant inhibitory effects while those from *V. rotundifolium* and *V. verrucosum* did not. In addition, CT from plants collected in 2009 were more potent in inhibition of larval development than those from plants collected in 2010. This variability could be due to differences in the chemical structure and the concentration of CT in different plant species (Meagher et al., 2004, Min et al., 2003). Furthermore, soil type, climate, cultivar, season (Waghorn et al., 2006) and other environmental differences may affect the bio-synthetic pathway of CT, which may impact their chemical structure and concentration (Athanasiadou et al., 2007). The chemical structure of CT plays an important role in determining the overall biological activity (Hoste et al., 2006). Structure-activity relationships could be deduced once the building blocks of CT polymer have been investigated. Thus, the results of the determination of the chemical structure of CT have demonstrated that these compounds from *V. verrucosum*, *T. oleifolius* and *G. flava* were predominantly procyanidin type (**Chapter 2**). Epicatechin was the prevalent extender unit. The chemical structure (PC-type CT) of these plants might be responsible for the anti-parasitic effects.

In addition to differences in anti-parasitic activity, there were differences in the inhibitory effects of CT on the different species of parasite tested. While the active extracts induced significant inhibitory effects on all the parasite species used, activity was significantly greater against the abomasal parasite *H. contortus*. These findings are in agreement with those of Ademola (2007), who found that *H. contortus* was very sensitive to plant extracts, although this study was performed *in vivo*. Other studies which investigated the activity of plant extracts against *T. colubriformis* and *T. circumcincta* using a larval migration inhibition assay, demonstrated that the effects of CT from *Querchus robur*, *Rubus fructicosus* and *Corylus avellana* varied between the nematode species (Paolini et al., 2004). However, in an *in vitro* study, there was no difference in the response of *T. circumcincta*, *H. contortus* and *Trichostrongylus vitrinus* to a quebracho CT extract. This was despite observed differences in susceptibility to quebracho CT extract between the species when tested *in vivo* (Athanasiadou et al., 2001). Most studies on the effects of CT on GIN, particularly using LDA, have focussed on one species of GIN (Hounzangbe-Adote et al., 2007, Molan et al., 2002). In the current study, different species of GIN were used, and the effects of CT from the forage plants differed between the parasite species. Furthermore, the variable anti-parasitic results suggest that the binding capacity of CT to the structural proteins of parasites differs significantly between species (Hounzangbe-Adote et al., 2005, Molan et al., 2000a).

The effect of CT from *T. oleifolius*, *V. verrucosum* and *G. flava* against different species of GIN from goat faeces has not been investigated. However, as sheep and goats have similar GIN species, it is expected that similar results would be obtained. However, further studies should establish whether the same species of parasites in goats could be impacted by CT.

The potential impact of CT extracts from *T. oleifolius* and *G. flava in vivo* on parasite burdens and egg production has not been investigated *in vivo*. Given the *in vitro* data presented in this study, in which CT from these two species had marked activity against a range of GIN species, such an experiment would be of value.

4.7. Conclusions

The results of this study suggest that plant extracts from Botswanan forage plants have anti-parasitic effects *in vitro*, which may translate into a role for these plants

in reducing the effects of parasitism in young sheep and goats. Consequently, pasture contamination with the third stage infective larvae may be reduced in some farming systems, as well dependence on anthelmintic drugs in animal health and productivity.

CHAPTER FIVE:

CONDENSED TANNINS FROM A SELECTED RANGE OF BOTSWANAN FORAGE PLANTS ARE EFFECTIVE PRIMING AGENTS OF γσ T CELLS IN RUMINANTS

5.1. Abstract

The potential impact of extracts from forage plants on γδ T cell activity in ruminants was evaluated using an *in vitro* immunoassay. This study investigated whether the plant extracts could prime γδ T cells via up-regulation of CD25 (interleukin-2 receptor alpha). Purified Sephadex LH-20 fractions, isolated from *Viscum rotundifolium, Viscum verrucosum, Tapinanthus oleifolius*, and *Grewia flava,* were screened against γδ T cells on kid, lamb and calf peripheral blood lymphocytes. Condensed tannins (CT) from *G. flava* significantly primed γδ T cells in kids up to 64.75% at 10 µg/mL, which was statistically significant relative to the negative control at 22.66% ($p=0.004$). CT from *T. oleifolius* also induced priming of γδ T cells in kids, while fractions from *V. rotundifolium* and *V. verrucosum* induced minimal priming of γδ T cells. In contrast, there was no significant priming of γδ T cells from lambs and calves for any of the tested fractions ($p>0.05$). These findings suggest that CT from a selected range of Botswanan forage plants can stimulate the immune system *in vivo* in selected ruminant species and may participate in enhancing host innate immune responses.

5.2. Introduction

Young cattle, sheep and goats possess relatively high proportions of gamma-delta (γδ) T lymphocytes, a subset of T lymphocytes, which play a major role in the innate immune response. These γδ T cells express γδ T cell receptors (TCR) which recognise an array of antigens and ligands (Hedges et al., 2005).

While γδ T cells represent a small fraction of T-lymphocytes in the human peripheral blood (0.5-5%), they can be present in much higher proportions (30-70%) in the blood stream of young ruminants, as well as in chickens (Gertner et al., 2007, Hedges et al., 2005, Nath, 2008). A significant difference in the γδ T cell proportions between animal species is observed only in the peripheral blood. γδ T cells represent the major lymphocyte subset in the intestinal mucosal linings, reproductive and the respiratory tracts of all species, where they could be strategically placed to have a role for innate (i.e. first line of defence) immune responses to pathogens (Williams, 2001), quick clearance of infected cells (Hayday, 2000), and/or the prevention of re-infection (Komori et al., 2006). They also guard the extracellular environment by ensuring tissue

restoration and by participating in wound healing of damaged epithelial surfaces (Chen et al., 2002).

In contrast to αβ T cells which recognise processed antigens presented on the major histocompatibility complex (MHC), γδ T cells recognise antigens independent of MHC presentation (O'Brien et al., 2007). As such, γδ T cells can directly recognise ligands such as the phosphoantigens, the alkyl amines, the aminobisphosphanates, and other non-peptide antigens, which are not presented on the MHC (Casetti and Martino, 2008). In addition, low and high molecular weight compounds such as phenolics and condensed tannins (CT) are recognised (Gertner et al., 2007). Holderness et al. (2007) reported that CT from unripe apple peel significantly primed bovine γδ T cells *in vitro* as measured via up-regulation of CD25. Similarly, CT from forage plant extracts primed lamb and calf γδ T cells *in vitro*, although the activity was not as significant as in CT from apple peel (Schreurs et al., 2010). Ramirez-Restrepo et al. (2010) reported that lambs fed temperate willow blocks containing CT had a higher proportion of γδ T cells in their blood than those fed lucerne. Conversely, in a consecutive *in vivo* study, young sheep fed willow did not exhibit different levels of primed γδ T lymphocytes, although their worm burdens were lower than in the relevant control animals (Mupeyo et al., 2010b).

The priming capacity of CT from forage plants on goat γδ T cells *in vitro* has not been investigated. Hence, in addition to priming of bovine and ovine γδ T cells, the effects of CT on the expression of CD25 on γδ T cells in goats and their contribution to the innate immune responses warrants further investigation.

Smallholding farmers in Botswana have reported feeding a range of parasitic and browse plants to small ruminants temporarily over a short period of time (about 1-2 months) to meet their nutritional requirements and to improve their health (Madibela et al., 2000). However, there is limited information on the effects of CT from these forage plants on the ruminant immune system. Therefore, the main aim of this study was to investigate the potential impact of purified CT from Botswanan forage plants on the innate immunity, particularly on the priming of γδ T cells in kids, calves and lambs via up-regulation of CD25.

5.3. Materials and Methods

5.3.1. Plant material

Fresh samples of four different plants (*Viscum rotundifolium, Viscum verrucosum, Tapinanthus oleifolius* and *Grewia flava*) were collected at the Botswana College of Agriculture (BCA) farm in Gaborone, Botswana in February 2009 (summer). These samples, consisting of leaves and small stems, were freeze-dried and couriered to New Zealand under Ministry of Agriculture and Forestry (MAF) approval.

5.3.2. Plant extraction

Freeze-dried and ground leaves and small stems (100 g) were extracted with acetone:water (3:7; v/v; 3L) containing ascorbic acid (1 g/L) and strained through cheesecloth to remove plant debris. The filtered extract was concentrated *in vacuo* using a rotary evaporator at 40 ^{o}C to remove acetone and the aqueous solution was subsequently defatted with dichloromethane. The aqueous layer was then concentrated *in vacuo* to remove residual dichloromethane and freeze-dried to yield a brown CT crude extract. Solvent % (v/v) is for % solvent in water unless otherwise stated.

5.3.3. Step fractionation

The fractionation on Sephadex LH-20 of CT crude extract was carried out according to the method of Sivakumaran et al. (2006). Briefly, the CT extract (5 g) was dissolved in methanol:water (1:1, 30 mL), loaded onto an XK 26/40 Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) and equilibrated with aqueous methanol (1:1). Four fractions (SF1-I-SF4-I; 150 mL each) were obtained after elution with methanol:water (1:1). Elution with aqueous acetone:water (7:3) yielded two fractions (SF5-I;150 mL) and (SF6-I;350 mL), which were concentrated *in vacuo* and freeze-dried. In summary, fractionation yielded purified CT containing fractions (represented by SF6-I) from each plant: *V. verrucosum* (acetone:water; 7:3), *T. oleifolius* (acetone:water; 7:3), and *G. flava* (acetone:water; 7:3) and these fractions were used in the immunoassays. SF5 could not be used for the assays due to insufficient yield. For *V. rotundifolium,* CT were present in the first fraction (SF1, methanol:water; 1:1). Second

batch (SF1 to 6-II) of fractions for each plant was obtained when fractionation was repeated and the immunoassay results of goat, calf and lamb are shown in **Appendix 5-1, 5-2 and 5-3**. These results were in complete agreement with those of the first batch.

5.3.4. Condensed tannin content

The extractable, protein-bound and fibre-bound CT concentrations were determined by the butanol-HCl colorimetric method described by Jackson et al. (1996). All CT concentrations were determined using CT extracted from *Lotus pedunculatus* as a standard reference (Sivakumaran et al., 2006).

The plant collection to condensed tannin content determination sections above and their results in **section 5.4** below were repeated and taken from **Chapter 2**.

5.3.5. Animals and experimental design

All animal experiments were approved by the Massey University Animal Ethics Committee (AEC). Two replicate were run for each concentration of each CT (5 and 10 µg/mL) with cells of the blood collected from three kids, three calves and three lambs into sodium heparin tubes (n=6). The results of each CT at 5 and 10 µg/mL for each animal were adjusted by the removal of results with few events or counts in the analysis and hence n=4 was adopted for the statistical analysis. Blood samples from different animals were used in all the experiments. For pilot studies, Vacutainers (10 mL) with EDTA as anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) were used for collection of whole blood from the animals.

5.3.6. Isolation of peripheral blood mononuclear lymphocytes (PBMCs)

Peripheral blood mononuclear lymphocytes were isolated from the blood samples as previously (Ramírez-Restrepo et al., 2010). Blood samples were centrifuged to collect the buffy coat. Cells were then overlaid over Histopaque and PBL separated by density gradient centrifugation. The cells were washed twice and re-suspended to the desired concentration (1×10^6 cells/mL) in RPMI culture media containing antibiotics, L-glutamine, HEPES, and 5 % foetal calf serum.

5.3.7. Cell culture

Two purified CT extracts (5 mg) from each of *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *G. flava* were dissolved in phosphate buffered saline (PBS, 1 mL), sterile filtered and kept at -20 ºC until used. Stock solutions were further diluted to give a 500 µg/mL concentration. PBMCs from each animal were cultured with each Sephadex LH-20 CT containing fractions at 5 and 10 µg/mL or Concanavalin A (positive control) or PBS (negative control) at 5µg/mL at 37 ºC and 5% CO_2 for 48 hours. The optimal concentrations of 5 and 10 µg/mL were established prior to tests at higher concentrations of 20 and 50 µg/mL, which were found to be toxic to the cells (data not shown).

5.3.8. Cell analysis

Cell analysis to measure the priming of γδ T cells was performed according to the modified method of Ramirez-Restrepo et al. (2010). Briefly, cells were stained with a pool of monoclonal antibodies (mAbs) specific for surface antigens for γδ T cells (anti-γδ TCR; clone H1-176) and CD25 (anti-interleukin-2 receptor alpha IL-2Rα, clone CACT116A) (all obtained from VMRD, USA). MAb (clone H1-176) was originally produced and characterised by the sheep immunology group at the Basel Institute for Immunology, while MAb (clone H1-176) was obtained from the Veterinary Medical Research and Development (VMRD, Pulman, WA, USA). These mAbs were conjugated to appropriate fluorochromes Alexa 488 or Alexa 647 (Molecular Probes, Invitrogen, Eugene, USA). The cells were incubated at room temperature for 15 minutes, washed twice and re-suspended in PBS containing 1% (v/v) of 7-amino-actinomycin D (7AAD). 7AAD was included in all staining procedures to exclude dead cells from the analysis. Cells were analysed on a flow cytometer (Beckton Dickson, FACSCalibur, NJ, USA). Data were analysed and figures produced by using FlowJo software (Tree Star, Inc. Ashland, USA). Thirty thousand events were routinely acquired for analysis. Gating strategy was employed to differentiate lymphocytes on light scatter (side scatter-SSC and forward scatter-FSC) profiles from monocytes, granulocytes, and debris followed by exclusion of dead cells with 7AAD (**Figure 5-1A and 5-1B**). Finally, the proportion of γδ T cells which are CD25 positive (γδ $TCR^+/CD25^+$) relative to the total γδ T cell population was calculated and the mean

fluorescent intensity (MFI) of CD25 on γδ T cells was identified (**Figure 5-1C**). Detailed procedure for PMBC extraction, cell culture and analysis is shown in **Appendix 5-4** and **5-5**.

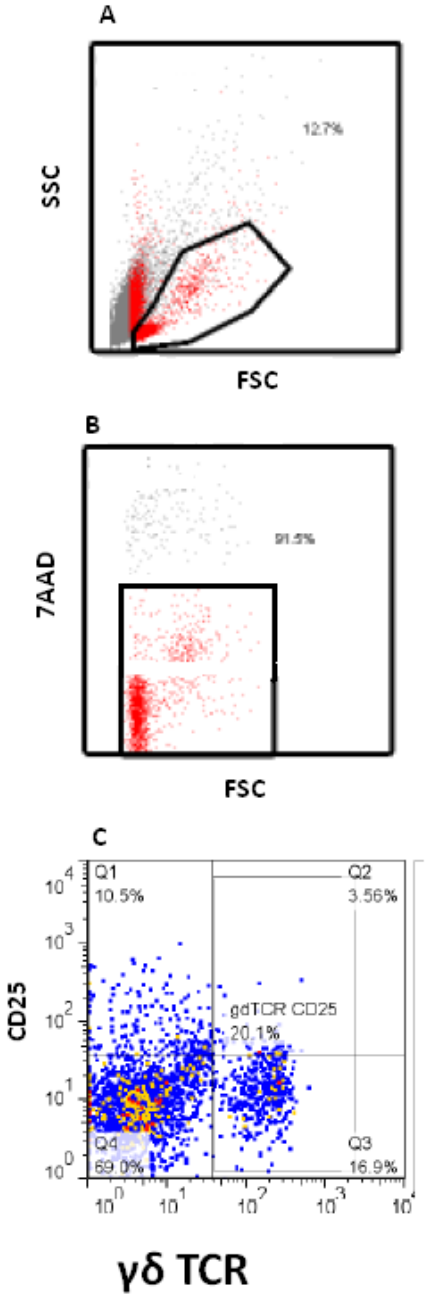

Figure 5–1 Gating strategy was employed in: A) Differentiation of lymphocytes by their characteristic FSC (forward scatter)/SSC (side scatter) profiles from monocytes, granulocytes, and debris B) Identification of viable cells by exclusion of 7AAD positive cells C) Identification of MFI (mean fluorescent intensity of CD25 on γδ T cells and proportion of γδ T cells expressing CD25 on γδ T cells.

5.3.9. Statistical analysis

Data on the priming of γδ T cells and their mean fluorescent intensity of kids, calves and lambs by the plant extracts were analysed using Minitab 15 statistical software (v15.1). Differences between treatments for each animal were analysed using one-way ANOVA procedures. The means from two pooled experiments were considered significantly different when $p<0.05$. The expression of CD25 on γδ T cells induced by the tested fractions was compared to that induced by PBS and Con A. In addition, data were subjected to an analysis of variance based on a 4 (CT sources) x 2 (concentration) x 3 (lamb, kid and calf) factorial design (General Linear Model, Minitab v15.1) to investigate for interaction effects. The model included plant-animal, plant-concentration, concentration-animal and plant-animal-concentration interaction effects. The model included the fixed effects of concentration. The plant and animal species (calf, lamb and kid) were incorporated in the model as random variables to control variation because the analysis was repeated across the four animals. The least square difference (LSD) test was used to separate least square means and were considered significantly different when $p<0.05$. The results for the factorial design are reported as the least square means from the two final pooled experiments.

5.4. Results

5.4.1. Condensed tannin concentration

CT content results from four forage plants are shown in **Table 5-1**. The total CT concentrations ranged from 0.5 to 12.7 (g/100g DM). *G. flava* leaves had the highest CT concentration (12.7%) followed by *V. verrucosum* (6.0%) and *T. oleifolius* (5.1%). *V. rotundifolium* contained trace CT content of 0.5%.

Table 5-1 Condensed tannin concentration (g/100g DM) of forage plants collected in February 2009 (summer) by the butanol-HCl colorimetric assay

Plant name	Free	Protein-bound	Fibre-bound	Total
Viscum rotundifolium	0.1	0.4	<0.1	0.5
Viscum verrucosum	3.9	2.0	0.1	6.0
Tapinanthus oleifolius	2.0	2.9	0.2	5.1
Grewia flava	6.9	5.0	0.7	12.7

5.4.2. Effects of plant extracts on priming of γσT cells from kids

The response of γδ T cells derived from the blood of kids was dependant on the plant species from which the CT were purified (**Table 5-2)**. CT from *G. flava* gave the highest proportion of primed γδ T cells from kids up to 64.75% at 10 µg/mL relative to primed γδ T cells at 22.66% by the negative control (p=0.004). *G. flava* had the highest CT content (12.7%). These results are in agreement with their MFI of CD25 expression on γδ T cells with 69.65 (p=0.002). CT from *T. oleifolius* showed increase in the proportion of primed γδ T cells up to 45.70% at 10 µg/mL, although not statistically significant (p = 0.102). Although there was no significant difference in priming of the cells from kids at 5 µg/mL, there was an indication of priming by CT from *T. oleifolius* (45.30%) (p = 0.110).

Second batch (SF1 to 6-II) of fractions for each plant was obtained when fractionation was repeated and the immunoassay results of goat, calf and lamb are shown in **Appendix 5-1**. These results were in complete agreement with those of the first batch.

Table 5-2 Mean percentage of primed γδ T cells in kids relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and the results are for two experiments or n=4 with comparison made to the non-stimuli.

Plant species extract and controls	Concentration (µg/mL)	Mean % of primed γδ T cells ($CD25^+$γδ TCR^+)±SEM	Mean MFI of CD25 on γδ $T cells^+$)±SEM
Controls			
non-stimuli		22.66±6.32	18.77±1.38
Con A	5	95.60±1.02 (**)	461.50±28.5 (**)
Plant species and extraction solvent			
V. rotundifolium (SF1-II, 50% methanol)	5	28.40±11.0(NS)	24.60±6.60 (NS)
	10	29.90±10.2(NS)	23.50±5.12 (NS)
V. verrucosum (SF6-I, 70% acetone)	5	25.57±9.65 (NS)	26.92±3.85 (NS)
	10	31.32±6.57 (NS)	20.93±2.89 (NS)
T. oleifolius (SF6-I, 70% acetone)	5	36.50±15.1(NS)	40.20±18.7 (NS)
	10	45.70±10.1 (NS)	49.20±12.5 (NS)
G. flava (SF6-I, 70% acetone)	5	45.30±10.3 (NS)	43.10±13.8 (NS)
	10	64.75±6.61 (*)	69.65±9.24 (*)
Significance (overall)	5	0.591	0.481
	10	0.018	0.001

Abbreviations and symbols : MFI (mean fluorescent intensity) and SF(step fraction), (** P <0.001), (* P<0.05), NS: not significant, P>0.05)

CT from *V. rotundifolium* and *V. verrucosum* induced a minimal increase in the proportion of primed γδ T cells, although not statistically significant. The results of priming of γδ T cells from kids by CT from *G. flava* and *T. oleifolius* are also represented in **Figure 5-2**. A sub-fraction of γδ T cells (32.2%) in kids was primed by CT from *G. flava* at 10 µg/mL, while non-γδ T cells were not primed by CT from *G. flava*. CT from *T. oleifolius* increased expression of CD25 on γδ T cells in kids at 10 µg/mL. CT from *T. oleifolius* and *G. flava* exclusively primed γδ T cells in the blood of kids in contrast to polyclonal activation by the mitogen Con A.

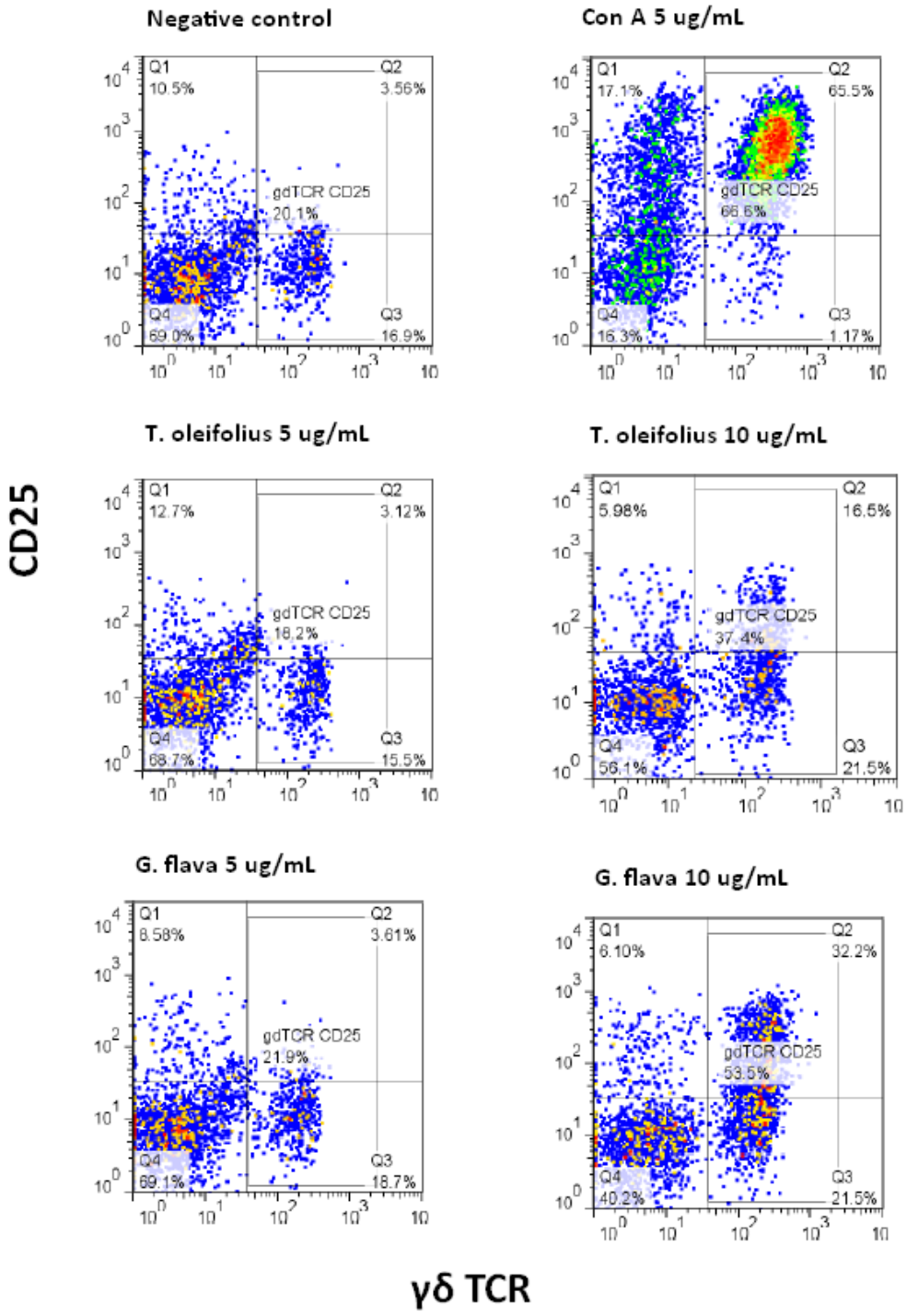

Figure 5–2 Results are representative of plant CT extracts priming γδ T cells in kids: priming in kids by *T. oleifolius* and *G. flava* at 10 µg/mL and by Con A at 5 µg/mL. No priming in kids by *T. oleifolius*, and *G. flava* at 5 µg/mL

5.4.3. Effects of plant extracts on priming of γσ T cells in calves

While significant priming of γδ T cells in the blood of kids by CT from *G. flava* and *T. oleifolius* was obtained, the plant extracts did not prime γδ T cells in the blood from calves (**Table 5-3**). The proportion of primed γδ T cells by the plant extracts was not statistically significant relative to the negative control ($p=0.308$ at 5 µg/mL and $p=0.475$ at 10 µg/mL) ($p<0.05$). These results are consistent with their MFI of CD25 on γδ T cells. However, there was an indication of an increase in the proportion of primed γδ T cells by CT from *G. flava* at 18.62% relative to the negative control with primed cells at 9.94%. In all the tested fractions, unprimed γδ T cells were predominant (**Figure 5-3**). In addition, the tested extracts did not prime the non-γδ T cells. In the positive control nearly all γδ T cells and non-γδ T cells were primed, while for the negative control all γδ T cells were not primed.

Second batch (SF1 to 6-II) of fractions for each plant was obtained when fractionation was repeated and the immunoassay results of goat, calf and lamb are shown in **Appendix 5-2**. These results were in complete agreement with those of the first batch.

Table 5-3 Mean percentage of primed γδ T cells in calves relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and the results are for two experiments or n=4 with comparison made to the negative stimuli.

