Isolation, Characterisation and Biological evaluation of *Tithonia diversifolia* and *Tephrosia vogelii* as sources of Anti-oxidant and Pesticide

University of Fort Hare
Together in Excellence

Thesis submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

By

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August, 2022

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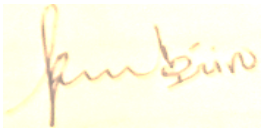
Student's signature  Date signed 04/August/2022

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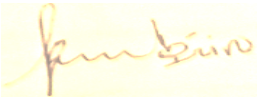
Student's signature 

Dedication

To my dear friend the late Professor Benard Kiremire (RIP)

Acknowledgement

First, I thank the Almighty God for His all-time favours in all dimensions of life upon me during this study.

I hereby express my heartfelt gratitude to my PhD supervisors: Professor Opeoluwa Oyedeji, Professor Adebola O Oyedeji, Professor Robert Byamukama and Doctor SK. Kuria for the support, advice and encouragement extended to me. I am sincerely humbled by your kindness and all concerns expressed leading to the success of writing this Thesis.

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Table of Content

List of Table

List of Figures

Acronyms and abbreviations

mg/mL	milligram per millilitre
mg/g	milligram per gram
µL	microlitre
ANOVA	analysis of variance
Abs	absorbance
amu	atomic mass unit
ATR	attenuated total reflection
^{1,3}A	fragment formed through retrocyclization RDA cleavage of O—C-2 and C-3—C-4 bonds of the C-ring of the aglycone i.e bonds 1 and 3
BHT	2,6-di-tert-butyl-4-methylphenol
br	broad resonance
^{13}C-NMR	carbon nuclear magnetic resonance
4-CHBA	4-*O*-caffeoyl-2-hydroxybutanoic acid
4-CMHBA	4-*O*-caffeoyl-2-hydroxy-3-methylbutanoic acid
CID	collision inductive dissociation
COSY	correlation spectroscopy
CRD	completely randomized design
DA	Dalton
DAD	diode array detector
EtOH	ethanol
FT-IR	Fourier transform infra-red
GC-MS/MS	gas chromatography mass spectrometry/ mass spectrometry
HBA	2-hydroxybutanoic acid
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
HRESIMS	high-resolution electrospray ionization mass spectrometry
IC_{50}	inhibitory concentration with 50% Inhibition
LC-MS/MS	liquid chromatography-mass spectrometry/mass sperctrometry
LD_{50}	lethal dose 50%
MHz	megaHertz

^{1}H-NMR	proton nuclear magnetic resonance
RDA	Retro Diels Aldo fission/cleavage/ reaction
STL	sesquiterpene lactone
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultra violet
UHPLC	ultra high performance liquid phase chromatography
w/w	weight per weight
WHO	World health organisation

List of Research Output/Publications

Articles extracted from the Thesis

i) Kerebba, N., Oyedeji, A.O., Byamukama, R., Kuria, S.K., Oyedeji, O.O., 2019. Pesticidal activity of *Tithonia diversifolia* (Hemsl.) A. Gray and *Tephrosia vogelii* (Hook f.); phytochemical isolation and characterization: A review. South African Journal of Botany **121**(2019), 366-376. https://doi.org/10.1016/j.sajb.2018.11.024.

ii) **Kerebba, N.,** Oyedeji, A.O., Byamukama, R., Kuria, S.K., Oyedeji, O.O., 2020. Chemical Variation and Implications on Repellency Activity of *Tephrosia vogelii* (Hook f.) Essential Oils Against *Sitophilus zeamais* Motschulsky**.** Agriculture, 10(5), 164. https://doi.org/10.3390/agriculture10050164.

iii) **Kerebba, N.,** Oyedeji, A.O., Byamukama, R., Kuria, S.K., Oyedeji, O.O., 2022. Evaluation for Feeding Deterrents Against *Sitophilus zeamais* (Motsch.) from *Tithonia diversifolia* (Hemsl.) A. Gray, Journal of Biologically Active Products from Nature, 12:1, 77-93**,** https://doi.org/10.1080/22311866.2021.2023046.

iv) **Kerebba, N.,** Oyedeji, A.O., Byamukama, R., Kuria, S.K., Oyedeji, O.O., 2022. UHPLC-ESI-QTOF-MS/MS Characterisation of Phenolic compounds from *Tithonia diversifolia* (Hemsl.) A. Gray and Anti-oxidant activity. ChemistrySelect,7, e202104406. https://doi.org/10.1002/slct.202104406.

Conferences and symposium

1) The South African Chemical institute Eastern Cape Postgraduate Seminar, held at Nelson Mandela University 25th-October, 2019. Oral presentation title: 'Chemical variation and implications on pesticidal activity of *Tephrosia vogelii* (Hook f.) Essential oils against *Sitophilus zeamais* Motschulsky'.

2) International Conference on Pure and Applied Chemistry (ICPAC)-2018, held on 2-6 July 2018, at University of Mauritius, Réduit, Mauritius. Oral presentation title: 'Pesticidal activity of *Tithonia diversifolia* (Hemsl.) A. Gray and *Tephrosia vogelii* (Hook f.); phytochemical isolation and characterization'.

3) 2nd International Webinar on "Mass Spectrometry & Analytical Techniques" held during November 11-12, 2021 | Virtual Conference organised by the Coalesce Research Group. Oral presentation title: 'New hydroxycinnamic acid derivatives from *Tithonia diversifolia* (Hemsl.) A. Gray and anti-oxidant activity'.

Abstract

This study aimed at evaluating chemically