Plant species extract and controls	Concentration (µg/mL)	Mean % of primed γδ T cells ($CD25^+$γδ $TCR^+)^+$)±SEM	Mean MFI of CD25 on γδ T $cells^+$)±SEM
Controls			
non-stimuli (PBS)		9.94±2.36	22.82±4.96
Con A	5	75.95±18.3 (*)	656.00±270 (*)
Plant species and extraction solvent			
V. rotundifolium (SF1-I, 50% methanol)	5	15.81±2.37 (NS)	27.45±4.96 (NS)
	10	13.43±1.08 (NS)	26.20±3.41 (NS)
V. verrucosum (SF6-I, 70% acetone)	5	10.21±2.17 (NS)	22.90±5.20 (NS)
	10	14.42±1.48 (NS)	27.70±3.59 (NS)
T. oleifolius (SF6-I, 70% acetone)	5	10.58±2.34 (NS)	23.70±4.42 (NS)
	10	11.06±3.15 (NS)	23.45±5.50 (NS)
G. flava (SF6-I, 70% acetone)	5	8.54±2.34 (NS)	26.48±5.21 (NS)
	10	18.62±6.54 (NS)	32.27±5.58 (NS)
Significance (overall)	5	0.308	0.960
	10	0.475	0.634

Abbreviations: MFI (mean fluorescent intensity) and Fr (fraction), NS: not significant; P>0.05, (*P<0.05
, **P<0.001)

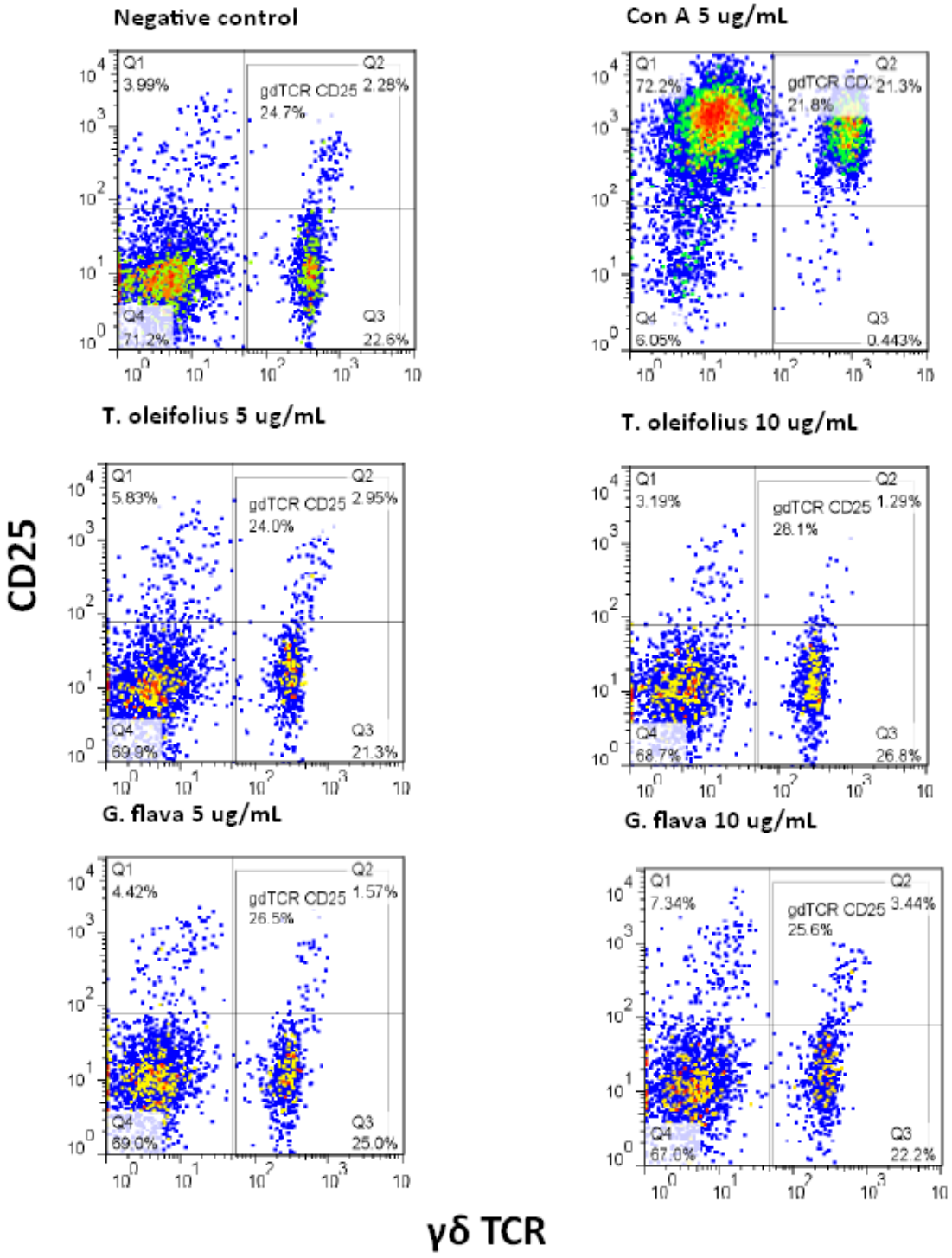

Figure 5–3 Results are representative of plant CT extracts priming γδ T cells in calves: No priming in calves at 5 and 10 µg/mL of the extracts.

5.4.4. Effects of plant extracts on priming of γσ T cells in lambs

The priming of γδ T cells from lambs was minimal in comparison to response from goats (**Table 5-4**). There were no significant differences in the proportion of primed γδ T cells by CT from all the tested extracts (p=0.966 at 5 µg/mL and p=0.844 at 10 µg/mL). However, there was an indication of elevation of primed γδ T cells (43.45%) by CT from *G. flava* relative to the negative control (32.49%). The mean percentage expression of CD25 on γδ T cells of lambs induced by the plant extracts was in agreement with MFI of CD25 on γδ T cells and was not statistically significant (p=0.922 at 5 µg/mL and p=0.659 at 10 µg/mL).

Second batch (SF1 to 6-II) of fractions for each plant was obtained when fractionation was repeated and the immunoassay results of goat, calf and lamb are shown in **Appendix 5-3**. These results were in complete agreement with those of the first batch.

Table 5-4 Mean percentage of primed γδ T cells in lambs relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and the results are for two experiments or n=4.

Plant species extract and controls	Concentration (µg/mL)	Mean % of primed γδ T cells (CD25+γδ TCR+)$^{+}$)±SEM	Mean MFI of CD25 on γδ T cells^{+})±SEM
Controls			
non-stimuli (PBS)		32.49± 13.2	27.40± 11.6
Con A	5	34.11± 18.2 (NS)	179.40± 67.5(*)
Plant species and extraction solvent			
V. rotundifolium (Fr-1, 50% methanol)	5	37.52± 13.3 (NS)	28.20±11.6 (NS)
	10	35.02±11.7 (NS)	23.83±9.24 (NS)
V. verrucosum (Fr-6, 70% acetone)	5	24.08± 13.0 (NS)	22.90±11.3 (NS)
	10	20.15±12.5 (NS)	20.70±11.9 (NS)
T. oleifolius (Fr-6, 70% acetone)	5	34.57±16.8 (NS)	31.50±13.8 (NS)
	10	32.75±14.3 (NS)	29.40±12.8 (NS)
G. flava (Fr-6, 70% acetone)	5	37.47± 17.8 (NS)	40.40±19.1 (NS)
	10	43.45±18.4 (NS)	47.90±19.8 (NS)

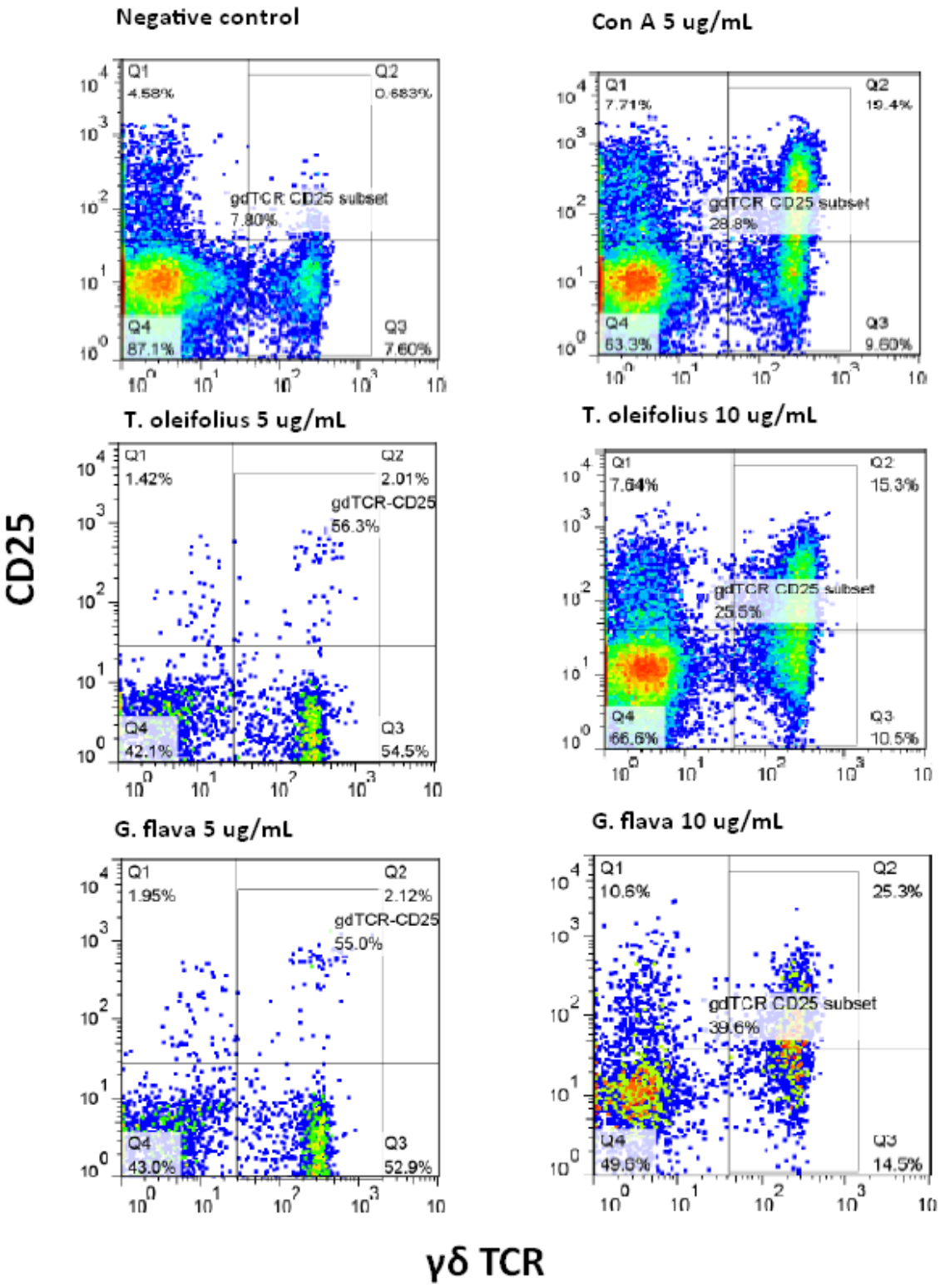

Figure 5–4 Results are representative of plant CT extracts priming γδ T cells in lambs: No priming of cells of lambs by the negative control, *T. oleifolius* and *G. flava* at 5 µg/mL. Priming in lambs by *T. oleifolius* and *G. flava* at 10 µg/mL.

5.4.5. Effects of animal species, concentration and their interaction on priming γσ T cells

In addition to the effects of plant species on priming of γδ T cells, the cellular response was also dependent on the animal species. The mean proportion of primed γδ T cells in the blood of kids (35.95%) was higher than those in lambs (28.80%) and calves (12.14%) (p=0.015) at both these concentrations. The increase in the concentration of the CT extracts in the cell culture did not induce significant impact on the priming of γδ T cells (p=0.357). However, the priming of γδ T cells in kids was induced at higher concentration of 10 µg/mL, suggesting concentration-dependence and cell proliferation induction. No plant x animal (p=0.072) (**Table 5-5**), plant x concentration (p=0.107), concentration x animal (p=0.173), and plant x animal x concentration (p=0.833) interactions on priming γδ T cells occurred. In addition, no plant x concentration (p=0.275), plant x animal (p=0.317), concentration x animal (p=0.453), no plant x concentration x animal (p=0.064) interactions occurred in the MFI of CD25 on γδ T cells.

Table 5-5 Least square means (%) on plant x animal interaction (p=0.072) on primed γδ T cells (or expressing CD25) and MFI of CD25 on γδ T cells by CT extracts at 5 and 10 µg/mL (means of two experiments or n=8)

Animal species	Plant species extract and mean proportion of primed γδ T cells (or $CD25^+/\gamma\delta$ TCR^+)			
	V. rotundifolium CT extract	*V. verrucosum* CT extract	*T. oleifolius* CT extract	*G. flava* CT extract
Goat	27.37	28.01	35.64	52.78
Calf	12.32	12.25	10.91	13.07
Lamb	25.75	19.86	32.19	37.30
Significance	NS	NS	NS	NS
Abbreviations: NS (not significant)				

5.5. Discussion

The results obtained demonstrate that CT containing extracts from Botswanan forage plants, particularly *G. flava* and *T. oleifolius*, prime γδ T cells derived from the circulating blood of young goats. This is in agreement with reports in the literature that CT from broadleaf dock (*Rumex obtusifolius*) and sulla (*Hedysarum coronarium*) significantly primed γδ T cells from the circulating blood of calves *in vitro* (Schreurs et al., 2010). In the same study (Schreurs et al., 2010), there was minimal stimulation of γδ T cells from lambs following the addition of the plant extracts. According to Ramirez-Restrepo et al. (2010), the numbers of γδ T cells in the peripheral blood was elevated in sheep fed temperate willow blocks. In addition, CT from unripe apple peel significantly primed γδ T cells and augmented cell division (Holderness et al., 2007). This activity has been observed in humans and mice (Jutila et al., 2008). It has been proposed that the variability in the response of γδ T cells to stimulation with the plant extracts could be due to differences in the chemical structure and concentrations of CT (Hummer and Schreier, 2008, Spencer et al., 2007).

In the present study, CT from *G. flava* and *T. oleifolius* significantly primed γδ T cells derived from the blood of kids, but not those derived from lambs or calves. This indicates that the γδ T cell responses of related animal species such as goat, sheep and cattle may vary considerably (Hein and Mackay, 1991). It is proposed, therefore, that goat γδ T cells may have been evolved to recognise selected range of CT from forage plants. It is noted that goats are browsers in contrast to lambs and cattle, which are grazers (Alonso-Díaz et al., 2010, Hoste et al., 2005). Browsers may ingest a wide range of plants containing high CT, which may stimulate innate immune cells in the gut mucosa. Therefore, further studies should be conducted to establish whether γδ T cells in other browsing species such as deer could be stimulated by CT from these forage species. Other factors that could have contributed to observed species differences in the priming of cells are the history of antigen exposure, diet and age, and the physiological state of the animal.

In addition to the observed differences in plant and animal species effects on the priming of γδ T cells, the *in vitro* cellular response in the kids was induced at low CT concentrations, apoptosis (programmed cell-death) occurring at high concentrations. Normally, *in vivo*, high concentrations of CT are regulated and toxic levels are inhibited from reaching the bloodstream (Jutila et al., 2008). A small proportion of CT are

absorbed in the upper gastrointestinal tract, although the mechanisms of absorption are still unclear (Stevenson and Hurst, 2007). Therefore, the observed priming of γδ T cells in the kids at these low concentrations suggests that the results may have physiological significance.

The stimulation of γδ T cells by CT is receptor-mediated, and is subtle, direct and antigen-independent (Holderness et al., 2008). The priming model that describes this mechanism is shown in **Appendix 5-6**. CT have been shown to be ligands for γδ T cell receptors and this receptor binding results in functional changes of the cell such as CD25 expression and synthesis of interleukin IL-2 (Ferrero et al., 2007). Primed γδ T cells may be able to respond more quickly and robustly in downstream responses to pathogens. The priming event induced by CT enables the interaction of IL-2 with IL-2R, which in turn initiates cell proliferation (Parkin and Cohen, 2001). In addition, the activated γδ T cell can migrate, and home into sites of infection, where they produce pro-inflammatory effector cytokines such as interferons (IFNγ) and tumor necrosis factor (TNFα) (Jutila et al., 2008).

The γδ T cells are the predominant lymphocytes in the mucosal linings of all animal species and play a significant role in innate immunity (Holderness et al., 2008). The priming of γδ T cells *in vitro* implies a similar process may occur in the gut mucosa of goats, which is commonly exposed to pathogens such as bacteria, viruses and gastrointestinal nematodes (Hein and Mackay, 1991). However, the potential impact of the extracts from *G. flava* and *T. oleifolius* on the ruminant immune system and on gastrointestinal nematodes *in vivo* has not been investigated.

5.6. Conclusions

The current study presents new information on the priming of γδ T cells from young goats following exposure to CT from *G. flava* and *T. oleifolius*. Should this priming also occur *in vivo*, the feeding of these plants may have a positive impact on the ability of the animals to respond to pathogens, such as gastrointestinal nematodes. It remains to been if priming is restricted to specific subsets of γδ T cells such as those represented in workshop cluster 1 (WC1), and if the stimulated cells could reduce the effects of parasitism.

CHAPTER SIX:

ISOLATION AND CHARACTERISATION OF PHENOLICS AND CONDENSED TANNINS FROM BOTSWANAN FORAGE PLANTS AND THEIR BIOLOGICAL SIGNIFICANCE- SUMMARY AND FUTURE DIRECTIONS

6.1. Plant samples

The objectives of this study were to isolate and characterise phenolics and condensed tannins (CT) from a selected Botswanan forage plants and to investigate their anti-parasitic and immunostimulatory effects in small ruminants *in vitro*. The plants (*V. rotundifolium, V. verrucosum, T. oleifolius, G. flava* and *I. sinensis*) were harvested in summer (February 2009 and 2010) and voucher specimens were deposited at the Botswana College of Agriculture (BCA) herbarium. These samples were then couriered to New Zealand under Ministry of Agriculture and Forestry (MAF) approval. A flow chart of the experimental plan of the entire project is shown in **Figure 6-1**.

6.2. Nutrient content of plant samples

The nutritional composition of the plants was investigated from freeze-dried plant samples, which were submitted to Mr. Grant Taylor, Feedtech, AgResearch Grasslands, by a near infrared reflectance spectroscopy (NIRS systems 6500). The samples were not found not to be suitable for NIRS because the sample types were too different from the validation points used in the calibration curves made available for each parameter. Also, the CT content was not obtained by NIRS. However, the CT content was determined by the butanol-HCl method.

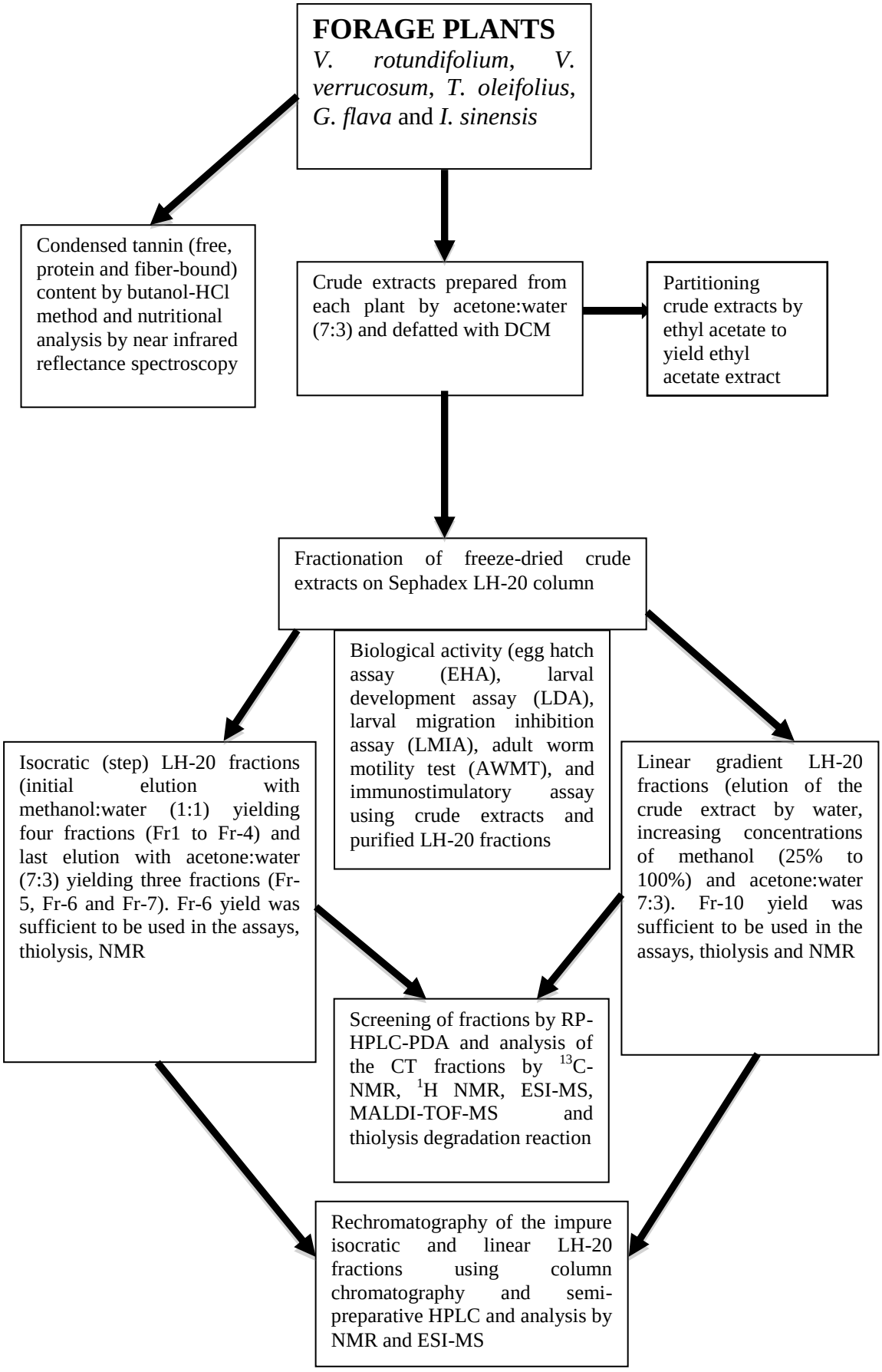

Figure 6–1 A flow chart of the experimental plan of the whole project

6.3. Condensed tannin content determination

The extractable, protein and fibre-bound CT concentrations of a selected Botswanan forage plants were determined by the butanol-HCl colorimetric method. All CT contents were determined using CT extracted from *Lotus pedunculatus* as a reference standard. The results indicated that CT (protein-bound and free) from *V. verrucosum, T. oleifolius* and *G. flava* were present in significant amounts, while trace amounts of CT was found in *V. rotundifolium* and *I. sinensis*.

6.4. Plant extraction and purification

Crude extracts were prepared from each plant using an acetone:water (7:3) solution and subsequently purified on a Sephadex LH-20 matrix, which employs an adsorption and size-exclusion chromatography. Step (conventional using methanol:water 1:1 and acetone: water 7:3) and linear gradient (non-conventional using water, increasing concentrations of methanol, and acetone:water 7:3) methods were used in the purification of the extracts. These compounds were monitored by a reverse-phase high performance liquid chromatography (RP-HPLC). The results indicated that methanolic fractions contained low Mw phenolics which were predominant as evidenced by sharp peaks in the chromatograms. Aqueous acetone fractions had broad unresolved humps which were indicative of CT. This phenomenon was observed in fractions from *V. verrucosum*, *T. oleifolius* and *G. flava*. The methanolic fractions from *V. rotundifolium* showed a broad hump, suggesting the presence of CT, and were re-chromatographed to separate the medium or high molecular weight CT (HMWCT or MMWCT) from the low molecular weight CT (LMWCT).

6.5. The effect of purified condensed tannins from Botswana forage plants on the free-living stages of gastrointestinal nematode livestock parasites

The effect of condensed tannins (CT) extracted from Botswanan forage plants on the free-living stages of a number of species of gastrointestinal nematode parasites derived from infected sheep were investigated using a number of *in vitro* assays. Fresh samples of five different plants (*Viscum rotundifolium, Viscum verrucosum, Tapinanthus oleifolius, Grewia flava and Ipomoea sinensis*) were collected in summer

(February 2009 and 2010). Anti-parasitic activity with some positive leads was achieved. The CT isolated from *V. verrucosum*, *T. oleifolius* and *G. flava* collected in 2009 completely inhibited the development of all parasite species. These CT fractions were more potent in inhibiting larval development of *H. contortus* than fractions from the same plant species collected in 2010. None of the CT fractions inhibited egg hatch or motility in *Caenorhabditis elegans*. However, a slight effect on larval migration was observed with some CT extracts. These results suggest that CT extracts from Botswanan forage plants have anti-parasitic properties *in vitro*, and that further research is required to determine whether the findings are also observed in goats fed the plants in a field situation. Future work should also focus on culturing the faecal samples from goats fed these plants and infected with different species of parasites. The study should be carried out to investigate for anti-parasitic effects of CT in reducing pasture contamination with infective larvae. Therefore, if pasture contamination with the infective larvae is reduced in some farming systems, dependence on the anthelmintic drug may also be reduced.

A summary of the plants (*V. verrucosum*, *T. oleifolius* and *G. flava)* with significant anti-parasitic activity and the chemicals they contained are shown in **Table 6-1**. The fractions that contained CTs or flavonoids but lacked LDA are also included. **Table 6-2** shows the linear gradient gractions tested for antiparasitic effects. These fractions were not used in the immunoassays.

6.6. Condensed tannins from Botswanan forage plants are effective priming agents of γσ T cells

The potential impact of CT extracts from *V. rotundifolium*, *V. verrucosum*, *T. oleifolius* and *G. flava* on γδ T cell activity was evaluated using an *in vitro* immunoassay. This study investigated whether plant extracts could prime γδ T cells via up-regulation of CD25 (interleukin-2 receptor alpha). Sephadex LH-20 fractions, isolated from *Viscum rotundifolium*, *Viscum verrucosum*, *Tapinanthus oleifolius* and *Grewia flava*, were screened against γδ T cells on kid, lamb and calf peripheral blood lymphocytes. The CT from *G. flava* and *T. oleifolius* significantly primed γδ T cells in kids in contrast to those of lambs and calves. These findings suggest that CT from a selected Botswanan forage plants can stimulate the innate immune system *in vivo* in selected ruminant species. However, the effect of CT from Botswanan forage plants in the stimulation of the innate immunity of ruminants *in vivo* has not been investigated.

Should this priming also occur *in vivo*, the feeding of these plants may have a positive impact on the ability of the animals to respond to pathogens, such as gastrointestinal nematodes. Once positive *in vivo* results are obtained, these plants may be integrated into some farming systems and extracts may be administered as a drench to small ruminants. In the future, fractions containing low molecular weight phenolics from the forage plants should also be evaluated for potential immunostimulatory properties *in vitro*. These fractions could not be evaluated because it took considerable time to carry out cell cultures and analysis. More studies are needed to search for other surface markers that could be stimulated. The impact of these phenolics of forage plants from Botswana on increasing the stability of mRNA and cell proliferation has not been investigated. Antioxidant and antibacterial assays were not conducted due to time constraints and are thus recommended for future studies. A summary of the plants (*V. verrucosum, T. oleifolius* and *G. flava)* with significant anti-parasitic activity and the chemicals they contained are shown in **Table 6-1**. The fractions that contained CTs or flavonoids but lacked immune effects are also included.

6.7. Characterisation of CT by NMR and thiolysis degradation reaction

A summary of the isolated CT from *V. verrucosum, T. oleifolius* and *G. flava* which exhibited anti-parasitic activity are shown in **Tables 6-1** and **6-2**. The CT from *T. oleifolius* and *G. flava* were also effective in the stimulation of innate immunity in ruminants, particularly in goats. The biological activity of CT depends on the concentration and the chemical structure. Thus, the chemical characteristics of the CT from *V. verrucosum, T. oleifolius* and *G. flava* were investigated by ^{13}C-NMR spectroscopy and thiolysis degradation reaction. Both methods provided information on the PC:PD ratio and the stereochemical information of the CT polymers. Thus, the results demonstrated that CT from *V. verrucosum, T. oleifolius* and *G. flava* were similar, with PC and *cis* dominant structures and variable chain lengths. Prodelphinidin (PD)-type CT were not present in all the fractions. Further purification of the thiolysis adducts of the degraded CT led to the isolation of (-)-epicatechin which was the dominant compound in the extension units of the CT polymer. The dominance of the *cis* stereochemistry may be one of the contributing factors to their biological activity. For further studies, biological activity should be investigated with the epicatechin monomer units. Anti-parasitic and immunostimulatory activity of the individual units of CT

polymers have not been investigated. It is hoped that these results would encourage extensive cultivation and commercialisation of these plants into Botswanan farming systems. Furthermore, the CT compounds may offer opportunities in development of nutraceutical and pharmaceutical products. Further studies should also be carried out on the chemical synthesis of the isolated molecules and also tested for biological activity.

6.8. Isolation and characterisation of flavonoids from Botswanan forage plants

Although the biological activity was not observed in fractions containing low Mw phenolics, these fractions were purified by a semi-preparative RP-HPLC and characterized by ^{1}H and ^{13}C-NMR and other MS techniques such as ESI-MS/MS. The following compounds were isolated and characterised: Quercetin-3-*O*-apiosyl-glucoside were tentatively identified from *Tapinanthus oleifolius*. Furthermore, a condensed tannin (CT) dimer such as epicatechin-4β→8-epicatechin and 2,3-*cis*-dihydroquercetin. Quercetin 3-O-(1→6)-α-rhamnosyl (1→2)-apiosyl-β-glucoside and quercetin 3-O-β-rhamnosyl-(1→6) glucoside were isolated from *V. verrucosum*. These chemical structures were confirmed by the COSY, HMQC, HSQC, DEPT and TOCSY experiments. For further confirmation of the chemical structures, flavonoids should be derivatised and subsequently analysed by ^{1}H and ^{13}C-NMR and GC-MS. The sugar adducts should also be confirmed by acid hydrolysis and the retention times of the products compared to those of authentic standards. This experiment could not be carried out due to unavailability of standards. However, the results showed that the sugars such as glucose, rhamnose and apiose were attached to a quercetin moiety. Pure isolates were not obtained from *G. flava*, *V. rotundifolium* and *I. sinensis* as further purification was not conducted due to time constraints. Functionalisation (i.e. methylation or galloylation) of the active components and subsequent anti-parasitic and immunostimulatory activity of the products were not conducted due to time constraints. Further studies should also be carried out on the chemical synthesis of the isolated molecules and tested for biological activity.

Table 6-1 A summary of the step LH-20 fractions from plants collected in 2009 and 2010 and their anti-parasitic (AP) and immune system (IS)stimulation

Plant sample and date collection	Extract code and fractions of interest	First batch	AP	Second batch	AP	Eluting solvent	IS	CT or flavonoids	Monomer unit
V. rotundifolium									
27/02/2009	OTC00301	OTC02101	0	OTC02201	0	MeOH:H2O (1:1)	not active	CT	epicatechin
		OTC02102	0	OTC02202	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC02103	0	OTC02203	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC02104	0	OTC02204	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC02105	0	OTC02205	0	Acetone:H2O (7:3)	n.d	flavonoid	n.d
10/02/2010	OTD01801	OTD02901	0	OTD03001	0	MeOH:H2O (1:1)	n.d	CT	n.d
		OTD02902	0	OTD03002	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD02903	0	OTD03003	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD02904	0	OTD03004	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
V. verrucosum	OTC00601	OTC02901	0	OTC03101	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
27/02/2009		OTC02902	0	OTC03102	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC02903	0	OTC03103	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC02904	0	OTC03104	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTC02905	0	OTC03105	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTC02906	1	OTC03106	0	Acetone:H2O (7:3)	not active	CT	epicatechin
	SF7	OTC02907	1	OTC03107	0	Acetone:H2O (7:3)	nd	CT	epicatechin
12/02/2010	OTD01901	OTD03101	0	OTd03201	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03102	0	OTD03202	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03103	0	OTD03203	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03104	0	OTD03204	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03105	0	OTD03205	1	Acetone:H2O (7:3)	n.d	CT	n.d
		OTD03106	0	OTD03206	1	Acetone:H2O (7:3)	not active	CT	epicatechin
T. oleifolius	OTC00801	OTC03401	0	OTC03501	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
27/02/2009		OTC03402	0	OTC03502	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC03403	0	OTC03503	0	MeOH:H2O (1:1)	n.d	flavonoid	quercetin-sugars
		OTC03404	0	OTC03504	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTC03405	0	OTC03505	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTC03406	1	OTC03506	1	Acetone:H2O (7:3)	minimal active	CT	epicatechin
	SF7	OTC03407	1	OTC03507	1	Acetone:H2O (7:3)	minimal active	CT	epicatechin
2/02/2010	OTD02001	OTD03301	0	OTD03401	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03302	0	OTD03402	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03303	0	OTD03403	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03304	0	OTD03404	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTD03305	0	OTD03405	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTD03306	0	OTD03406	0	Acetone:H2O (7:3)	n.d	CT	epicatechin
	SF7	OTD03307	0	OTD03407	0	Acetone:H2O (7:3)	nd	CT	epicatechin
G. flava	OTC00901	OTC03701	0	OTC04001	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
27/02/2009		OTC03702	0	OTC04002	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC03703	0	OTC04003	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC03704	0	OTC04004	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTC03705	0	OTC04005	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTC03706	1	OTC04006	1	Acetone:H2O (7:3)	active	CT	epicatechin
	SF7	OTC03707	1	OTC04007	1	Acetone:H2O (7:3)	active	CT	epicatechin
2/02/2010	OTD02101	OTC03501	0	OTD03601	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC03502	0	OTD03602	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC03503	0	OTD03603	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC03504	0	OTD03604	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTC03505	1	OTD03605	1	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTC03506	1	OTD03606	1	Acetone:H2O (7:3)	n.d	CT	epicatechin
	SF7	OTC03507	1	OTD03607	1	Acetone:H2O (7:3)	nd	CT	epicatechin
I. sinensis	OTD01101	OTC04201	0	OTC04401	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
3/03/2009		OTC04202	0	OTC04402	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC04203	0	OTC04403	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTC04204	0	OTC04404	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTC04205	0	OTC04405	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTC04206	0	OTC04406	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF7	OTC03707	0	OTC04007	0	Acetone:H2O (7:3)	nd	CT	n.d
2/02/2010	OTD02201	OTD03701	0	OTD03601	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03702	0	OTD03602	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03503	0	OTD03603	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
		OTD03504	0	OTD03604	0	MeOH:H2O (1:1)	n.d	flavonoid	n.d
	SF5	OTD03505	0	OTD03605	0	Acetone:H2O (7:3)	n.d	CT	n.d
	SF6	OTD03506	0	OTD03606	0	Acetone:H2O (7:3)	not active	CT	n.d

Larval scoring for inhibition of larval development: 0= 0% inhibition, 1= complete inhibition (100%). All crude extracts were not active and their results not included. CT containing fractions from *T. oleifolius* (OTC03306 and OTC03406) and *G. flava* (OTC03706 and OTC04006) primed γδ T cells, anti-parasitic (AP) and immune stimulating (IS) activity, n.d not determined

Table 6-2 A summary of the linear gradient LH-20 fractions from plants collected in 2009 and 2010 and their anti-parasitic activity from a two-fold dilution. Immunoasay was not conducted for these fractions.

Plant name	Date of collection	Sample code	Description	Two-fold serial dilution 500	250	125-0.488
V. verrucosum	27/02/2009	GF-10	Acetone:water (7:3)	**1**	0	0
T. oleifolius	27/02/2009	GF-8	100% methanol	0	0	0
		GF-9	100% methanol	**1**	0	0
		GF-10	Acetone:water (7:3)	**1**	**1**	0
		GF-11	Acetone:water (7:3)	**1**	**1**	0
G. flava	27/02/2009	GF-9	100% methanol	**1**	**1**	0
		GF-10	Acetone:water (7:3)	**1**	**1**	0
		GF-11	Acetone:water (7:3)	**1**	**1**	0

Abbreviations: larval scoring: 0 (not dead); 1 (100% inhibition or completely dead); nd-not determined, GF- gradient fraction

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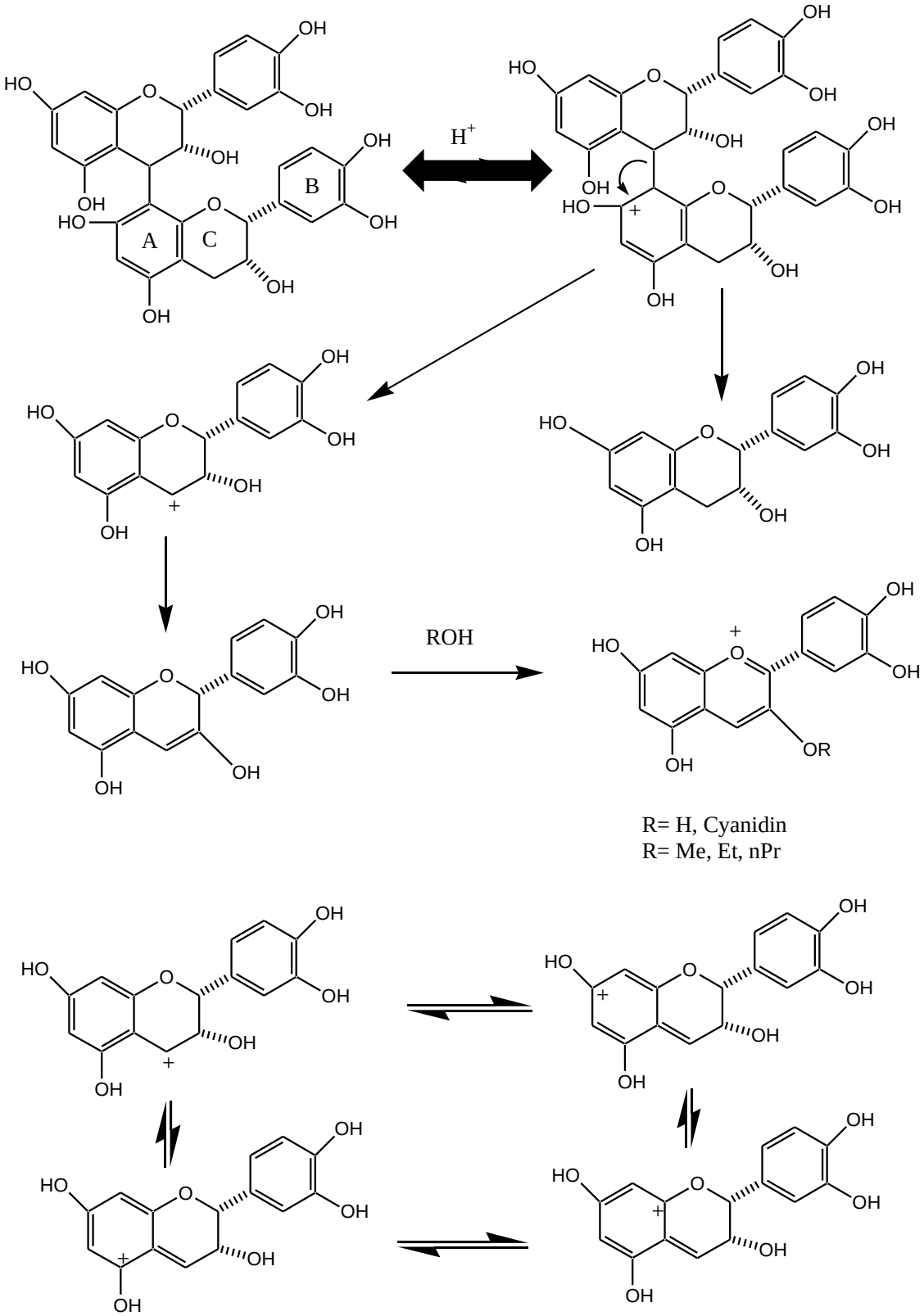
CHAPTER SEVEN: APPENDICES FOR CHAPTER 1-6

Appendix 1-1 Acid catalysed degradation of the CT dimeric procyanidin B-2 and resonance stabilisation of the carbocation



Appendix 2-1 Pictures of plants harvested in Botswana in 2009 and 2010

Grewia flava

Viscum rotundifolium

Ipomoea sinensis

Viscum verrucosum

Tapinanthus oleifolius

Appendix 2-2 General physico-chemical characteristics and chromatographic performance of Sephadex LH-20 powder

Matrix	Hydroxypropylated, cross-linked dextran
Bead form	spherical, porous
Average particle size	
dry	70 µm
in methanol	103 µm (1 g dry powder/4 mL)
pH stability	
Working	2-13
Cleaning	2-13
Chemical stability	Stable in most aqueous and organic eluent systems
	Not stable below pH 2 nor to strong oxidizing agents
Maximum linear flow rate	700 cm/h (volumetric flow: 58.8 mL/min)
Recommended linear flow rate	60 cm/h (volumetric flow: 5.0 mL/min)
Operating temperature	4 ºC to 40 ºC
Maximum operating back pressure is solvent dependent	
Sample loading volumes	
Adsorption mode	Depends on the resolution required
Molecular sizing	< 2% total medium volume
Partition mode	< 15 total medium volume
Exclusion limit	4000-5000 (depends on solvent)

(Adopted from GE Healthcare website New Zealand on 15th June 2009; http://www5.gelifesciences.com/aptrix/upp01399.nsf/Content/Laboratory)

Appendix 2-3 Conversion of linear flow (cm/hour) to volumetric flow rates (mL/min) and vice versa (adopted from GE Healthcare homepage)

From linear flow (cm/hour) to volumetric flow rate (mL/min)

Volumetric flow rate = Linear flow (cm/h)/60 x cross sectional area (cm^2)

= $Y/60 \times \pi x d^2/4$

where Y = linear flow in cm/h, d = column inner diameter in cm ($\pi d^2/4$ is the same as πr^2)

Example 1

The recommended flow rate of Sephadex LH-20 column is 60 cm/h and diameter of the column = 2.5 cm. What would be the recommended volumetric flow rate in mL/min?

Volumetric flow rate = 60 cm/h x 3.14 x 2.5 x 2.5/60 x 4

= 4.9 mL/min approximately 5.0 mL/min used with FPLC for LH-20 columns

Appendix 2-4 Running method for the use of Sephadex LH-20 column coupled to FPLC and using aqueous methanol (1:1, v/v) as the eluting solvent for step fractionation

Time	**Parameter**	**Action**
0.0	Conc %B	0.0
0.0	Port	4.0 (deactivated/default)
0.0	mL/min	5.0
0.1	Conc %B	50.0 Column equilibration
60.0	Con %B	50.0
60.1	Conc %B	0.0
60.2	mL/min	0.0
60.3	Port	4.1 (Activated for sample inject)
60.4	Alarm	0.1
60.4	Hold	

Manual injection of the sample

60.5	Port	4.0
60.6	mL/min	5.0
60.7	Conc %B	0.0 Sample elution
60.8	Conc %B	50.0
250.0	Conc %B	50.0
250.1	Conc %B	0.0 Column equilibration
250.2	Conc %B	0.0
250.2	Conc %B	0.0

Appendix 2-5 Running method for the use of Sephadex LH-20 column coupled to FPLC and using aqueous acetone (3:7, v/v) as the eluting solvent for step fractionation.

Time	**Parameter**	**Action**
0.0	Conc %B	0.0
0.0	Port	4.0 (deactivated/default)
0.0	mL/min	5.0
0.1	mL/min	0.0
0.1	Port	4.1 (Activated for sample inject)
0.2	Alarm	0.1
0.2	Hold	
Manual injection of the sample		
0.3	Port	4.0
0.4	mL/min	5.0
0.5	Conc %B	0.0
0.6	Conc %B	70.0
250.0	Conc %B	70.0
250.1	Conc %B	0.0
300.0	Conc %B	0.0
300.1	Conc % B	0.0

Appendix 2-6 Weights of the step LH-20 fractions from plants collected in 2009 and 2010

Plant sample	Date collected	Extract code	First batch	Amount (mg)	Second batch	Amount (mg)	Eluting Solvent
V. rotundifolium	27/02/2009	OTC00301	OTC02101	1660	OTC02201	3052	MeOH:H2O (1:1)
			OTC02102	1019	OTC02202	439	MeOH:H2O (1:1)
			OTC02103	32.4	OTC02203	56	MeOH:H2O (1:1)
			OTC02104	68.1	OTC02204	5.8	MeOH:H2O (1:1)
			OTC02105	4.7	OTC02205	4.7	Acetone:H_2O (7:3)
	10/02/2010	OTD01801	OTD02901	2950	OTD03001	3030	MeOH:H_2O (1:1)
			OTD02902	675	OTD03002	583	MeOH:H_2O (1:1)
			OTD02903	153	OTD03003	141	MeOH:H_2O (1:1)
			OTD02904	17	OTD03004	12	MeOH:H_2O (1:1)
V. verrucosum	27/02/2009	OTC00601	OTC02901	2206	OTC03101	2560	MeOH:H_2O (1:1)
			OTC02902	661	OTC03102	447	MeOH:H_2O (1:1)
			OTC02903	65.9	OTC03103	56.4	MeOH:H_2O (1:1)
			OTC02904	44.6	OTC03104	25.2	MeOH:H_2O (1:1)
			OTC02905	2.4	OTC03105	2.5	Acetone:H_2O (7:3)
			OTC02906	378	OTC03106	385.6	Acetone:H_2O (7:3)
			OTC02907	14.6	OTC03107	10.6	Acetone:H_2O (7:3)
	12/02/2010	OTD01901	OTD03101	2268	OTD03201	2600	MeOH:H_2O (1:1)
			OTD03102	331	OTD03202	330	MeOH:H_2O (1:1)
			OTD03103	43	OTD03203	50	MeOH:H_2O (1:1)
			OTD03104	17	OTD03204	20	MeOH:H_2O (1:1)
			OTD03105	5	OTD03205	1	Acetone:H_2O (7:3)
			OTD03106	176	OTD03206	170	Acetone:H_2O (7:3)
T. oleifolius	27/02/2009	OTC00801	OTC03401	2041	OTC03501	2950	MeOH:H_2O (1:1)
			OTC03402	513	OTC03502	296	MeOH:H_2O (1:1)
			OTC03403	4.7	OTC03503	8	MeOH:H_2O (1:1)
			OTC03404	2.7	OTC03504	6.4	MeOH:H_2O (1:1)
			OTC03406	41.4	OTC03506	119	Acetone:H_2O (7:3)
			OTC03407	2.7	OTC03507	15.1	Acetone:H_2O (7:3)
	2/02/2010	OTD02001	OTD03301	2271	OTD03401	2264	MeOH:H_2O (1:1)
			OTD03302	306	OTD03402	131	MeOH:H_2O (1:1)
			OTD03303	37	OTD03403	38	MeOH:H_2O (1:1)
			OTD03304	13	OTD03404	5	MeOH:H_2O (1:1)
			OTD03305	3	OTD03405	3	Acetone:H_2O (7:3)
			OTD03306	581	OTD03406	588	Acetone:H_2O (7:3)
			OTD03307	10	OTD03407	12	Acetone:H_2O (7:3)

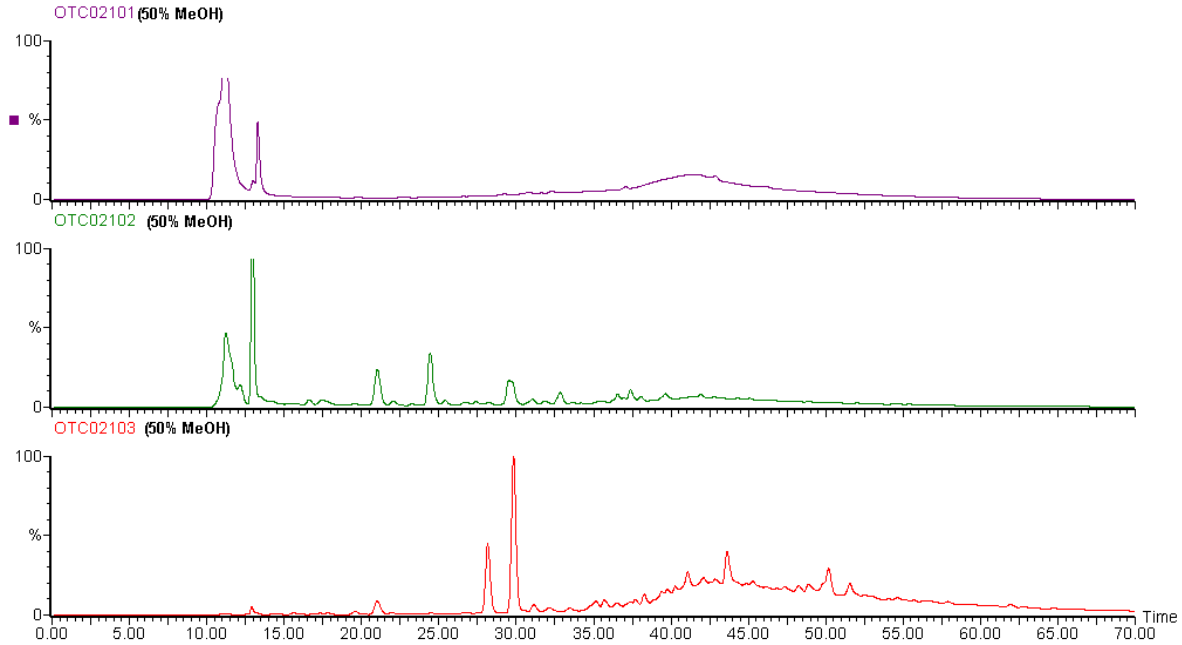
Appendix 2-7 Weights of step LH-20 fractions from plants collected in 2009 and 2010

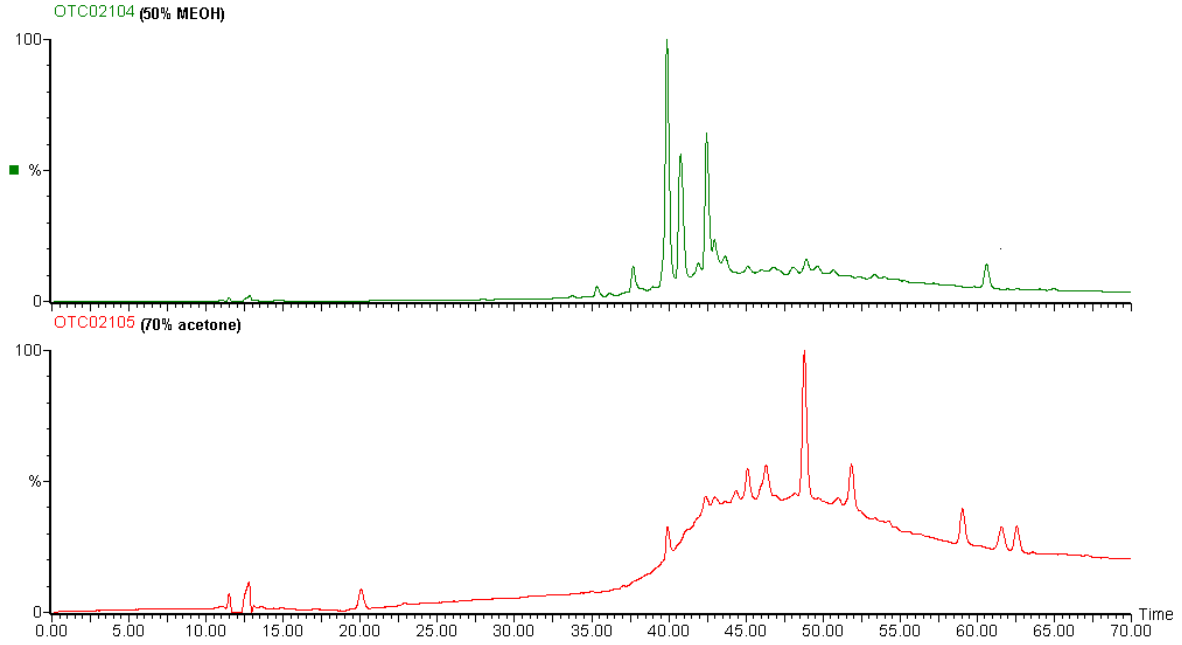
Plant sample	Date collected	Extract code	First batch	Amount (mg)	Second batch	Amount (mg)	Eluting Solvent
G. flava	27/02/2009	OTC00901	OTC03701	540	OTC04001	495	MeOH:H_2O (1:1)
			OTC03702	1440	OTC04002	1700	MeOH:H_2O (1:1)
			OTC03703	253	OTC04003	258	MeOH:H_2O (1:1)
			OTC03704	82.2	OTC04004	101	MeOH:H_2O (1:1)
			OTC03705	9	OTC04005	11.7	Acetone:H_2O (7:3)
			OTC03706	1230	OTC04006	1210	Acetone:H_2O (7:3)
			OTC03707	59.2	OTC04007	23.2	Acetone:H_2O (7:3)
	2/02/2010	OTD02101	OTD03501	540	OTD03601	626	MeOH:H_2O (1:1)
			OTD03502	1440	OTD03602	1772	MeOH:H_2O (1:1)
			OTD03503	253	OTD03603	281	MeOH:H_2O (1:1)
			OTD03504	82.2	OTD03604	84	MeOH:H_2O (1:1)
			OTD03505	9	OTD03605	12	Acetone:H_2O (7:3)
			OTD03506	1230	OTD03606	817	Acetone:H_2O (7:3)
			OTD03507	59.2	OTD03607	13	Acetone:H_2O (7:3)
I. sinensis	3/03/2009	OTD01101	OTC04201	738	OTC04401	2040	MeOH:H_2O (1:1)
			OTC04202	2440	OTC04402	1000	MeOH:H_2O (1:1)
			OTC04203	173	OTC04403	10.9	MeOH:H_2O (1:1)
			OTC04204	19.7	OTC04404	20.5	MeOH:H_2O (1:1)
			OTC04206	64.3	OTC04406	29.2	Acetone:H_2O (7:3)
	2/02/2010	OTD02201	OTD03701	1153	OTD03801	762	MeOH:H_2O (1:1)
			OTD03702	2005	OTD03802	2423	MeOH:H_2O (1:1)
			OTD03703	349	OTD03803	231	MeOH:H_2O (1:1)
			OTD03704	47	OTD03804	42	MeOH:H_2O (1:1)
			OTD03705	traces	OTD03805	7	Acetone:H_2O (7:3)
			OTD03706	89	OTD03806	53	Acetone:H_2O (7:3)

Appendix 2-8 Linear gradient LH-20 fractions from plants collected in 2009

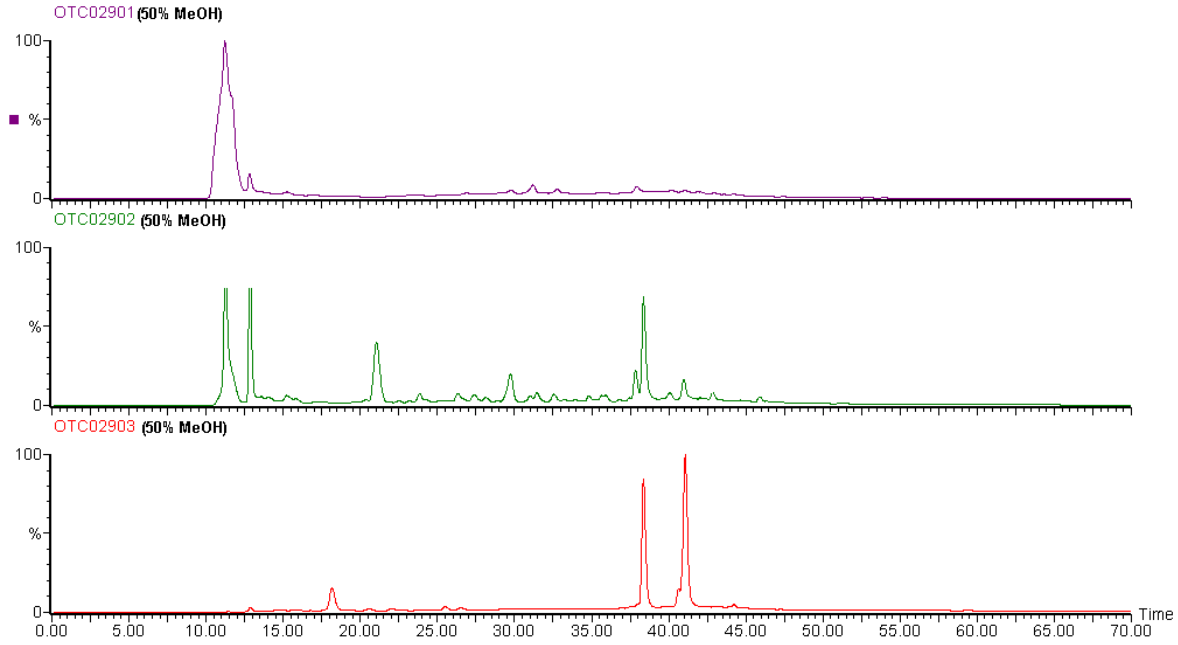
Plant sample	Date collected	Extract code	First batch	Amount (mg)	Eluting Solvent
V. rotundifolium	27/02/2009	OTD01801	OTD04801	6970	H_2O
			OTD04802	965	H_2O
			OTD04803	126	MeOH:H_2O (1:3)
			OTD04804	158	MeOH:H_2O (1:1)
			OTD04805	485	MeOH:H_2O (3:1)
			OTD04806	292	100% MeOH
			OTD04807	16	100% MeOH
V. verrucosum	27/02/2009	OTC00701	OTD04201	8620	H_2O
			OTD04202	496	H_2O
			OTD04203	91	MeOH:H_2O (1:3)
			OTD04204	72	MeOH:H_2O (1:1)
			OTD04205	198	MeOH:H_2O (3:1)
			OTD04206	238	100% MeOH
			OTD04207	144	100% MeOH
			OTD04208	113	100% MeOH
			OTD04209	94	100% MeOH
			OTD04210	267	Acetone:H_2O (7:3)
			OTD04211	52	Acetone:H_2O (7:3)
T. oleifolius	27/02/2009	OTD00701	OTD04401	8590	H_2O
			OTD04402	306	H_2O
			OTD04403	33	MeOH:H_2O (1:3)
			OTD04404	47	MeOH:H_2O (1:1)
			OTD04405	65	MeOH:H_2O (3:1)
			OTD04406	43	100% MeOH
			OTD04407	27	100% MeOH
			OTD04408	16	100% MeOH
			OTD04409	22	100% MeOH
			OTD04410	128	Acetone:H_2O (7:3)
			OTD04411	121	Acetone:H_2O (7:3)
G. flava	27/02/2009	OTC00901	OTD04601	2750	H_2O
			OTD04602	4380	H_2O
			OTD04603	827	MeOH:H_2O (1:3)
			OTD04604	102	MeOH:H_2O (1:1)
			OTD04605	100	MeOH:H_2O (3:1)
			OTD04606	145	100% MeOH
			OTD04607	404	100% MeOH
			OTD04608	670	100% MeOH
			OTD04609	794	100% MeOH
			OTD04610	538	Acetone:H_2O (7:3)
			OTD04611	661	Acetone:H_2O (7:3)
			OTD04612	111	Acetone:H_2O (7:3)

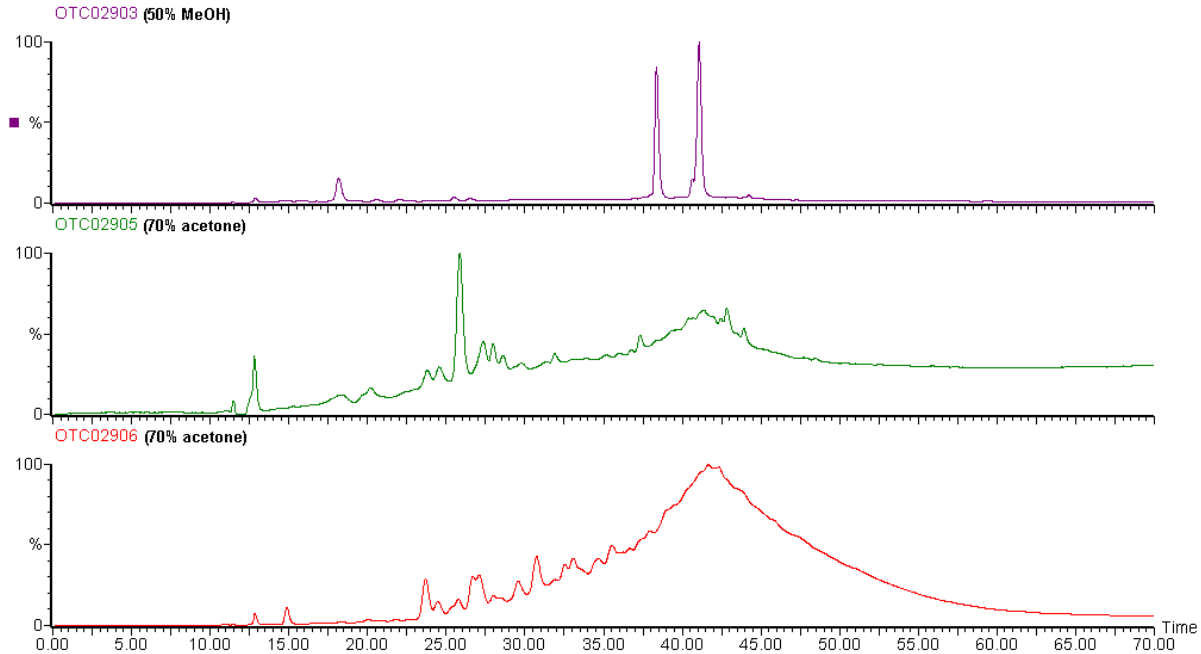
Appendix 2-9 Chromatograms of LH-20 fractions (OTC02101-05) of *V. rotundifolium* at 280 nm



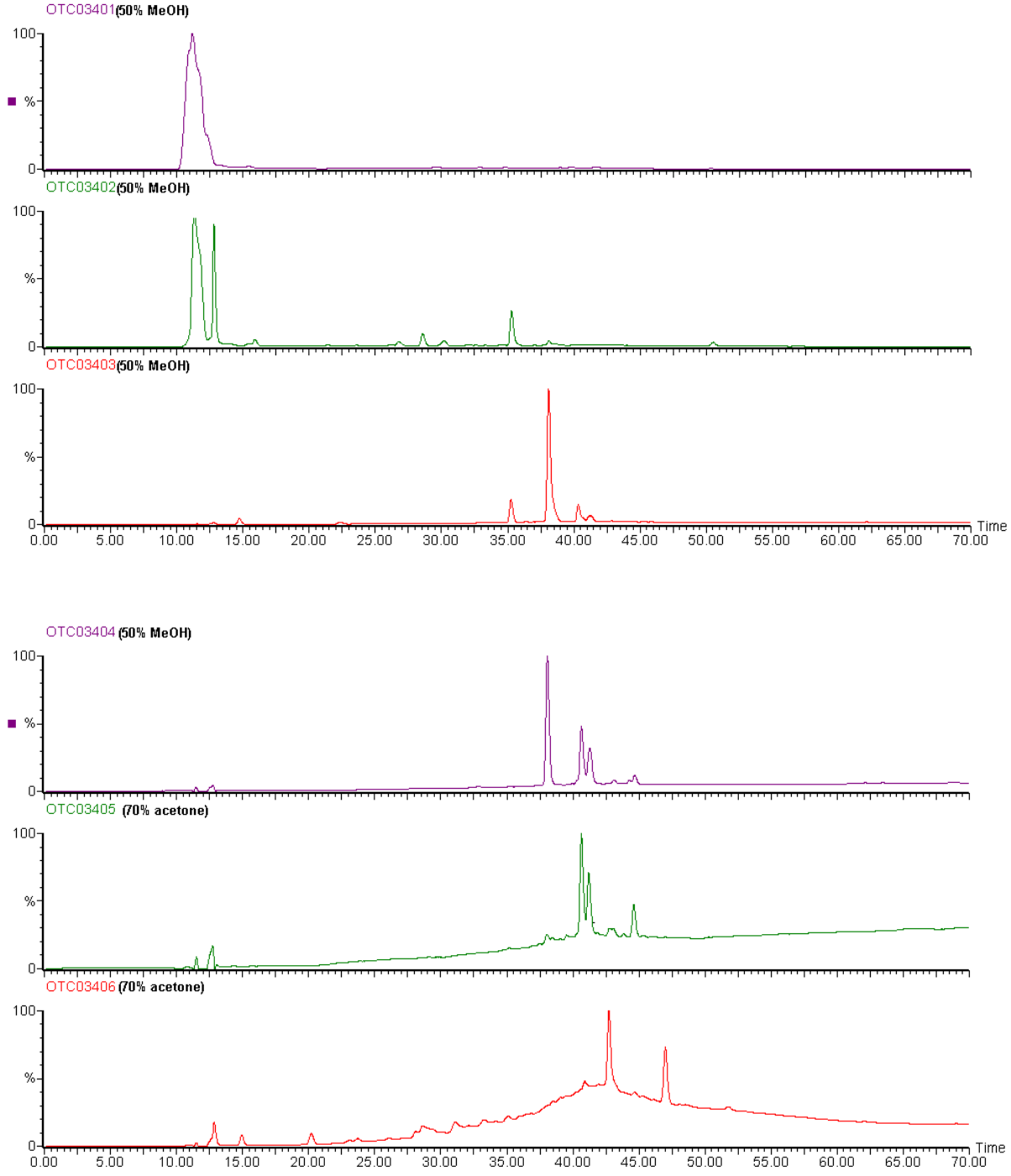


Appendix 2-10 Chromatograms of LH-20 fractions (OTC02901-06) of *V. verrucosum* at 280 nm

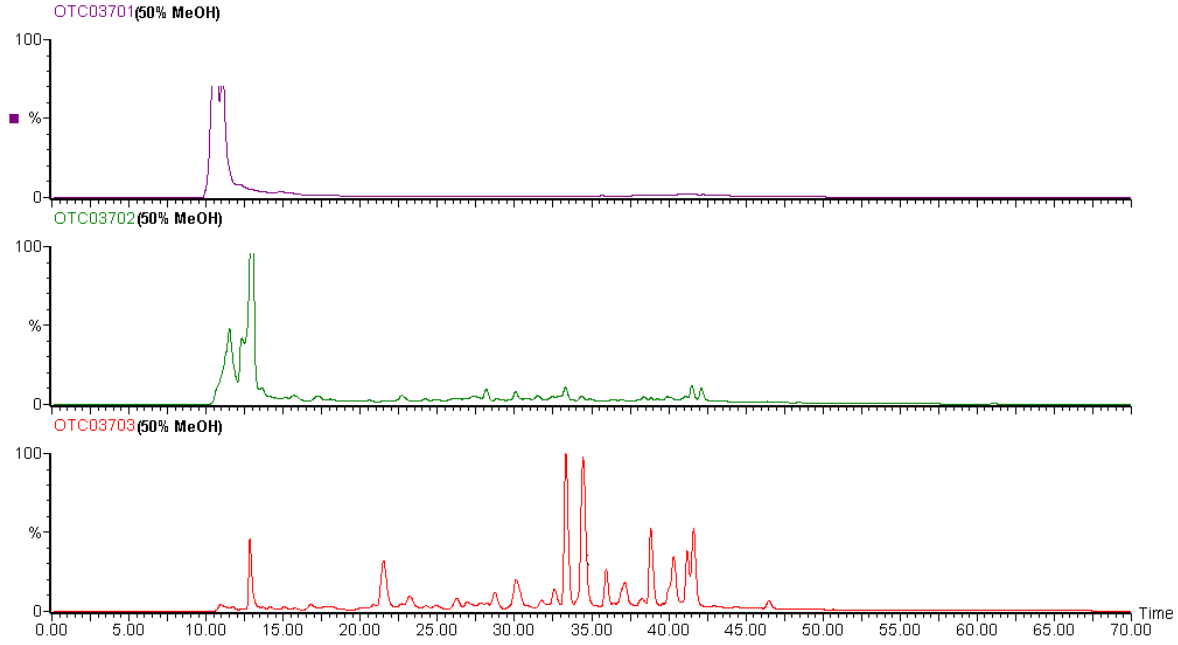


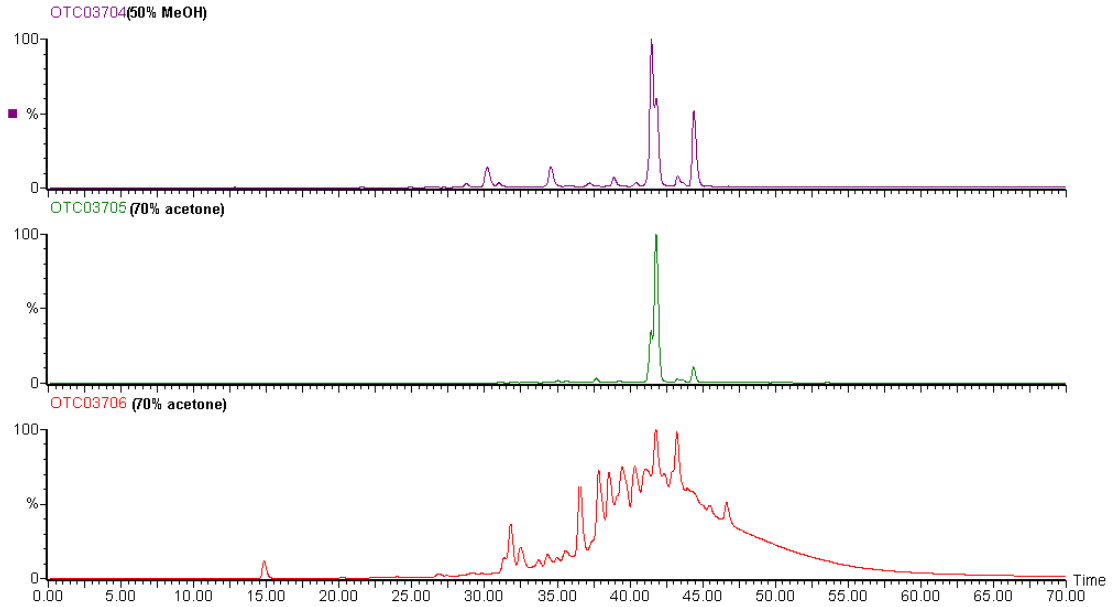


Appendix 2-11 Chromatograms of LH-20 fractions (OTC03401-06) of *T. oleifolius* at 280 nm

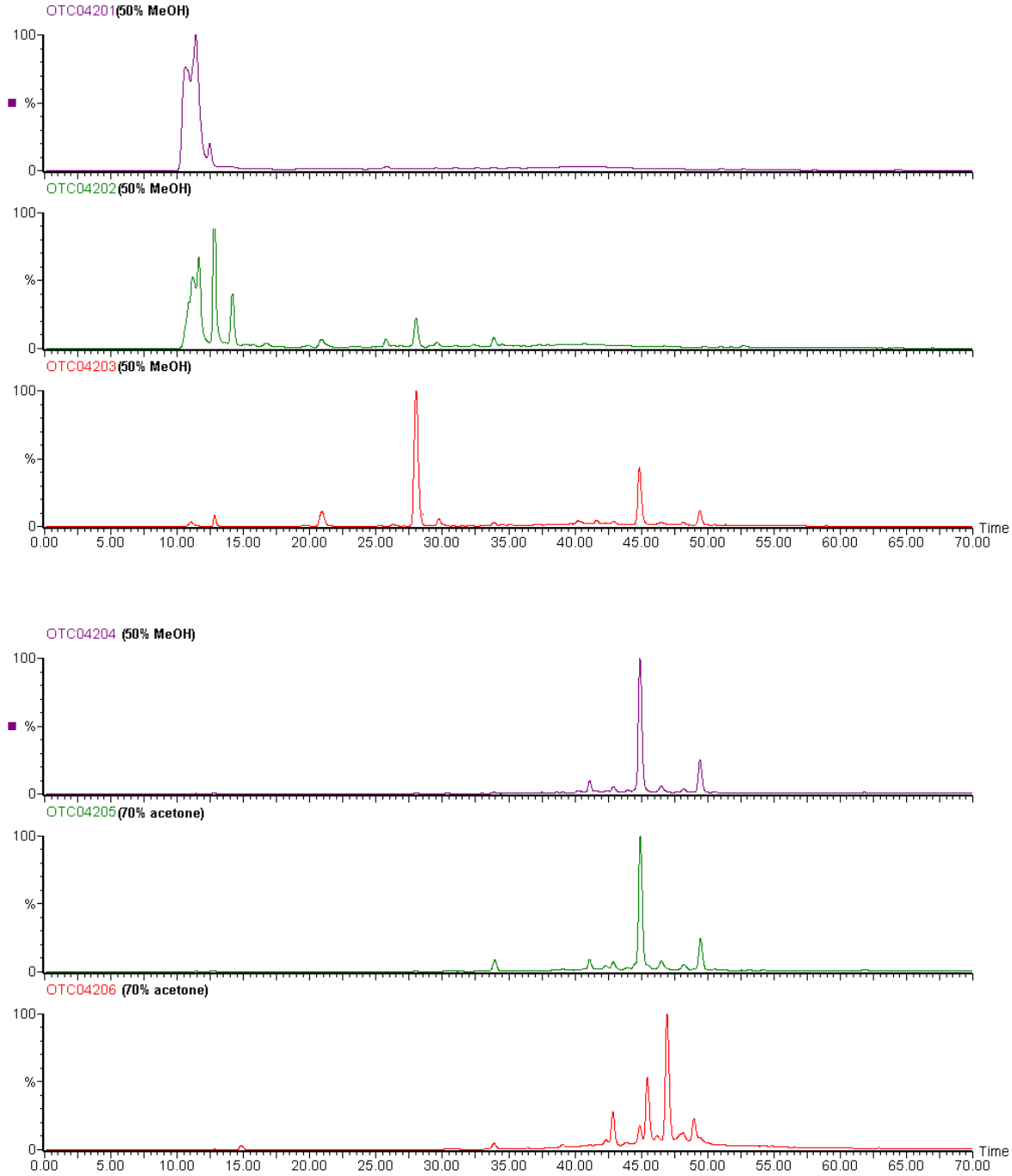


Appendix 2-12 Chromatograms of LH-20 fractions (OTC03701-06) of *G. flava* at 280 nm

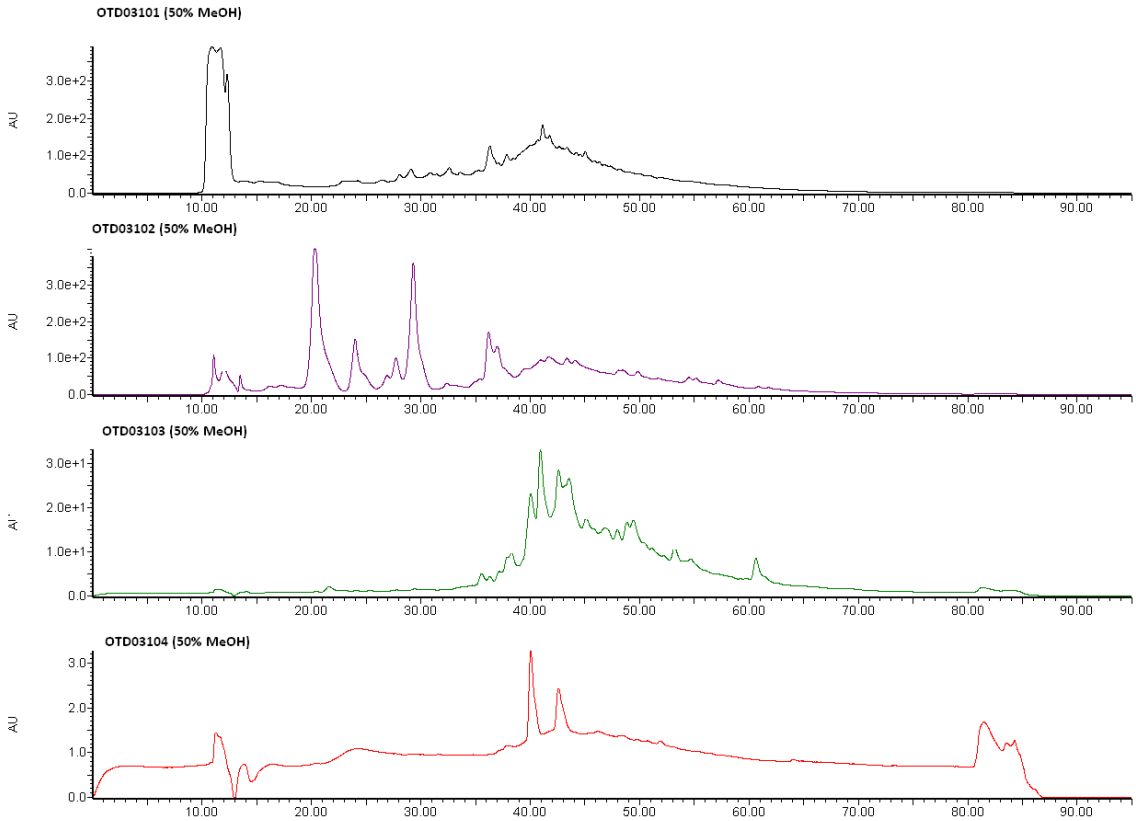




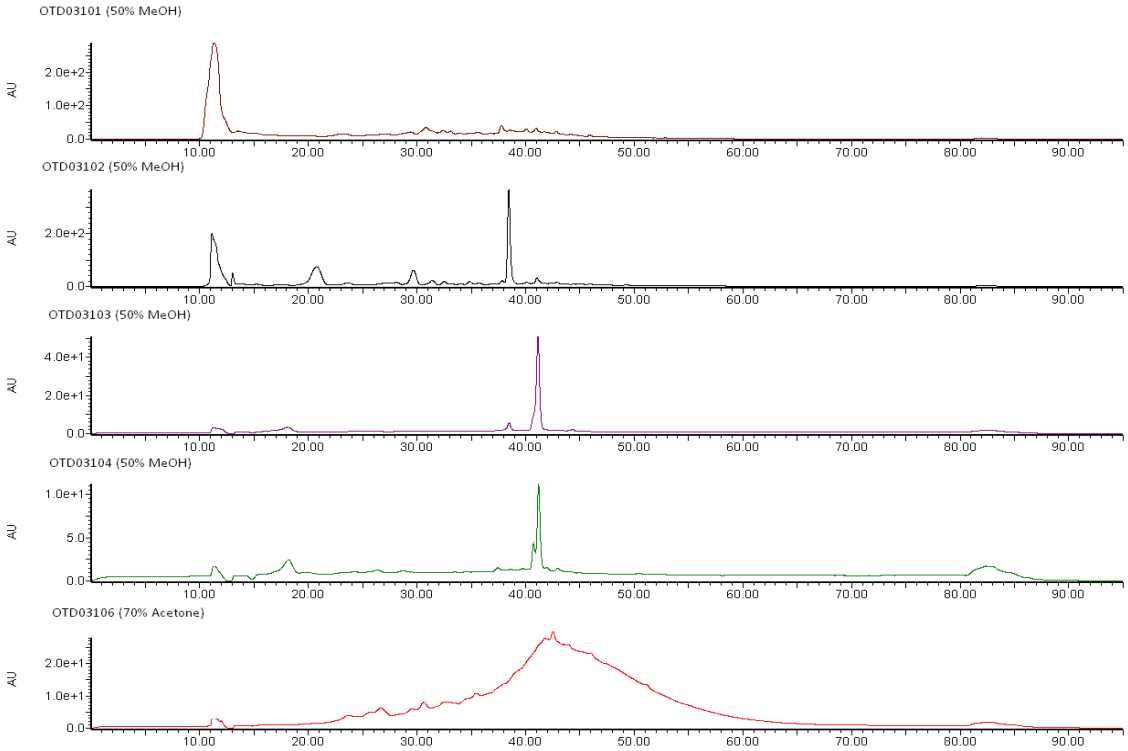
Appendix 2-13 Chromatograms of LH-20 fractions (OTC04201-06) of *I. sinensis* at 280 nm



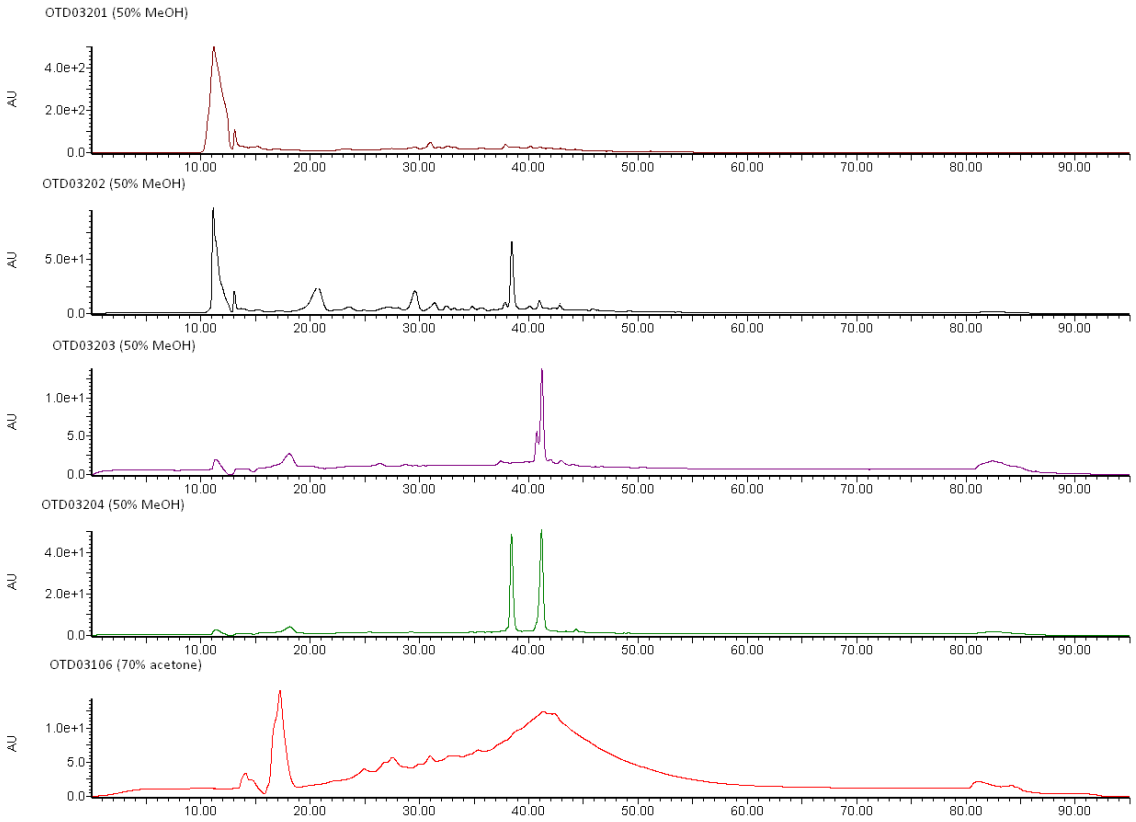
Appendix 2-14 HPLC chromatograms (50% MeOH and 70% acetone for step LH-20 fractionation) for fractions from plants collected in 2010 for *V. verrucosum*, *V. rotundifolium*, *T. oleifolius* and *G. flava* and *I. sinensis* from the step LH-20 method HPLC chromatograms of LH-20 fractions (OTD03101-04) of *V. rotundifolium* at 280 nm



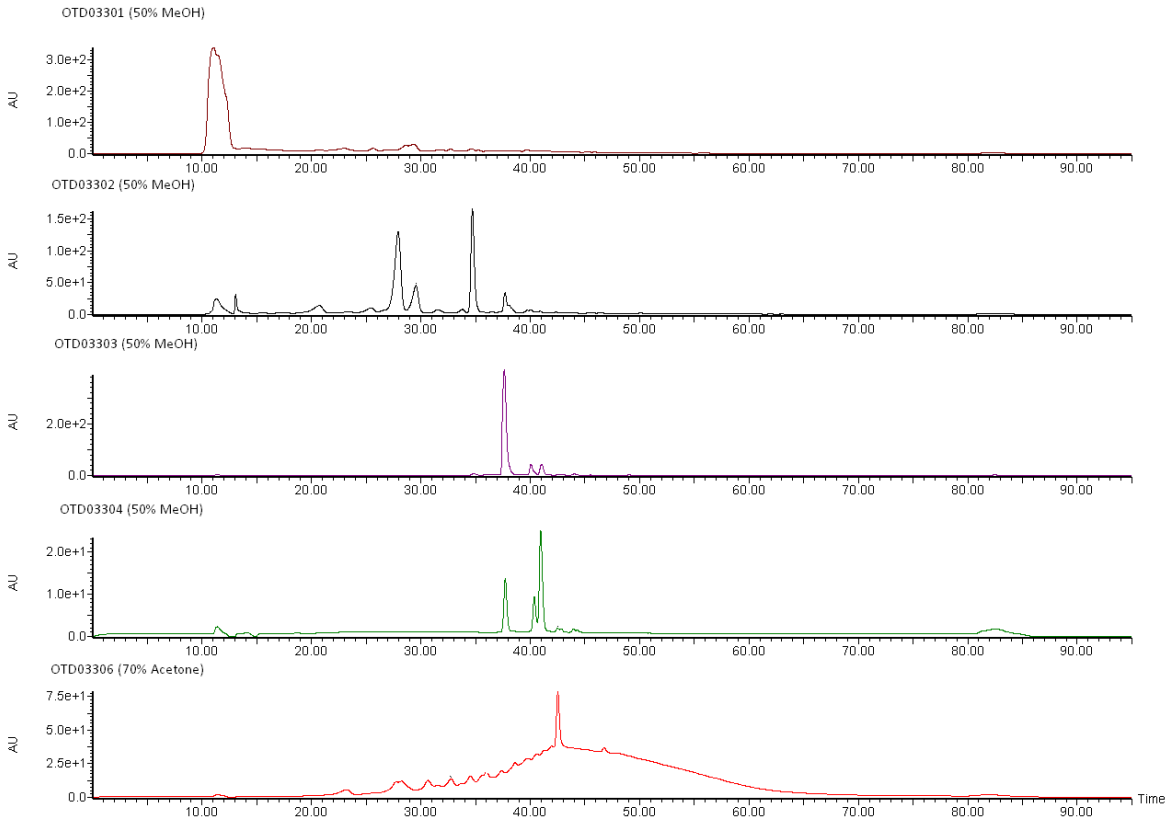
Appendix 2-15 HPLC-PDA chromatograms of LH-20 fractions (OTD03201-06) of *V. verrucosum* at 280 nm 1st batch



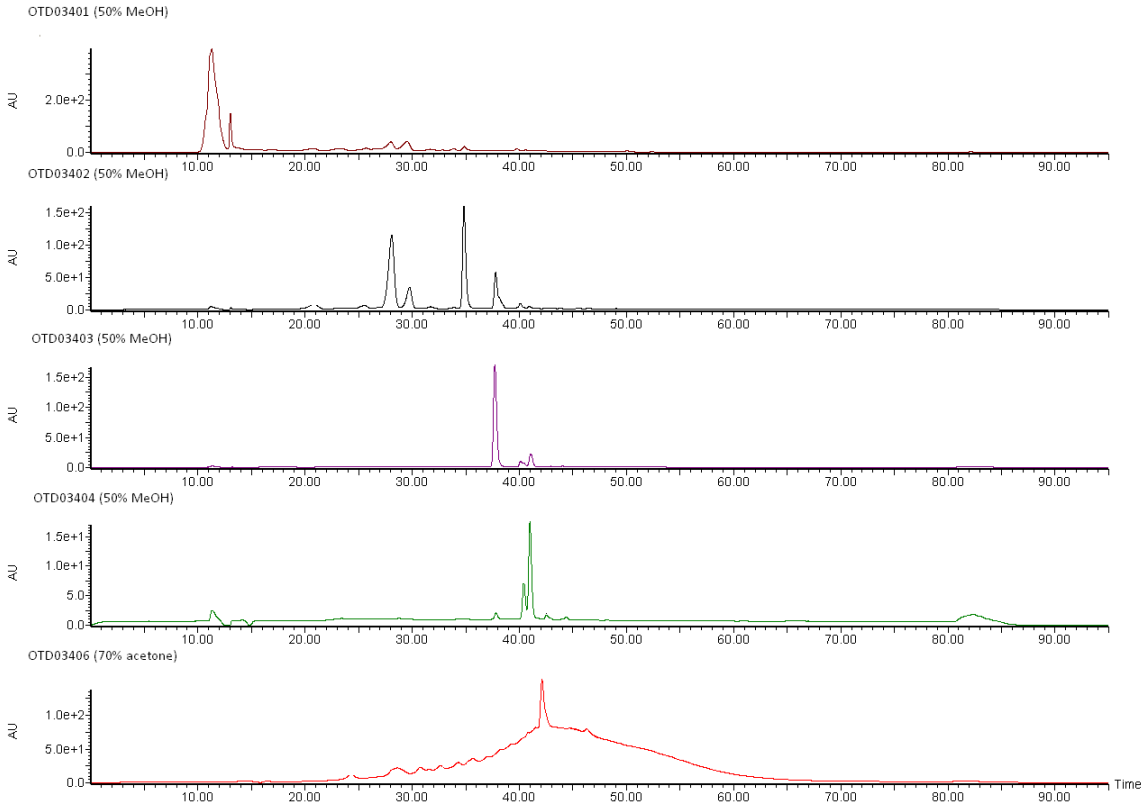
Appendix 2-16 HPLC-PDA chromatograms of LH-20 fractions (OTD03201-06) of *V. verrucosum* at 280 nm 2nd batch



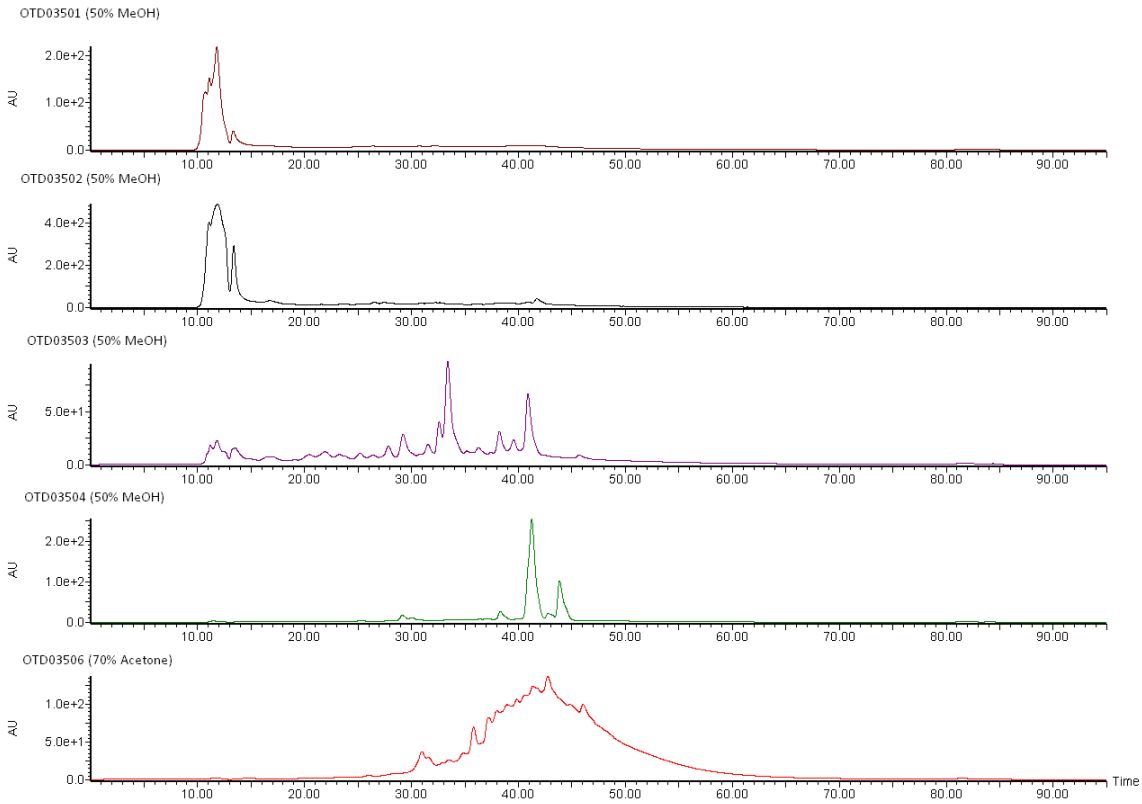
Appendix 2-17 HPLC-PDA chromatograms of LH-20 fractions *T. oleifolius* (OTD03301-06) at 280 nm 1st batch



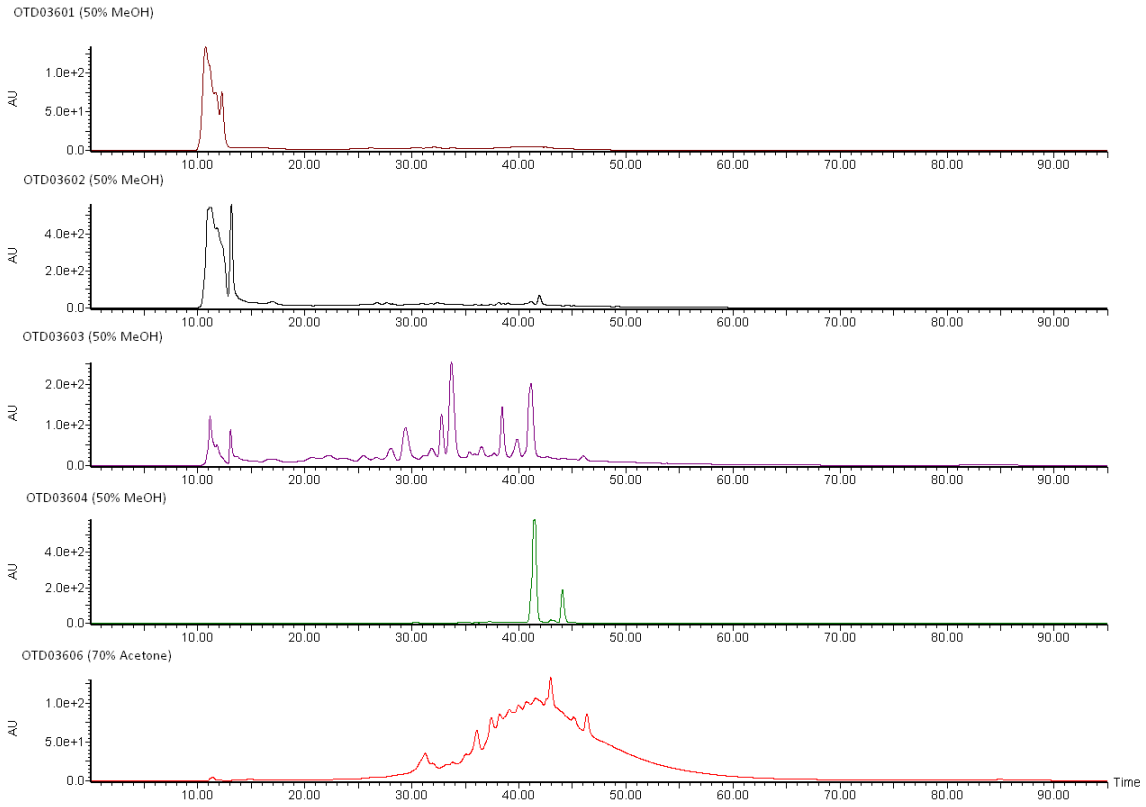
Appendix 2-18 HPLC-PDA chromatograms of LH-20 fractions *T. oleifolius* (OTD03401-06) at 280 nm 2nd batch



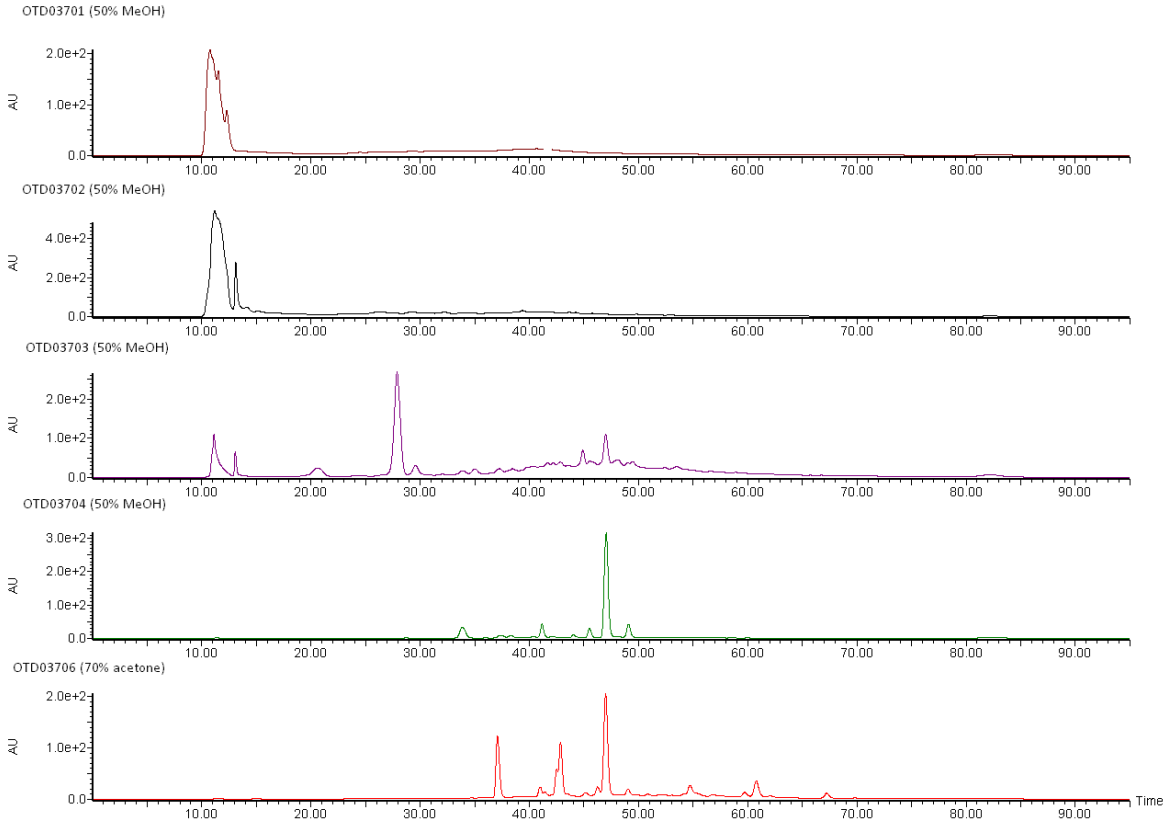
Appendix 2-19 HPLC-PDA chromatograms of LH-20 fractions *G. flava* (OTD03501-06) at 280 nm 1st batch



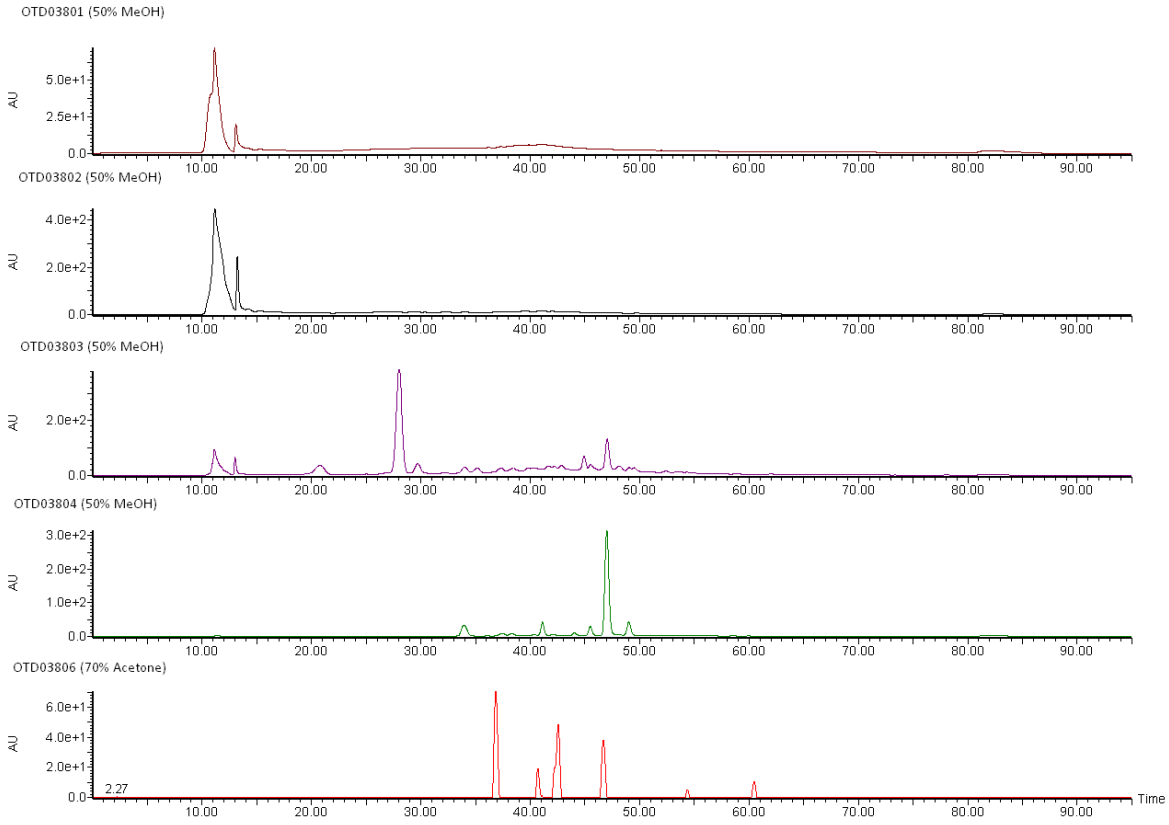
Appendix 2-20 HPLC-PDA chromatograms of LH-20 fractions *G. flava* (OTD03601-06) at 280 nm 2nd batch



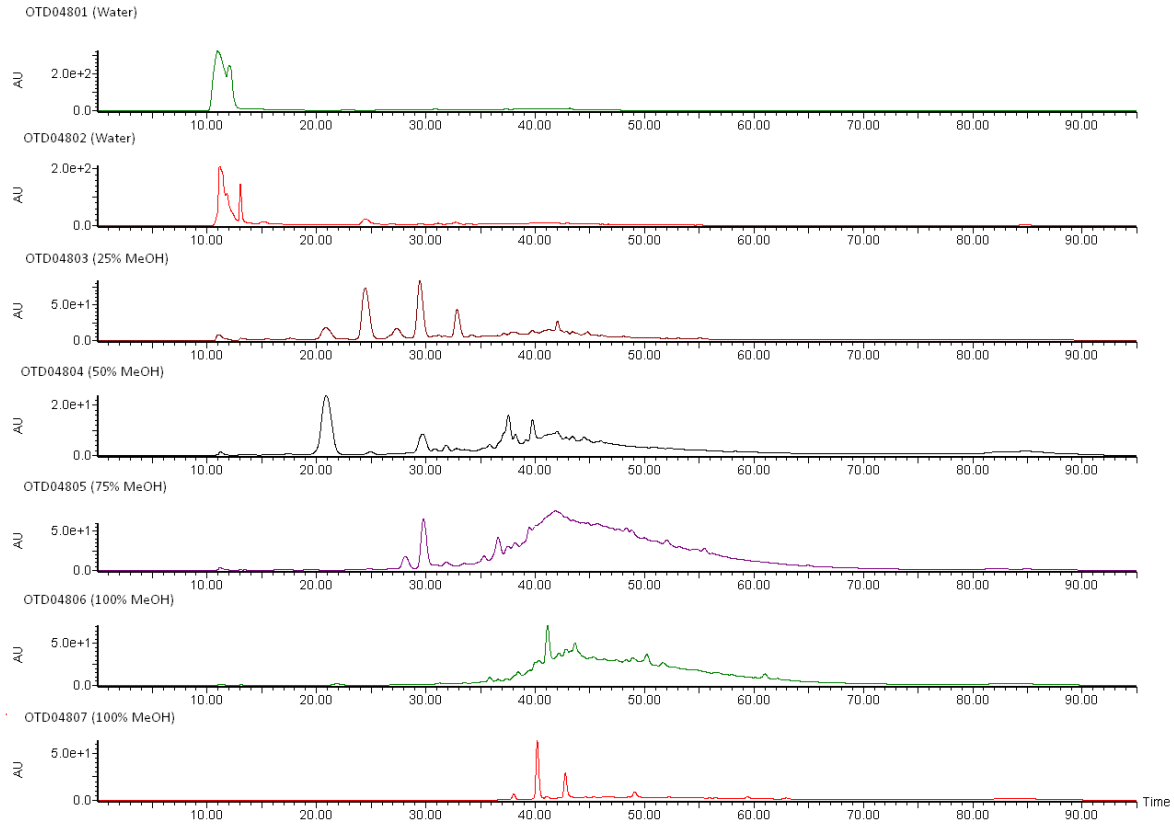
Appendix 2-21 HPLC-PDA chromatograms of LH-20 fractions *I. sinensis* (OTD03701-06) at 280 nm 1st batch



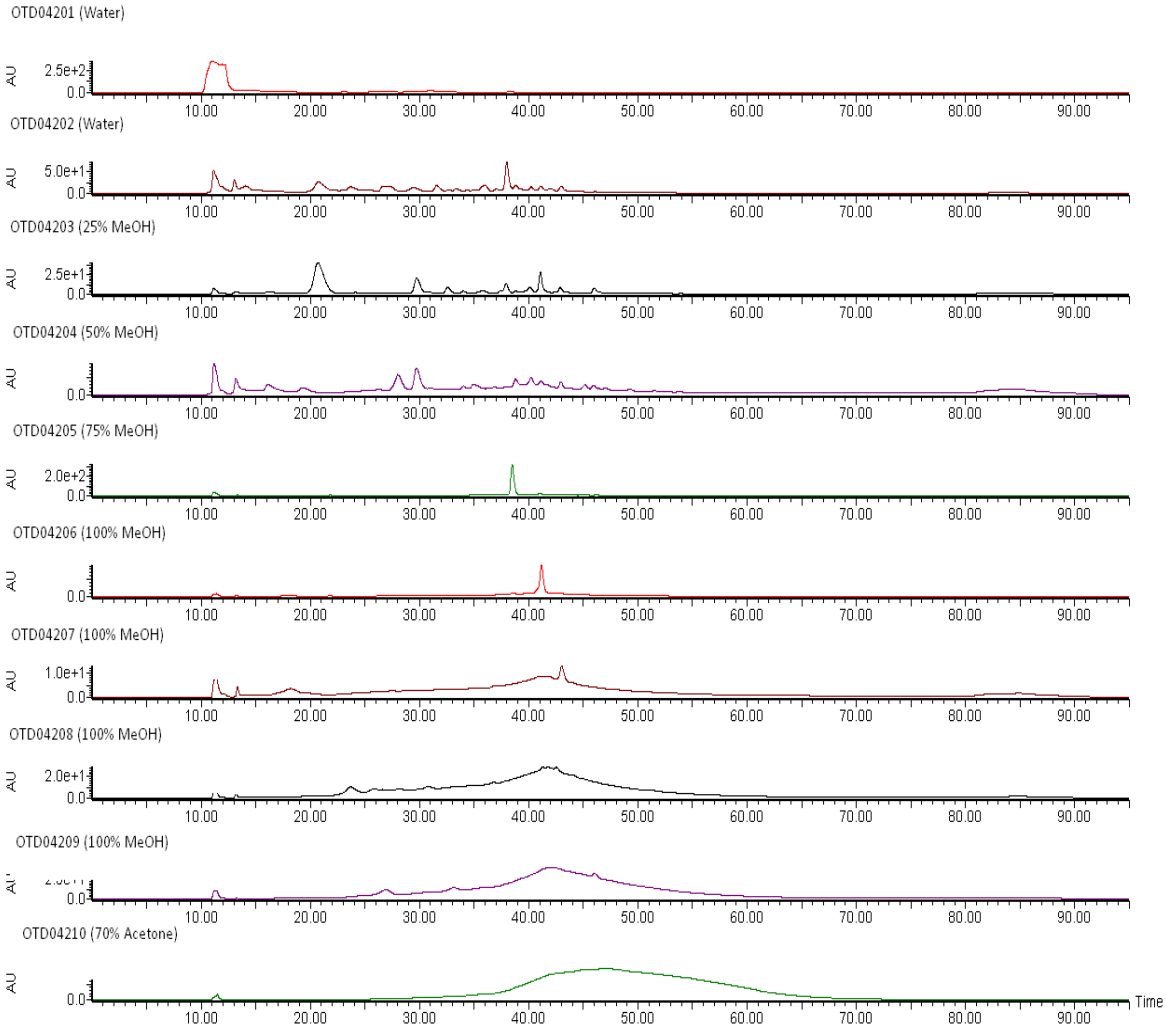
Appendix 2-22 HPLC-PDA chromatograms of LH-20 fractions *I. sinensis* (OTD03801-06) at 280 nm 2nd batch



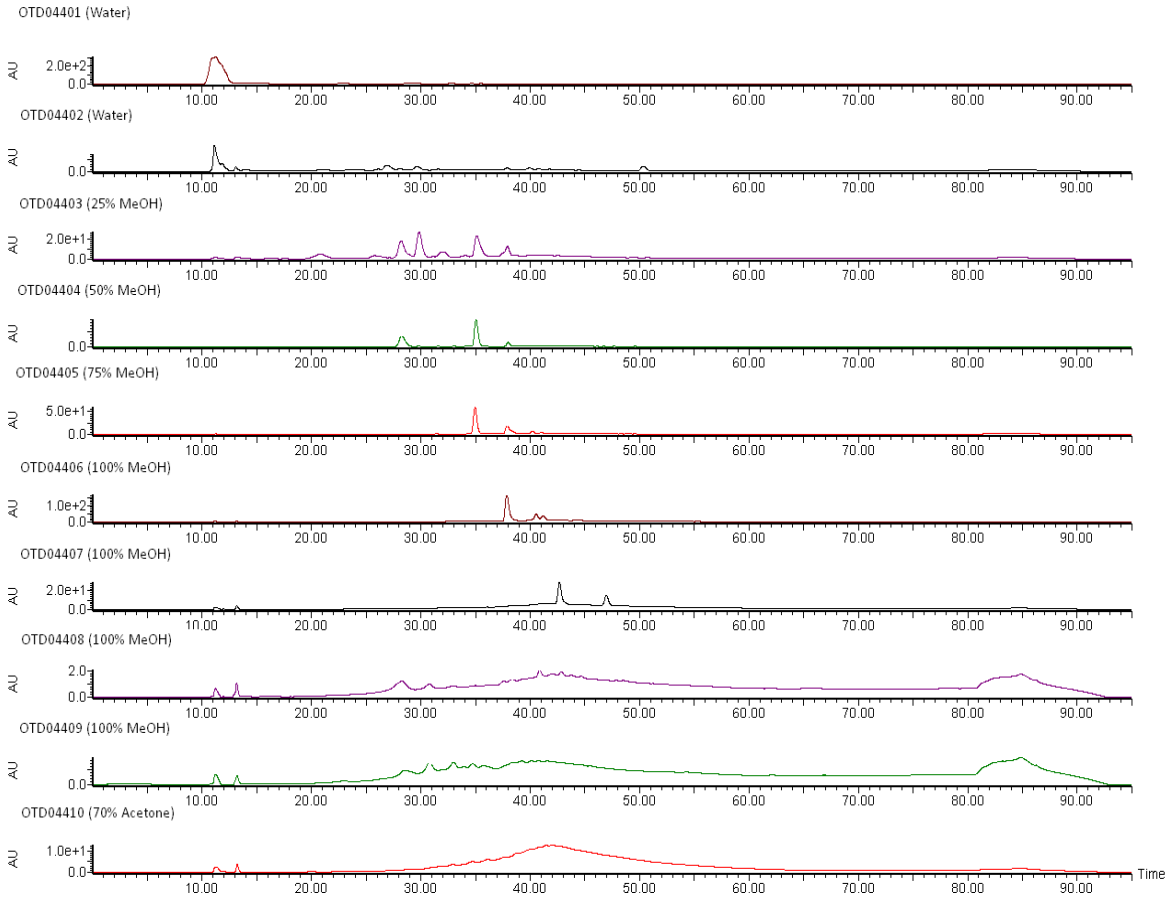
Appendix 2-23 HPLC chromatograms of fractions eluted by water (2 fractions), MeOH:H_2O (25%, 1 fraction), MeOH (50%, 1 fraction), MeOH (100% MeOH, 4 fractions) and acetone:H_2O (70%, 2 fractions) from plants collected in 2009 for *V. rotundifolium* from the linear LH-20 method



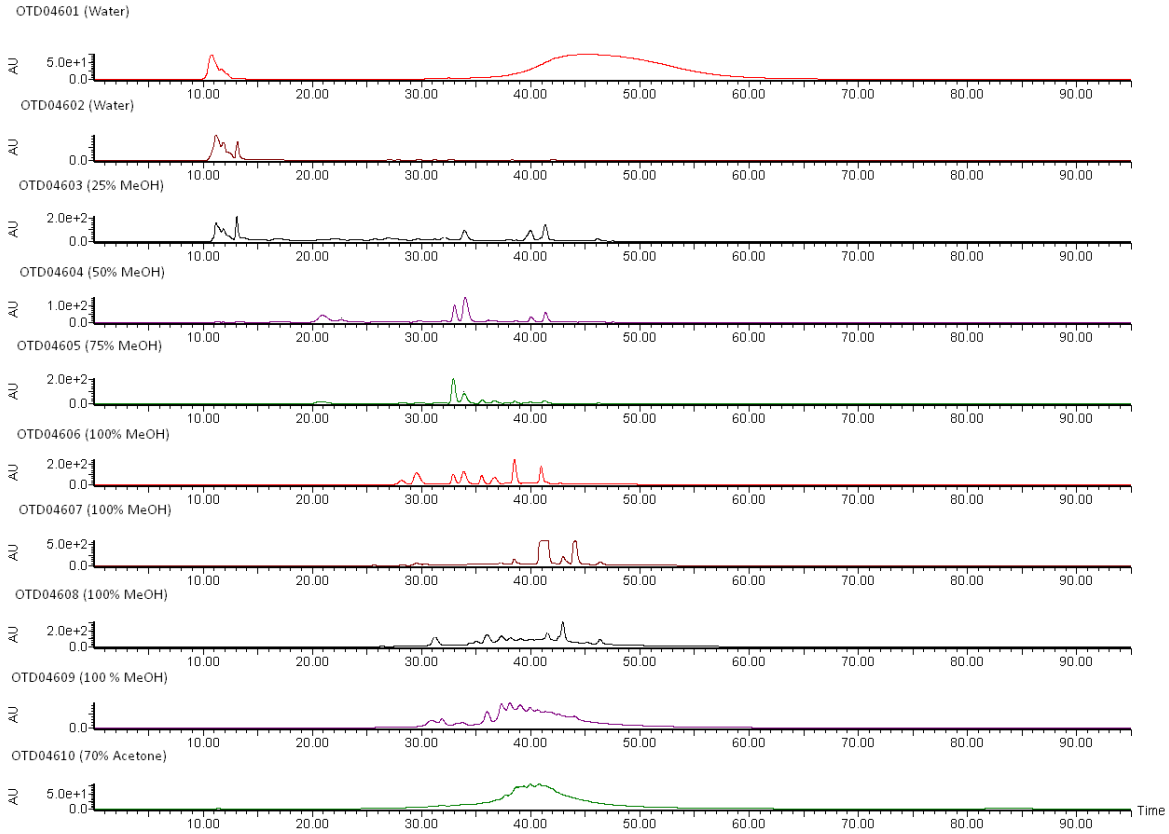
Appendix 2-24 HPLC chromatograms of fractions eluted by water (2 fractions), MeOH:H_2O (25%, 1 fraction), MeOH:H_2O (50%, 1 fraction), MeOH:H_2O (75%, 1 fraction), 100% MeOH (4 fractions) and acetone:H_2O (70%, 1 fraction) from plants collected in 2009 for *V. verrucosum* from the linear LH-20 method



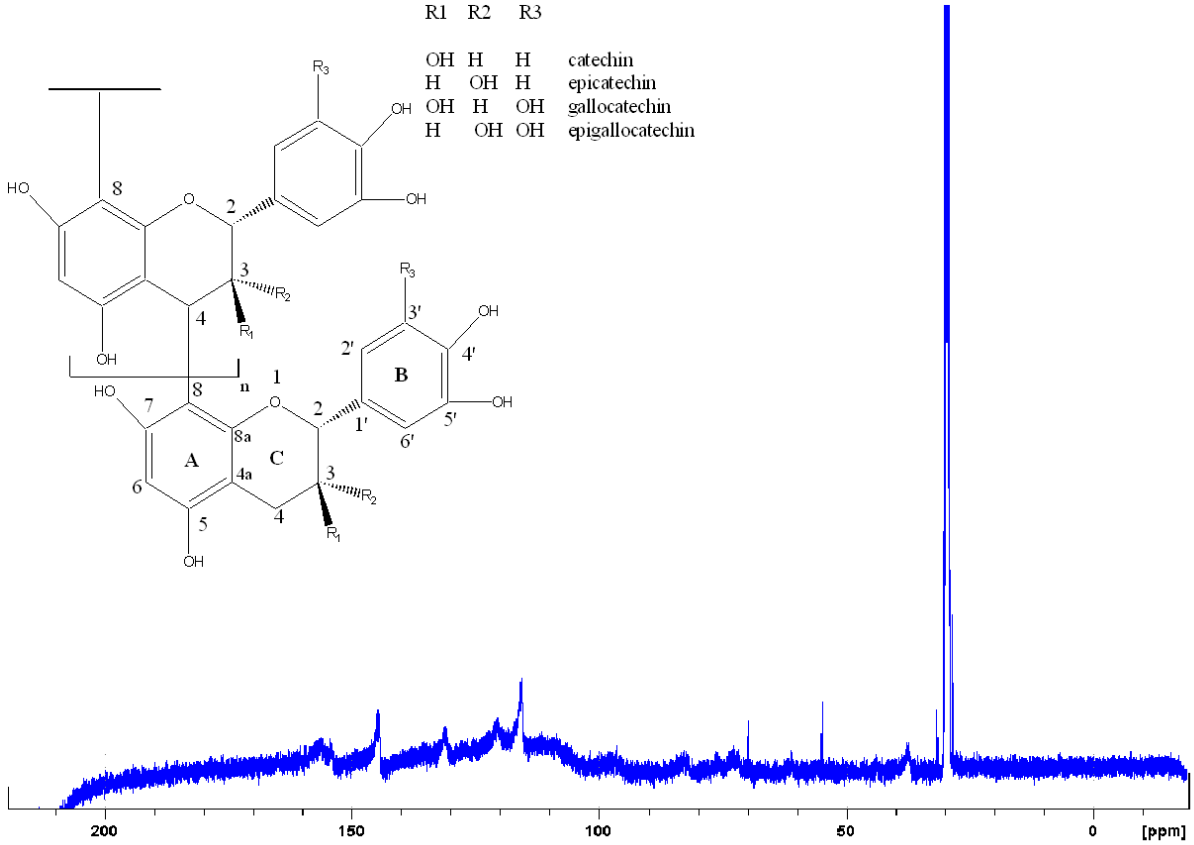
Appendix 2-25 HPLC chromatograms of fractions eluted by water (2 fractions), MeOH:H_2O (25%, 1 fraction), MeOH:H_2O (50%, 1 fraction), MeOH:H_2O (75%, 1 fraction), 100% MeOH (4 fractions) and acetone:H_2O (70%, 1 fraction) for fractions from plants collected in 2009 for *T. oleifolius* from the linear LH-20 method



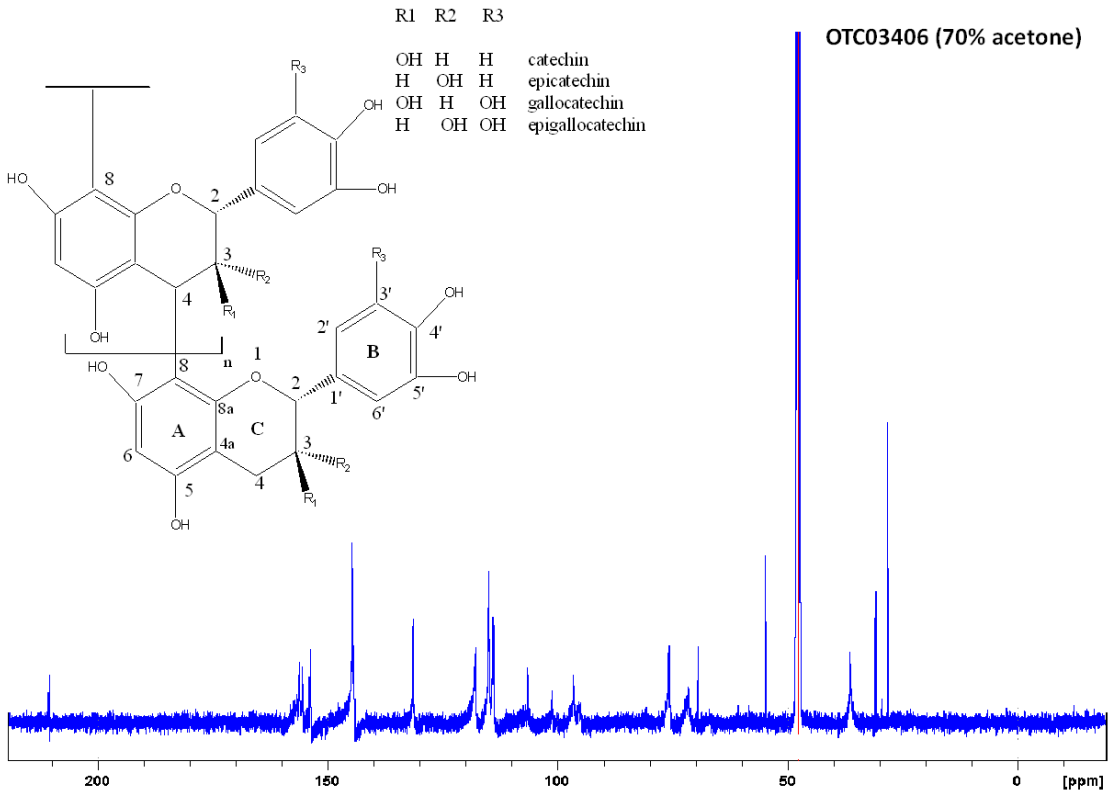
Appendix 2-26 HPLC chromatograms of fractions eluted by water (2 fractions), MeOH:H_2O (25%, 1 fraction), MeOH:H_2O (50%, 1 fraction), MeOH:H_2O (75%, 1 fraction), 100% MeOH (4 fractions) and acetone:H_2O (70%, 1 fractions) from plants collected in 2009 for *G. flava* from the linear LH-20 method



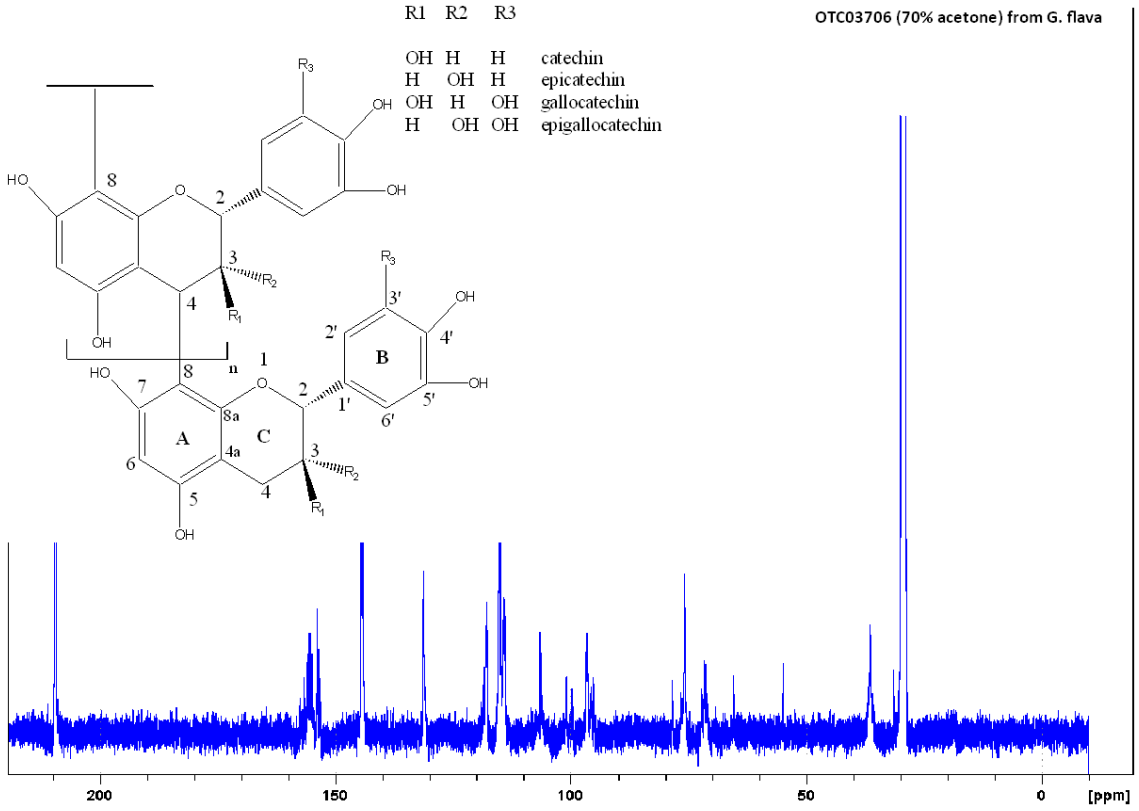
Appendix 2-27 ^{13}C-NMR spectrum of OTC02906 (70% acetone) from *V. verrucosum* collected in 2009 by the 500 MHz NMR



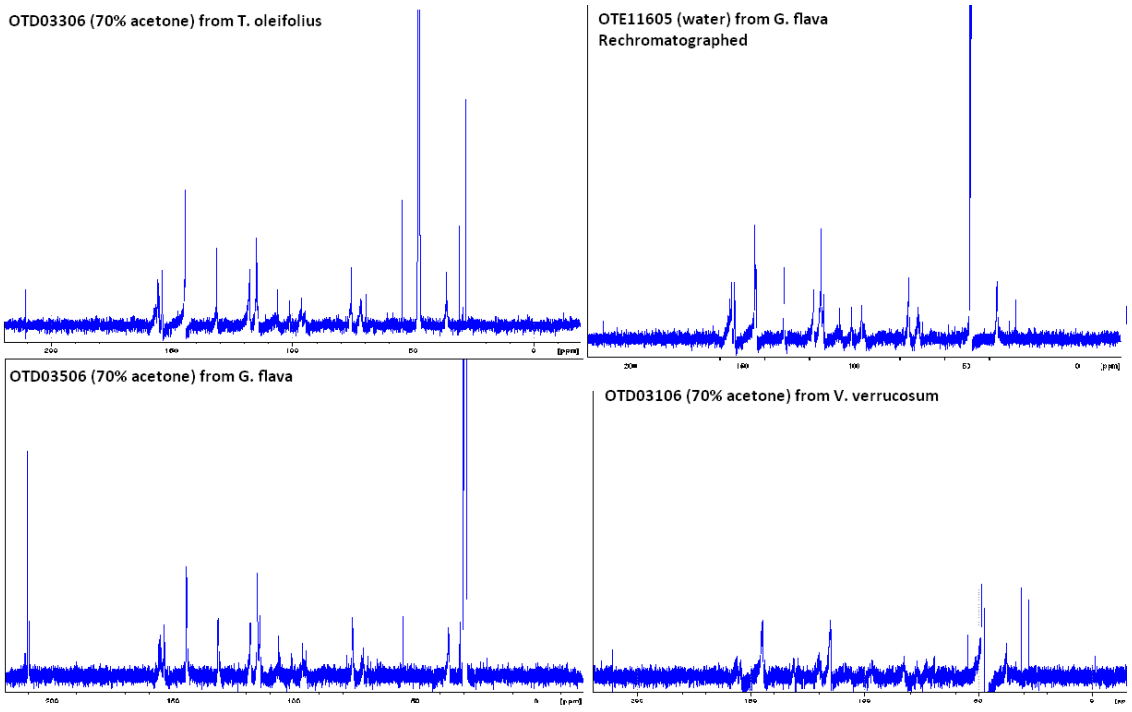
Appendix 2-28 ^{13}C-NMR spectrum (d_4-methanol) of OTC03406 (70% acetone) from *T. oleifolius* collected 2009



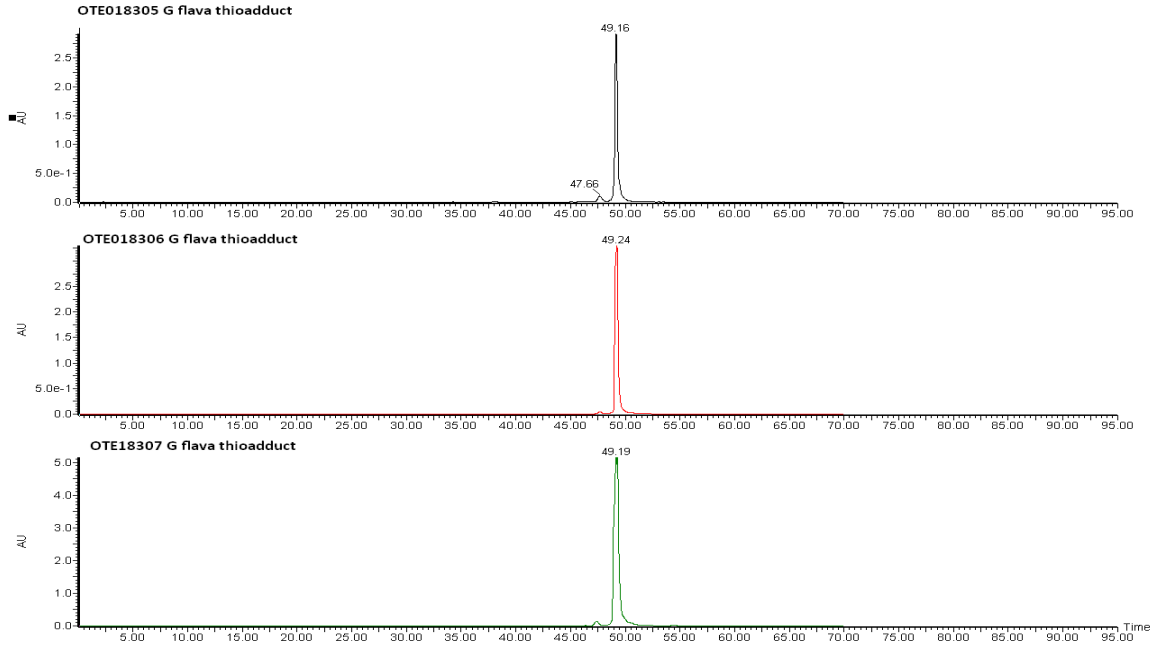
Appendix 2-29 ^{13}C-NMR spectrum (d_6-acetone) of OTC03706 (70% acetone) from *G. flava* collected in 2009



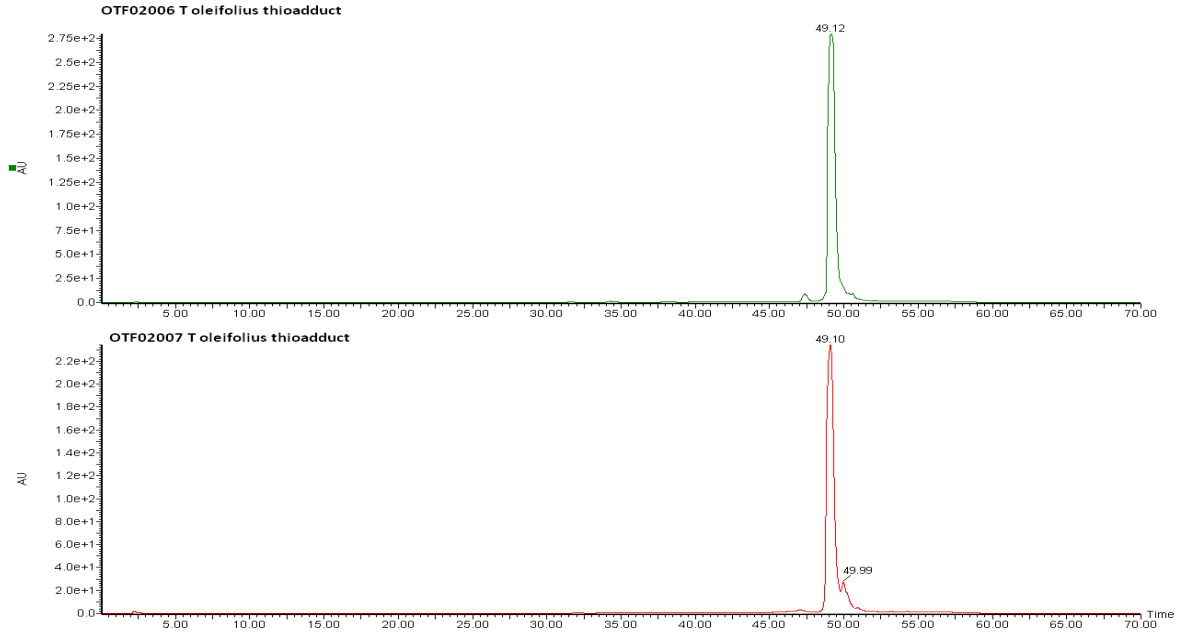
Appendix 2-30 ^{13}C-NMR spectra of the aqueous acetone fractions from *T. oleifolius*, *G. flava* and *V. verrucosum* for plants collected in 2010 by a 500 MHz NMR.



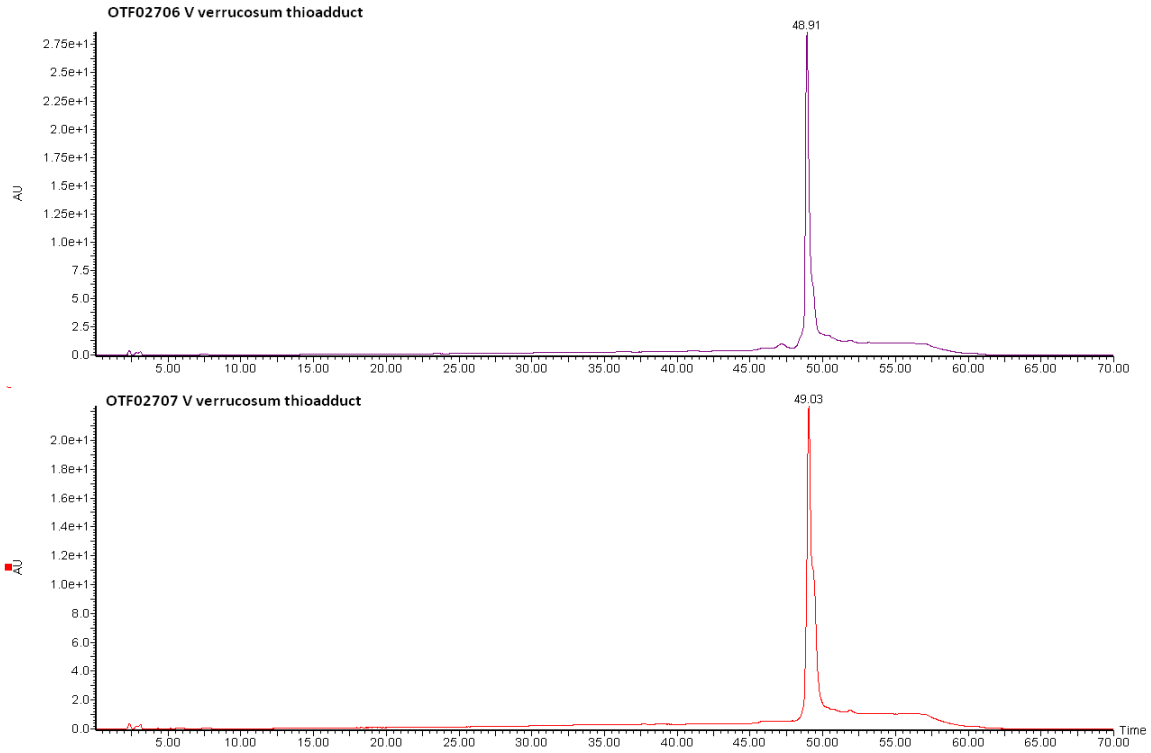
Appendix 2-31 HPLC-PDA chromatograms of the purified thiolysis adducts of CT from *G. flava*



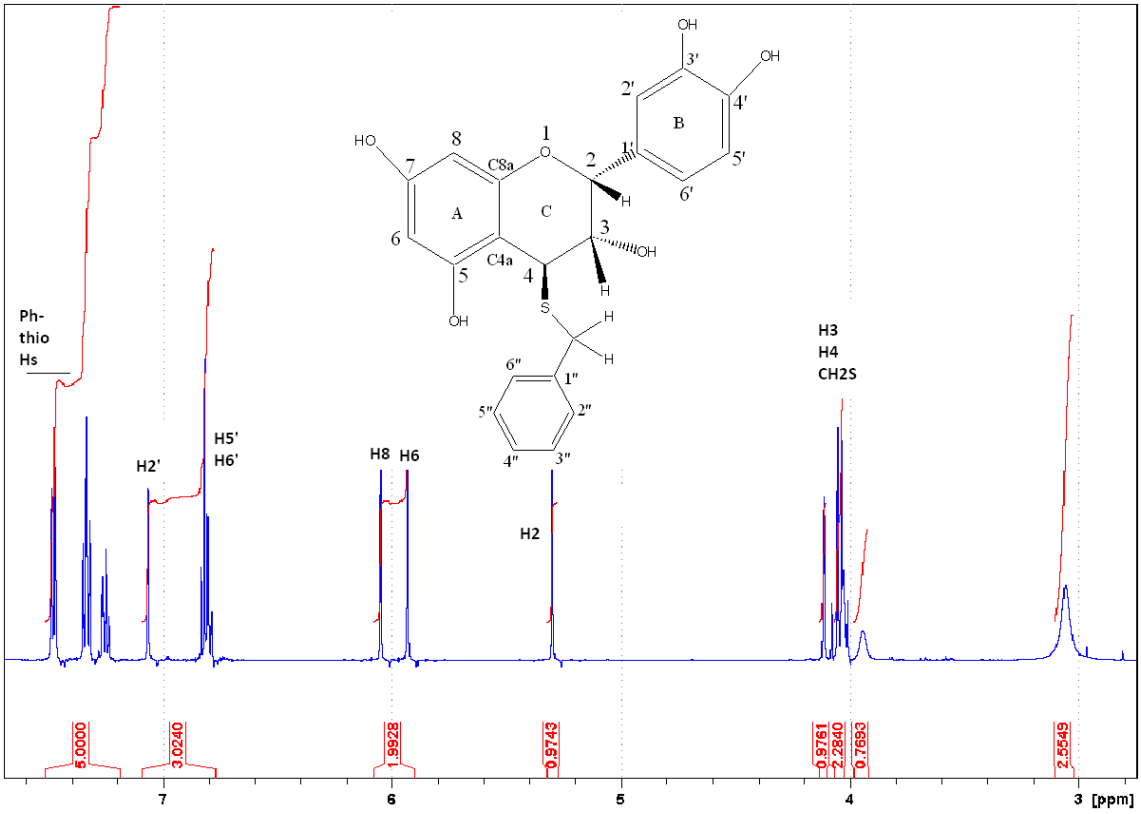
Appendix 2-32 HPLC-DA chromatograms of the purified thiolysis adducts of CT from *T. oleifolius*



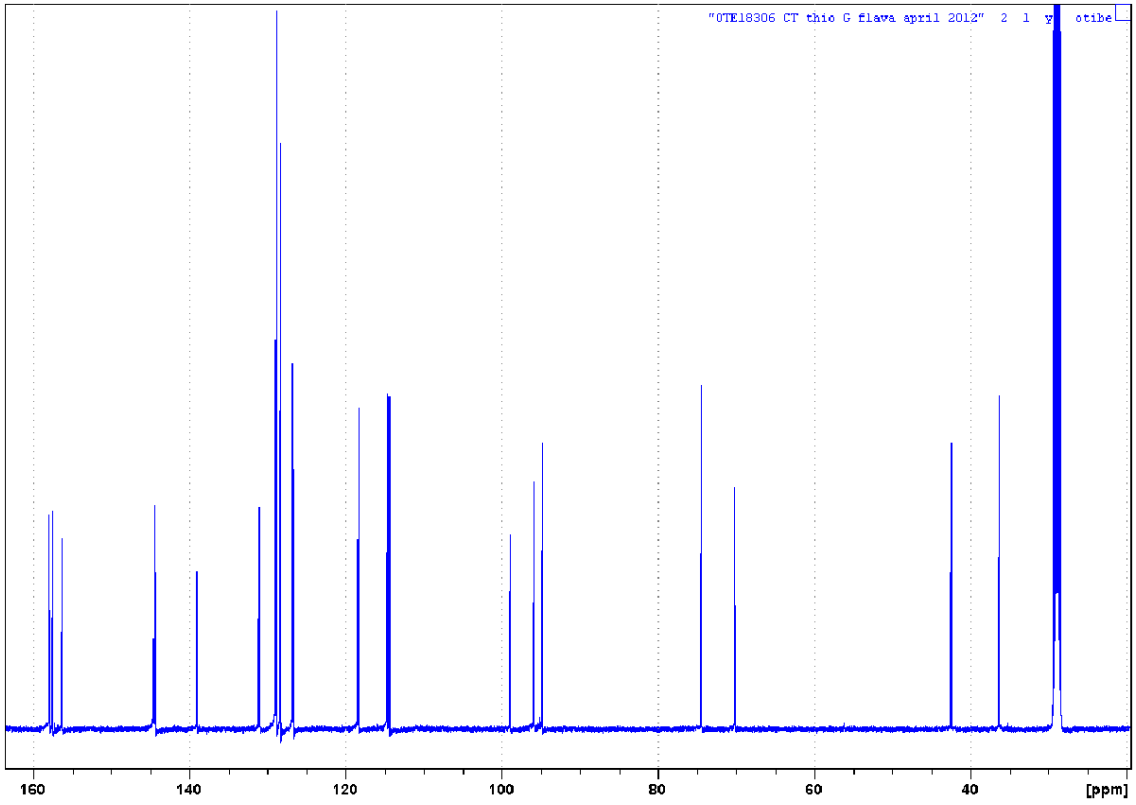
Appendix 2-33 HPLC-DA chromatograms of the purified thiolysis adducts of CT from *V. verrucosum*



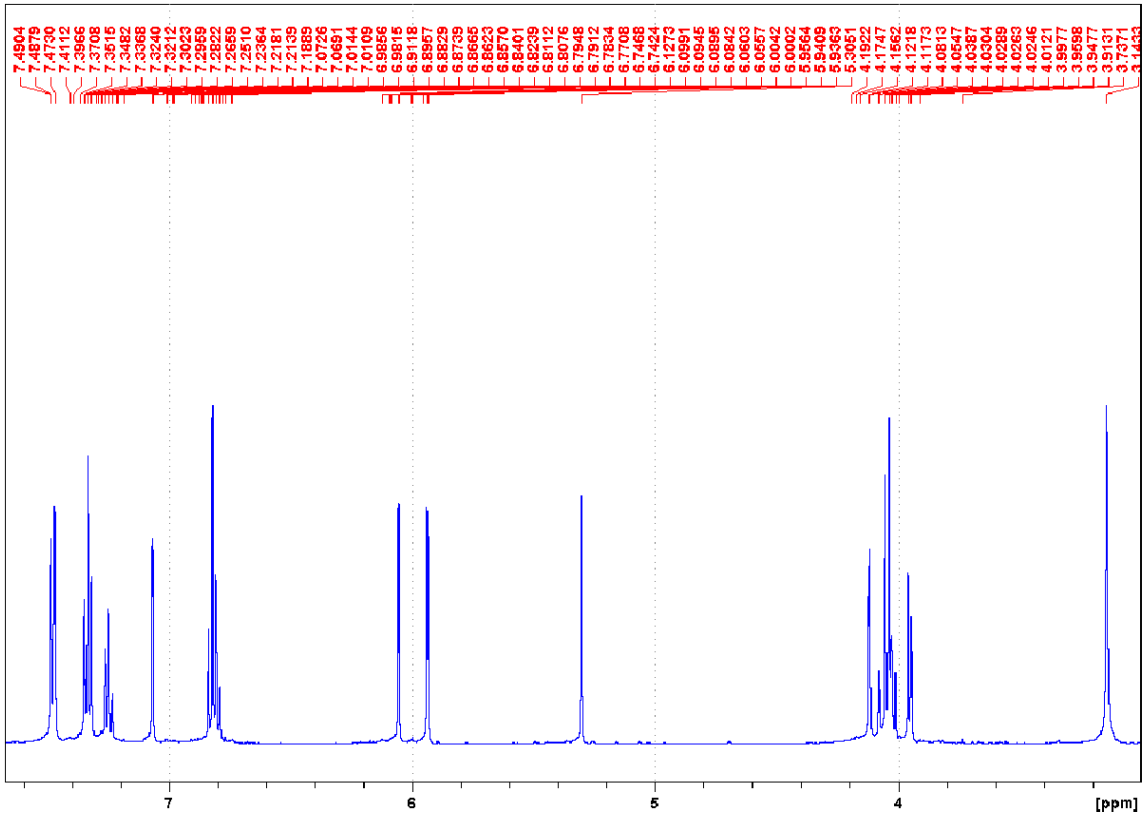
Appendix 2-34 ^{1}H NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT from *G. flava*



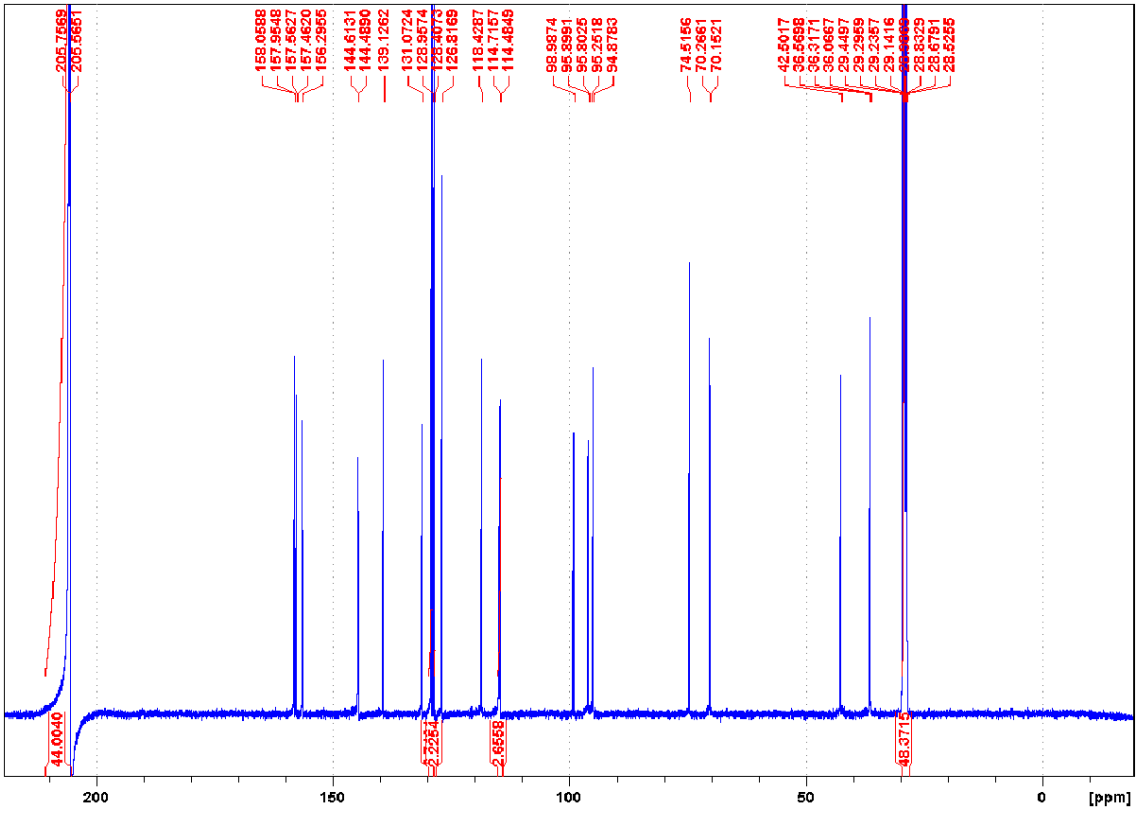
Appendix 2-35 ^{13}C-NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT from *G. flava*



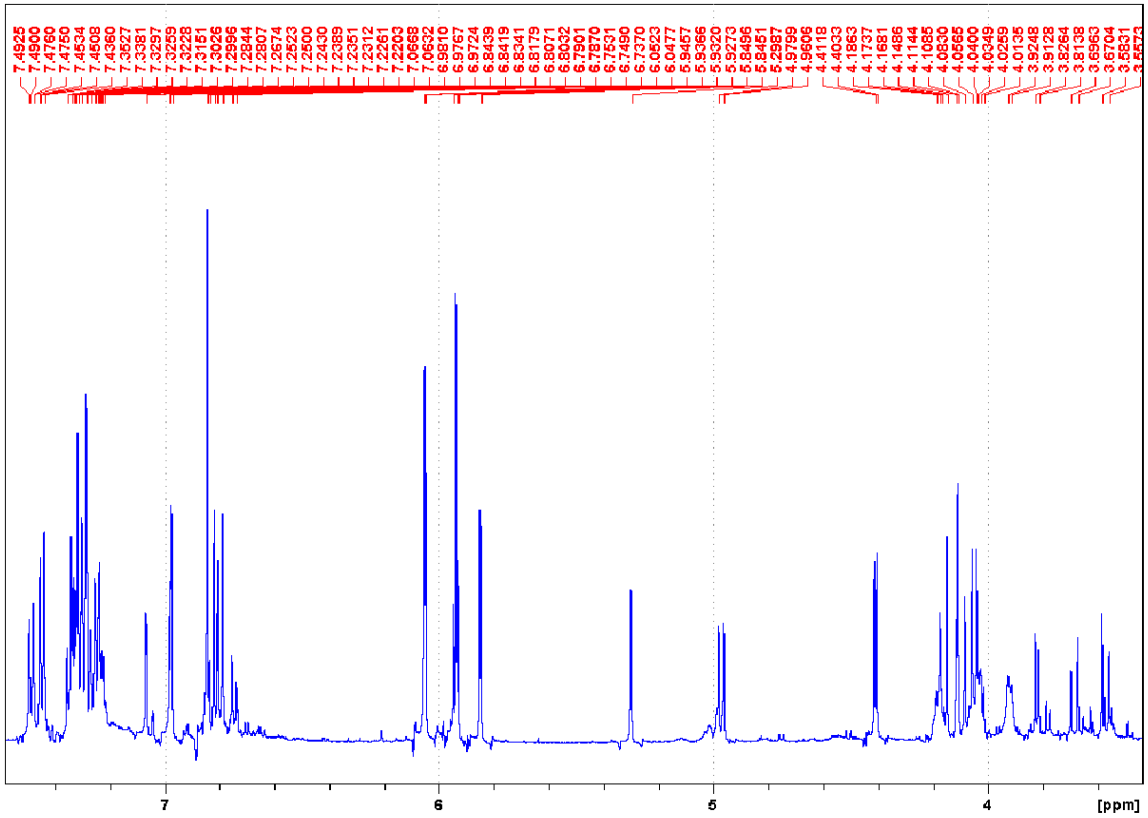
Appendix 2-36 ^{1}H spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT from *T. oleifolius*



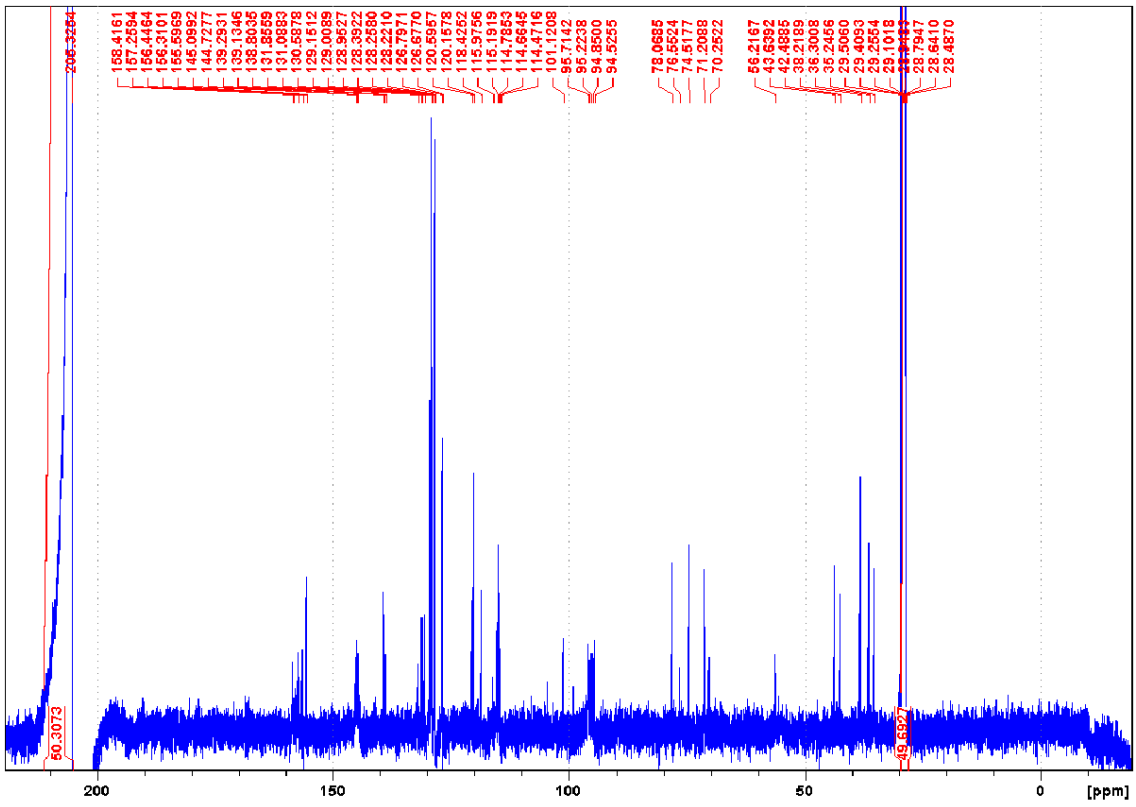
Appendix 2-37 ^{13}C-NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT from *T. oleifolius*



Appendix 2-38 ^{1}H NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT from *V. verrucosum*



Appendix 2-39 ^{13}C-NMR spectrum of the isolated thiolysis adduct (epicatechin-4β-benzylthioether) of CT from *V. verrucosum*



Appendix 2-40 Step LH-20 fractions from plants collected in 2009 and 2010 and their anti-parasitic activity

Plant sample	Date collected	Extract code	First batch SF-I	Activity	Second batch SF-II	Activity	Eluting Solvent
V. rotundifolium	27/02/2009	OTC00301	OTC02101	0	OTC02201	0	MeOH:H_2O (1:1)
			OTC02102	0	OTC02202	0	MeOH:H_2O (1:1)
			OTC02103	0	OTC02203	0	MeOH:H_2O (1:1)
			OTC02104	0	OTC02204	0	MeOH:H_2O (1:1)
			OTC02105	0	OTC02205	0	Acetone:H_2O (7:3)
	10/02/2010	OTD01801	OTD02901	0	OTD03001	0	MeOH:H_2O (1:1)
			OTD02902	0	OTD03002	0	MeOH:H_2O (1:1)
			OTD02903	0	OTD03003	0	MeOH:H_2O (1:1)
			OTD02904	0	OTD03004	0	MeOH:H_2O (1:1)
V. verrucosum	27/02/2009	OTC00601	OTC02901	0	OTC03101	0	MeOH:H_2O (1:1)
			OTC02902	0	OTC03102	0	MeOH:H_2O (1:1)
			OTC02903	0	OTC03103	0	MeOH:H_2O (1:1)
			OTC02904	0	OTC03104	0	MeOH:H_2O (1:1)
			OTC02905	1	OTC03105	1	Acetone:H_2O (7:3)
			OTC02906	1	OTC03106	1	Acetone:H_2O (7:3)
			OTC02907	1	OTC03107	1	Acetone:H_2O (7:3)
	12/02/2010	OTD01901	OTD03101	0	OTD03201	0	MeOH:H_2O (1:1)
			OTD03102	0	OTD03202	0	MeOH:H_2O (1:1)
			OTD03103	0	OTD03203	0	MeOH:H_2O (1:1)
			OTD03104	0	OTD03204	0	MeOH:H_2O (1:1)
			OTD03105	1	OTD03205	1	Acetone:H_2O (7:3)
			OTD03106	1	OTD03206	1	Acetone:H_2O (7:3)
T. oleifolius	27/02/2009	OTC00801	OTC03401	0	OTC03501	0	MeOH:H_2O (1:1)
			OTC03402	0	OTC03502	0	MeOH:H_2O (1:1)
			OTC03403	0	OTC03503	0	MeOH:H_2O (1:1)
			OTC03404	0	OTC03504	0	MeOH:H_2O (1:1)
			OTC03406	1	OTC03506	1	Acetone:H_2O (7:3)
			OTC03407	1	OTC03507	1	Acetone:H_2O (7:3)
	2/02/2010	OTD02001	OTD03301	0	OTD03401	0	MeOH:H_2O (1:1)
			OTD03302	0	OTD03402	0	MeOH:H_2O (1:1)
			OTD03303	0	OTD03403	0	MeOH:H_2O (1:1)
			OTD03304	0	OTD03404	0	MeOH:H_2O (1:1)
			OTD03305	1	OTD03405	1	Acetone:H_2O (7:3)
			OTD03306	1	OTD03406	1	Acetone:H_2O (7:3)
			OTD03307	1	OTD03407	1	Acetone:H_2O (7:3)

Larval scoring for inhibition of larval development: 0= 0% inhibition and 1= complete inhibition (100%). All crude extracts were not active. CT containing fractions from *T. oleifolius* and *G. flava* only induced gamma delta T cell activity.

Appendix 2-41 Step LH-20 fractions from plants collected in 2009 and 2010 and their anti-parasitic activity

Plant sample	Date collected	Extract code	First batch	Activity	Second batch	Activity	Eluting Solvent
G. flava	27/02/2009	OTC00901	OTC03701	0	OTC04001	0	MeOH:H_2O (1:1)
			OTC03702	0	OTC04002	0	MeOH:H_2O (1:1)
			OTC03703	0	OTC04003	0	MeOH:H_2O (1:1)
			OTC03704	0	OTC04004	0	MeOH:H_2O (1:1)
			OTC03705	1	OTC04005	1	Acetone:H_2O (7:3)
			OTC03706	1	OTC04006	1	Acetone:H_2O (7:3)
			OTC03707	1	OTC04007	1	Acetone:H_2O (7:3)
	2/02/2010	OTD02101	OTD03501	0	OTD03601	0	MeOH:H_2O (1:1)
			OTD03502	0	OTD03602	0	MeOH:H_2O (1:1)
			OTD03503	0	OTD03603	0	MeOH:H_2O (1:1)
			OTD03504	0	OTD03604	0	MeOH:H_2O (1:1)
			OTD03505	1	OTD03605	1	Acetone:H_2O (7:3)
			OTD03506	1	OTD03606	1	Acetone:H_2O (7:3)
			OTD03507	1	OTD03607	1	Acetone:H_2O (7:3)
I. sinensis	3/03/2009	OTD01101	OTC04201	0	OTC04401	0	MeOH:H_2O (1:1)
			OTC04202	0	OTC04402	0	MeOH:H_2O (1:1)
			OTC04203	0	OTC04403	0	MeOH:H_2O (1:1)
			OTC04204	0	OTC04404	0	MeOH:H_2O (1:1)
			OTC04206	0	OTC04406	0	Acetone:H_2O (7:3)
	2/02/2010	OTD02201	OTD03701	0	OTD03801	0	MeOH:H_2O (1:1)
			OTD03702	0	OTD03802	0	MeOH:H_2O (1:1)
			OTD03703	0	OTD03803	0	MeOH:H_2O (1:1)
			OTD03704	0	OTD03804	0	MeOH:H_2O (1:1)
			OTD03705	0	OTD03805	0	Acetone:H_2O (7:3)
			OTD03706	0	OTD03806	0	Acetone:H_2O (7:3)

Larval scoring for inhibition of larval development: 0= 0% inhibition and 1= complete inhibition (100%). All crude extracts were not active. CT containing fractions from *T. oleifolius* and *G. flava* induced gamma delta T cell activity.

Appendix 2-42 Linear gradient LH-20 fractions from plants collected in 2009

Plant sample	Date collected	Extract code	First batch	Eluting Solvent	Activity
V. rotundifolium	27/02/2009	OTD01801	OTD04801	H_2O	0
			OTD04802	H_2O	0
			OTD04803	MeOH:H_2O (1:3)	0
			OTD04804	MeOH:H_2O (1:1)	0
			OTD04805	MeOH:H_2O (3:1)	0
			OTD04806	100% MeOH	0
			OTD04807	100% MeOH	0
V. verrucosum	27/02/2009	OTC00701	OTD04201	H_2O	0
			OTD04202	H_2O	0
			OTD04203	MeOH:H_2O (1:3)	0
			OTD04204	MeOH:H_2O (1:1)	0
			OTD04205	MeOH:H_2O (3:1)	0
			OTD04206	100% MeOH	1
			OTD04207	100% MeOH	1
			OTD04208	100% MeOH	1
			OTD04209	100% MeOH	1
			OTD04210	Acetone:H_2O (7:3)	1
			OTD04211	Acetone:H_2O (7:3)	1
T. oleifolius	27/02/2009	OTD00701	OTD04401	H_2O	0
			OTD04402	H_2O	0
			OTD04403	MeOH:H_2O (1:3)	0
			OTD04404	MeOH:H_2O (1:1)	0
			OTD04405	MeOH:H_2O (3:1)	0
			OTD04406	100% MeOH	1
			OTD04407	100% MeOH	1
			OTD04408	100% MeOH	1
			OTD04409	100% MeOH	1
			OTD04410	Acetone:H_2O (7:3)	1
			OTD04411	Acetone:H_2O (7:3)	1
G. flava	27/02/2009	OTC00901	OTD04601	H_2O	0
			OTD04602	H_2O	0
			OTD04603	MeOH:H_2O (1:3)	0
			OTD04604	MeOH:H_2O (1:1)	0
			OTD04605	MeOH:H_2O (3:1)	0
			OTD04606	100% MeOH	1
			OTD04607	100% MeOH	1
			OTD04608	100% MeOH	1
			OTD04609	100% MeOH	1
			OTD04610	Acetone:H_2O (7:3)	1
			OTD04611	Acetone:H_2O (7:3)	1
			OTD04612	Acetone:H_2O (7:3)	1

Larval scoring for inhibition of larval development: 0= 0% inhibition and 1= complete inhibition (100%). All crude extracts were not active.

Appendix 4-1 Anti-parasitic activity of first batch (I) step LH-20 fractions

Plant sample	Date collected	First batch	Eluting Solvent	*H. contortus*		*T. circumcincta*		*T. colubriformis*	
				100	500	100	500	100	500
V. rotindifolium	27/02/2009	OTC02101	50% MeOH	0	0	0	0	0	0
		OTC02102	50% MeOH	0	0	0	0	0	0
		OTC02103	50% MeOH	0	0	0	0	0	0
		OTC02104	50% MeOH	0	0	0	0	0	0
		OTC02105	70% acetone	0	0	0	0	0	0
	10/02/2010	OTD02901	50% MeOH	0	0	0	0	0	0
		OTD02902	50% MeOH	0	0	0	0	0	0
		OTD02903	50% MeOH	0	0	0	0	0	0
		OTD02904	50% MeOH	0	0	0	0	0	0
V. verrucosum	27/02/2009	OTC02901	50% MeOH	0	0	0	0	nd	nd
		OTC02902	50% MeOH	0	0	0	0	0	0
		OTC02903	50% MeOH	0	0	0	0	nd	nd
		OTC02904	50% MeOH	0	0	0	0	nd	nd
		OTC02905	70% acetone	0	0	0	0	0	0
	SF6	**OTC02906**	**70% acetone**	**1**	**1**	**1**	**1**	**1**	**1**
	SF7	**OTC02907**	**70% acetone**	**1**	nd	0	nd	nd	nd
	12/02/2010	OTD03101	50% MeOH	0	0	0	0	nd	nd
		OTD03102	50% MeOH	0	0	0	0	nd	nd
		OTD03103	50% MeOH	0	0	0	0	nd	nd
		OTD03104	50% MeOH	0	0	0	0	0	0
		OTD03105	70% acetone	0	0	0	0	nd	nd
		OTD03106	70% acetone	0	0	0	0	nd	nd
T. oleifolius	27/02/2009	OTC03401	50% MeOH	0	0	0	0	nd	nd
		OTC03402	50% MeOH	0	0	0	0	nd	nd
		OTC03403	50% MeOH	0	0	0	0	0	0
		OTC03404	50% MeOH	0	0	0	0	0	0
	SF6	**OTC03406**	**70% acetone**	**1**	**1**	**1**	**1**	**1**	**1**
	SF7	**OTC03407**	**70% acetone**	**1**	nd	0	nd	nd	nd
	2/02/2010	OTD03301	50% MeOH	0	0	0	0	nd	nd
		OTD03302	50% MeOH	0	0	0	0	nd	nd
		OTD03303	50% MeOH	0	0	0	0	nd	nd
		OTD03304	50% MeOH	0	0	0	0	nd	nd
		OTD03305	70% acetone	0	0	0	0	nd	nd
		OTD03306	70% acetone	0	0	0	0	nd	nd
		OTD03307	70% acetone	0	0	0	0	nd	nd

Solvent % (v/v) is for % solvent in water unless otherwise stated, nd- not determined; larval scoring, red 1-active (100% inhibition of LD) and black 0- inactive

Appendix 4-2 Anti-parasitic activity of first batch (I) step LH-20 fractions

Plant sample	**Date collected**	**First batch**	**Eluting Solvent**	***H. contortus***		***T. circumcincta***		***T. colubriformis***	
				100	500	100	500	100	500
G. flava	27/02/2009	OTC03701	50% MeOH	0	0	0	0	0	0
		OTC03702	50% MeOH	0	0	0	0	0	0
		OTC03703	50% MeOH	0	0	0	0	0	0
		OTC03704	50% MeOH	0	0	0	0	0	0
		OTC03705	70% acetone	0	0	0	0	0	0
	SF-6	**OTC03706**	**70% acetone**	**1**	**1**	**1**	**1**	**1**	**1**
	SF-7	**OTC03707**	**70% acetone**	**1**	**1**	**1**	**1**	**1**	**1**
	2/02/2010	OTD03501	50% MeOH	0	0	0	0	nd	nd
		OTD03502	50% MeOH	0	0	0	0	nd	nd
		OTD03503	50% MeOH	0	0	0	0	nd	nd
		OTD03504	50% MeOH	0	0	0	0	nd	nd
		OTD03505	70% acetone	0	0	0	0	nd	nd
	SF-6	**OTD03506**	**70% acetone**	0	**1**	0	0	**1**	**1**
		OTD03507	70% acetone	0	0	0	0	nd	nd
I. sinensis	3/03/2009	OTC04201	50% MeOH	0	0	0	0	0	0
		OTC04202	50% MeOH	0	0	0	0	0	0
		OTC04203	50% MeOH	0	0	0	0	0	0
		OTC04204	50% MeOH	0	0	0	0	0	0
		OTC04206	70% acetone	0	0	0	0	0	0
	2/02/2010	OTD03701	50% MeOH	0	0	0	0	nd	nd
		OTD03702	50% MeOH	0	0	0	0	nd	nd
		OTD03703	50% MeOH	0	0	0	0	nd	nd
		OTD03704	50% MeOH	0	0	0	0	nd	nd
		OTD03705	70% acetone	0	0	0	0	nd	nd
		OTD03706	70% acetone	0	0	0	0	nd	nd

Solvent % (v/v) is for % solvent in water unless otherwise stated, nd- not determined; larval scoring, red 1-active (100% inhibition of LD) and black 0- inactive

Appendix 4-3 Anti-parasitic activity from second batch (II) of step LH-20 fractions

Plant sample	Date collected	Second batch	Eluting Solvent	*H. contortus*		*T. circumcincta*		*T. colubriformis*	
				100	500	100	500	100	500
V. rotindifolium	27/02/2009	OTC02201	50% MeOH	0	0	0	0	0	0
		OTC02202	50% MeOH	0	0	0	0	0	0
		OTC02203	50% MeOH	0	0	0	0	0	0
		OTC02204	50% MeOH	0	0	0	0	0	0
		OTC02205	70% acetone	0	0	0	0	0	0
	10/02/2010	OTD03001	50% MeOH	0	0	0	0	nd	nd
		OTD03002	50% MeOH	0	0	0	0	nd	nd
		OTD03003	50% MeOH	0	0	0	0	nd	nd
		OTD03004	50% MeOH	0	0	0	0	nd	nd
V. verrucosum	27/02/2009	OTC03101	50% MeOH	0	0	0	0	0	0
		OTC03102	50% MeOH	0	0	0	0	0	0
		OTC03103	50% MeOH	0	0	0	0	0	0
		OTC03104	50% MeOH	0	0	0	0	0	0
		OTC03105	70% acetone	0	0	0	0	0	0
		OTC03106	70% acetone	0	0	0	0	nd	0
		OTC03107	70% acetone	0	nd	0	nd	nd	nd
	12/02/2010	OTD03201	50% MeOH	0	0	0	0	nd	nd
		OTD03202	50% MeOH	0	0	0	0	nd	nd
		OTD03203	50% MeOH	0	0	0	0	nd	nd
		OTD03204	50% MeOH	0	0	0	0	nd	nd
		OTD03205	70% acetone	0	0	0	0	nd	nd
		OTD03206	70% acetone	0	0	0	0	nd	nd
T. oleifolius	27/02/2009	OTC03501	50% MeOH	0	0	0	0	0	0
		OTC03502	50% MeOH	0	0	0	0	0	0
		OTC03503	50% MeOH	0	0	0	0	0	0
		OTC03504	50% MeOH	0	0	0	0	0	0
	SF-6	**OTC03506**	**70% acetone**	0	**1**	**1**	**1**	nd	**1**
	SF-7	**OTC03507**	**70% acetone**	**1**	nd	0	nd	nd	nd
	2/02/2010	OTD03401	50% MeOH	0	0	0	0	nd	nd
		OTD03402	50% MeOH	0	0	0	0	nd	nd
		OTD03403	50% MeOH	0	0	0	0	0	0
		OTD03404	50% MeOH	0	0	0	0	nd	nd
		OTD03405	70% acetone	0	0	0	0	nd	nd
		OTD03406	70% acetone	0	0	0	0	nd	nd
		OTD03407	70% acetone	0	0	0	0	nd	nd

Solvent % (v/v) is for % solvent in water unless otherwise stated, nd- not determined; larval scoring, highlighted 1-active (100% inhibition of LD) and black 0- inactive

Appendix 4-4 Anti-parasitic activity of second batch (II) of step fractions

Plant sample	Date collected	Second batch	Eluting Solvent	*H. contortus*		*T. circumcincta*		*T. colubriformis*	
G. flava	27/02/2009	OTC04001	50% MeOH	0	0	0	0	0	0
		OTC04002	50% MeOH	0	0	0	0	0	0
		OTC04003	50% MeOH	0	0	0	0	0	0
		OTC04004	50% MeOH	0	0	0	0	0	0
		OTC04005	70% acetone	0	0	0	0	0	0
	SF-6	**OTC04006**	**70% acetone**	**1**	**1**	**1**	**1**	**1**	**1**
	SF-7	**OTC04007**	**70% acetone**	0	**1**	**1**	**1**	**1**	**1**
	2/02/2010	OTD03601	50% MeOH	0	0	nd	nd	nd	nd
		OTD03602	50% MeOH	0	0	nd	nd	nd	nd
		OTD03603	50% MeOH	0	0	nd	nd	nd	nd
		OTD03604	50% MeOH	0	0	nd	nd	nd	nd
		OTD03605	70% acetone	0	0	nd	nd	nd	nd
		OTD03606	70% acetone	0	**1**	0	0	0	**1**
		OTD03607	70% acetone	0	0	nd	nd	nd	nd
I. sinensis	3/03/2009	OTC04401	50% MeOH	0	0	0	0	0	0
		OTC04402	50% MeOH	0	0	0	0	0	0
		OTC04403	50% MeOH	0	0	0	0	0	0
		OTC04404	50% MeOH	0	**1**	0	0	0	0
		OTC04406	70% acetone	0	0	0	0	0	0
	2/02/2010	OTD03801	50% MeOH	0	0	nd	nd	nd	nd
		OTD03802	50% MeOH	0	0	nd	nd	nd	nd
		OTD03803	50% MeOH	0	0	nd	nd	nd	nd
		OTD03804	50% MeOH	0	**1**	nd	nd	nd	nd
		OTD03805	70% acetone	0	0	nd	nd	nd	nd
		OTD03806	70% acetone	0	0	nd	nd	nd	nd

Solvent % (v/v) is for % solvent in water unless otherwise stated, nd- not determined

Appendix 4-5 Anti-parasitic activity of the linear gradient LH-20 fractions

Plant sample	Date collected	First batch	Eluting Solvent	*H. contortus*		*T. circumcincta*		*T. colubriformis*	
				100	500	100	500	100	500
V. rotundifolium	27/02/2009	OTD04801	water	0	0	nd	nd	nd	nd
		OTD04802	water	0	0	nd	nd	nd	nd
		OTD04803	25% MeOH	0	0	nd	nd	nd	nd
	GF-4	**OTD04804**	**50% MeOH**	0	**1**	nd	nd	nd	nd
		OTD04805	75% MeOH	0	0	nd	nd	nd	nd
	GF-6	**OTD04806**	**100% MeOH**	0	**1**	nd	nd	nd	nd
		OTD04807	100% MeOH	0	0	nd	nd	nd	nd
V. verrucosum	27/02/2009	OTD04201	water	0	0	nd	nd	nd	nd
		OTD04202	water	0	0	nd	nd	nd	nd
	GF-3	**OTD04203**	**25% MeOH**	0	**1**	nd	nd	nd	nd
		OTD04204	50% MeOH	0	0	nd	nd	nd	nd
		OTD04205	75% MeOH	0	0	nd	nd	nd	nd
	GF-6	**OTD04206**	**100% MeOH**	0	**1**	nd	nd	nd	nd
		OTD04207	100% MeOH	0	0	nd	nd	nd	nd
	GF-8	**OTD04208**	**100% MeOH**	0	**1**	nd	nd	nd	nd
		OTD04209	100% MeOH	0	0	nd	nd	nd	nd
	GF-10	**OTD04210**	**70% acetone**	0	**1**	nd	nd	nd	nd
		OTD04211	70% acetone	0	0	nd	nd	nd	nd
T. oleifolius	27/02/2009	OTD04401	water	0	0	nd	nd	nd	nd
		OTD04402	water	0	0	nd	nd	nd	nd
		OTD04403	25% MeOH	0	0	nd	nd	nd	nd
		OTD04404	50% MeOH	0	0	nd	nd	nd	nd
	GF-5	**OTD04405**	**75% MeOH**	0	**1**	nd	nd	nd	nd
		OTD04406	100% MeOH	0	0	nd	nd	nd	nd
	GF-7	**OTD04407**	**100% MeOH**	0	**1**	nd	nd	nd	nd
	GF-8	**OTD04408**	**100% MeOH**	0	**1**	0	0	0	**1**
	GF-9	**OTD04409**	**100% MeOH**	0	**1**	0	0	0	0
	GF-10	**OTD04410**	**70% acetone**	0	**1**	0	0	0	**1**
	GF-11	**OTD04411**	**70% acetone**	0	0	0	0	0	**1**
G. flava	27/02/2009	OTD04601	water	0	0	nd	nd	nd	nd
		OTD04602	water	0	0	nd	nd	nd	nd
		OTD04603	25% MeOH	0	0	nd	nd	nd	nd
		OTD04604	50% MeOH	0	0	nd	nd	nd	nd
		OTD04605	75% MeOH	0	0	nd	nd	nd	nd
		OTD04606	100% MeOH	0	0	nd	nd	nd	nd
		OTD04607	100% MeOH	0	0	nd	nd	nd	nd
		OTD04608	100% MeOH	0	0	0	0	nd	nd
	GF-9	**OTD04609**	**100% MeOH**	0	**1**	0	0	nd	nd
	GF-10	**OTD04610**	**70% acetone**	0	**1**	0	0	0	**1**
	GF-11	**OTD04611**	**70% acetone**	0	**1**	0	0	0	**1**
	GF-12	**OTD04612**	**70% acetone**	0	**1**	0	0	0	**1**

Solvent % (v/v) is for % solvent in water unless otherwise stated; nd- not determined

Appendix 4-6 Egg extraction protocol

Faecal samples were collected from lambs housed indoors, experimentally infected with monospecific *Haemonchus contortus, T. colubriformis, T. axei and Telardosagia (Ostertagia) circumcinta*, and fed with lucerne and chaff at AgResearch. The faecal matter was mashed into a liquid suspension (slurry). The slurry was strained through a 250 µm and 100 µm sieves to remove debris and washings containing the eggs collected at the bottom with a 20 µm sieve. The slurry in the upper sieve was further washed with a small amount of distilled water to ensure that no eggs were lost with the debris. The debris in the upper sieves was discarded. The washings were passed through the 20 µm sieve by tapping the sieve against the sink. The particulate matter on the sieve was transferred into a petri-dish (10 cm in diameter) by washing with saturated sodium chloride solution. The petri-dish was filled with saturated sodium chloride solution until three-quarter full for egg flotation. The bottom part of another petri-dish was floated on top of the solution for five minutes to trap the eggs. The floated petri-dish was removed and eggs adhered under it were washed several times with a jet of distilled water through a 100 µm sieve for further cleaning. The washings were collected onto a 20 µm sieve placed on a 250 mL beaker. The dish was placed five times on the solution for one minute to complete egg extraction and with subsequent washing of the eggs onto a 20 µm sieve. The eggs were washed several times with distilled water to remove salt residues. The trapped eggs were further washed with the salt solution and transferred onto a smaller petri-dish and more salt solution added for further cleaning of the eggs. The trapped eggs were washed again with tap water onto a 20 µm sieve. The retained eggs were transferred onto a Falcon tube and centrifuged at 200 g (Centrifuge Eppendorf 5810R) for two minutes in order to concentrate the eggs. In water the eggs settle to the bottom of the tube. The supernatant was removed under vacuum, the pellet that settled at the bottom containing eggs was re-suspended in water and centrifuged for two minutes at 200 g and the supernatant was discarded again. After removing the supernatant, the eggs at the bottom were retained. The number of eggs in a 10 µL solution was estimated to be 42 viewed under a microscope (Nikon CW10X/22, Japan). The concentration of the eggs was adjusted to ensure that 100 µL of the egg solution contained 100 eggs. *C. elegans* adult worms, a model parasite, were obtained from Ms Sheralee and Susan at AgResearch, Immunology and Parasitology section, Palmerston North.

Appendix 4-7 Larval development assay (LDA) protocol

Each test fraction (2 µL) of the stock solution (making 1% in the well**)** was added into three wells. The stock solution was prepared by dissolving about 5 mg of the test fraction in 100 µL dimethyl sulfoxide (DMSO) to make a stock solution with a concentration of 50 000 µg/mL. DMSO was used as the negative control. Agar (2%) (100 µL) was added into each well. Egg solution (60 µL) containing about 50 eggs were added to each well. The nutrient medium (40 µL) was also added into each well to provide food for the larvae. This made a final volume of about 200 µL in each well. The nutrient medium was prepared by mixing 1% yeast solution, 0.015% *E. coli* solution and Amphotericin B solution (5 mg/mL). *E. coli* bacteria was also used as food for development of nematode larvae, while amphotericin B was used as the antifungal agent. The plates were incubated for seven days in an incubator set at 25 ºC in a humid chamber kept moist all the time with the lids kept open. The plates were placed in a plastic container containing moistened paper towels at the bottom. Hatching was checked using a microscope in the test and negative control wells, and all the eggs hatched out.

Appendix 4-8 Larval migration inhibition (LMI) assay protocol

This assay involves the incubation of test solutions with LH-20 fractions and phenolics with L3 larvae in the wells of tissue culture plates. The freeze-dried crude extracts and CT containing fractions are dissolved in phosphate buffered saline (PBS; 0.1 M, 0.05 M NaCl; pH 7.2) and serially diluted with PBS prior to incubation. Test concentrations ranged between 100 and 500 µg/mL (125, 250, 500 µg/mL). The larvae were exsheathed with sodium hypochlorite solution (0.025% chlorine), washed three times with water and subsequently concentrated in PBS to contain 1500 larvae/mL. The larvae suspension (30 mL) was placed in a weighing boat (10 mL) containing a magnetic flea and stirred. One hundred micro litres of larvae solution (150 larvae per 100 µL solution ; third stage larvae, L3) was added to the wells containing negative control (no CT) and a range of test fractions with increasing concentration in a 48-well culture plate containing 400 µL of PBS. All incubations were carried out in 48 well tissue plates for 2 hours at 37 ºC. After incubation, the solutions were transferred to sieves (7 mm ID with 20 µm mesh at the bottom) and left overnight (16-18 hours) to enable the active larvae to migrate through the sieves. The sieve is placed to ensure that the active larvae can migrate through the sieve and larvae passing through the sieve counted. The cross diameter of L3 larvae is 25 µm which is slightly larger than the mesh so that the larvae would not fall through the sieve. Two duplicates were run for each concentration.

Calculation of data and statistical analysis

The number of larvae which had migrated through the sieves is counted using 40x magnification and the % LMI was determined according to Rabel et al., 1994 using the following equation;

$$\%\text{inhibition} = (A-B)/A \times 100,$$

Where A is the number of L3 larvae migrated through the sieves in the LMI assay in control incubations, and B is the number of L3 larvae migrated in incubations containing different concentrations of crude extracts and LH-20 fractions. Differences between treatments will be analysed using GLM (general linear model; Minitab, version 15.1) and regression analysis.

Appendix 4-9 Culturing of larvae (L3)

Faecal samples were emptied into a large tray and shaken so that they were evenly distributed, and irrigated with tap water until were shiny. The tray was shaken a bit and the inside of the tray was washed. Irrigation was repeated daily for 7-10 days. A ring of beige-pink forming was formed above the level of the faeces, indicating the presence of L3 larvae. After seven to ten days, the samples were emptied into a mesh bottomed tray, with a wet single paper of interfold papers. Enough tap water was added until the faecal pellets were completely covered, and a second mesh tray was placed on top, and the assembly covered with another tray and left overnight. The contents of tray were poured into a beaker (3 L) and rinsed a number of times. The worms were allowed to settle for 3 hrs, and the top brown water decanted, retaining the bottom sludge. The sludge was washed three times, with subsequent decanting, until the solution was clear. The solution was poured into a sieve with facial tissues, and the bottom pegged and left overnight. The L3 larvae settled at the bottom and was decanted into a large tissue flask, then diluted and placed in a 10 ºC incubator until needed.

Appendix 4-10 Exsheathing of *Haemonchus contortus* L3 larvae

The L3 larvae solution was concentrated on a filter (8.0 µm) and re-suspended in warm water at 30-37 ºC. The L3 suspension was transferred into Falcon tubes (50 mL) and to each 50 ml aliquot was added bleach (0.625 mL) to produce with 0.05% available chlorine. The tubes were placed in the rotator in the 37 ºC room, left to mix for 25 minutes; exsheathment was checked under a microscope (should be 99-100%). The solution was centrifuged at 600 g for 10 minutes at room temperature; the pellet was washed in water and re-suspended in water. To separate the larvae from the sheath, L3s plus sheaths were poured on a wire mesh tray and left overnight (at RT or 37 ºC). Sheaths were retained on the tissue, while the L3 larvae were washed out of the tray and concentrated on a filter (8.0 µm). The exsheathed L3 larvae were resuspended in a small volume of water in Falcon tubes (50 mL) and concentrated to 1500 larvae per mL.

Appendix 5-1 Mean percentage primed γδ T cells in kids relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by two CT fractions (**second batch**) extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and results are from two experiments or n=4.

Plant species extract and controls	Concentration (µg/mL)	Mean % of primed γδ T cells (CD25+γδ TCR+)	Mean MFI of CD25 on γδ T cells
Controls			
non-stimuli		22.66±6.32	18.77±1.38
Con A	5	95.60±1.02(*)	461.50±28.5 (*)
Plant species and extraction solvent			
V. rotundifolium (SF1-II, methanol:water 1:1)	5	25.22±8.02	21.10±2.43
	10	26.00±10.5	22.93±5.24
V. verrucosum (SF6-II, 70% acetone)	5	27.94±8.68	23.95±3.62
	10	27.22±9.42	23.50±5.09
T. oleifolius (SF6-II, 70% acetone)	5	29.31±9.57	23.72±4.84
	10	31.00±10.8	26.38±5.97
G. flava (SF6-II, 70% acetone)	5	29.55±9.76	23.88±4.56
	10	71.51±5.20 (*)	93.00±16.2 (*)
Significance	5	NS (p=0.457)	NS
	10	*(p =0.000)	*(p =0.000)

Abbreviations and symbols : MFI (mean fluorescent intensity), * significant ($p < 0.05$) and NS (not significant, $p > 0.05$), SF-II Step fraction second batch(fraction)

Appendix 5-2 Mean percentage primed γδ T cells in calves relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by CT fractions (**second batch**) extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and results are from two experiments or n=4)

Plant species extract and controls	Concentration (µg/mL)	Mean % of primed γδ T cells (CD25+γδ TCR+)	Mean MFI of CD25 on γδ T cells
Controls			
non-stimuli (PBS)		9.94±2.36	22.82±4.96
Con A	5	75.95±18.3 (*)	656.00±270 (*)
Plant species and extraction solvent			
V. rotundifolium (SF1-II, methanol:water 1:1)	5	10.42±1.80	22.45±4.61
	10	13.43±1.08	22.88±4.90
V. verrucosum (SF6-II, acetone:water 7:3)	5	10.21±2.17	22.90±4.42
	10	14.42±1.48	27.70±3.59
T. oleifolius (SF6-II, acetone:water 7:3)	5	10.58±2.34	23.70±5.21
	10	11.06±3.15	23.45±5.50
G. flava (SF6-II, acetone:water 7:3)	5	11.41±3.94	23.02±5.83
	10	13.73±3.07	24.00±4.52
Significance	5	NS	NS
	10	NS	NS

Abbreviations: MFI (mean fluorescent intensity), NS (not significant, $p > 0.05$), * significant ($p < 0.05$)SF-II step fraction second batch (fraction)

Appendix 5-3 Mean percentage of primed γδ T cells in lambs relative to total γδ T cell population and MFI of CD25 on γδ T cells induced by two CT fractions (**second batch**) extracted with aqueous acetone (3:7) from each plant species at two concentrations, except *V. rotundifolium* extracted with aqueous methanol (1:1), and results are from two experiments or n=4)

Plant species extract and controls	Concentration (µg/mL)	Mean % of primed γδ T cells ($CD25^+$γδ TCR^+)	Mean MFI of CD25 on γδ T cells
Controls			
non-stimuli (PBS)		32.49±13.2	27.40±11.6
Con A	5	34.11±18.2	179.40±67.5(*)
Plant species and extraction solvent			
V. rotundifolium (SF1-II, methanol:water 1:1)	5	15.45±9.79	15.13±6.23
	10	15.00±10.2	14.32±6.10
V. verrucosum (SF6-II, acetone:water 7:3)	5	16.13±8.28	22.90±11.3
	10	19.08±11.0	33.95±12.8
T. oleifolius (SF6-II, acetone:water 7:3)	5	31.29±14.0	26.40±10.7
	10	30.17±14.9	29.60±13.6
G. flava (SF6-II, acetone:water 7:3)	5	31.89±15.0	28.00±12.0
	10	36.79±17.5	33.50±15.0
Significance	5	NS	NS
	10	NS	NS

Abbreviations: MFI (mean fluorescent intensity), NS (not significant), SF-II step fraction second batch (fraction) * significant

Appendix 5-4 Separation of peripheral mononuclear blood lymphocytes (PMBL)

Whole blood were collected from three lambs (labelled lamb/ovine 1, 2 and 3) and three calves (labelled 33, 34 and 39) in sodium heparin tubes, and kids blood samples (1, 2 and 3) were given by Dr. D. Leathwick at AgResearch, Palmerston North. Blood (ovine/bovine/caprine) was spun at 500 g for 10 minutes and 900 g for 15 minutes (20 °C, break on low) and buffy coat collected. A pipette (2 mL) was used to remove the serum above the buffy coat. Buffy coat was collected with pipette (5 mL) into 15 mL Falcon tubes and re-suspended in 2 mL of RPMI-1640 (Roswell Park Memorial Institute) medium (without foetal calf serum FCS). RPMI-1640 medium was developed by Moore and others at Rosswell Park Memorial Institute, hence the acronym RPMI**.** It is used for culturing of human, calf (bovine) and sheep (ovine) cells. The resuspended buffy coat was then overlaid gently over 3 mL of Histopaque (1.077 g/mL, Sigma Aldrich) using a pipette (5 mL). Histopaque is a solution of polysaccharride (Ficoll 400) and a radiopaque contrast medium (sodium diatrizoate). This was then centrifuged at 600 g for 40 minutes at 20 °C. Histopaque 1077 is a gradient solution made for cell separation techniques. The supernatant was removed and the white interface (buffy coat) collected by aspiration and washed twice in RPMI-1640 medium (400 g for 5 minutes and 200 g for 10 minutes). The supernatant was decanted. For the whole blood samples, red blood cells were removed by hypotonic lysis using tris ammonium chloride. The cells were resuspended to the desired concentration of 1x 10^6 cells/mL in RPMI-1640 (10 mL) suspended in 500 µL of foetal calf serum (FCS, 5%). The number of cells was counted with trypan blue in a counting chamber (90 µL trypan blue and 10 µL cells (1:10 dilution). The number of cells (10 µL) for calf 1 was 10 (1x10^6 cells), calf 2 was 25 (10 mL and 15 mL RPMI with FCS for dilution to give 1 x10^6 cells) and calf 3 was 25 (taking average of number of cells in 4 quadrants in the chamber). Dilution formula was used ($M_1V_1=M_2V_2$).

Appendix 5-5 Cell analysis

At the end of the incubation period, test fractions, negative controls (PBS) and positive controls (Con A) were transferred into tubes (10 mL) and centrifuged at 400 g for 5 minutes for the whole blood and isolated γδ T cells. The supernatant was decanted and the cell solution retained at the bottom of the tube. Staining agent-Conjugated A (20 µL), consisting of CD 25 (cluster of differentiation), TCR and 7AAD, was added into each tube, vortexed and incubated at room temperature for 15 minutes. 7AAD (7-amino-actinomycin D) is a fluorochrome that is used to discriminate viable cells from non-viable in flow cytometric analysis. The excitation wavelength is 488 nm and emits/fluorescence/scatter helium light at 647 nm (long pass filter) in the far red range of the electromagnetic spectrum. Tris ammonium chloride (2 mL) was added to the whole blood samples for lysis of the red blood cells and the supernatant decanted. PBS (2 mL) was added into each tube for the whole blood tubes and γδ T cell tubes. The tubes were spun for 5 minutes at 400 g and the supernatant discarded. PBS (100 µL) was added into each and the cells were analysed using FACScalibur instrument. γδ T cells were cross reacted or stained with CD 25, TCR, 7AAD (20 µl). CD 25 (cluster differentiation) acts as a receptor and binds to and express IL-2α cells. CD25 (bovine interleukin 2α receptor) VMRD CACT116A- species activity-cross reacts with sheep, cattle and goat gamma delta T cells. Antibody γδ TCR binds to the γδ T cells. Cells were analysed using FACSCalibur instrument. A flow cytometer instrument (FACSCalibur) is used for the measurement of flow (movement) of cells.

Appendix 5-6 Priming model of γδ T cell receptors in interaction with CT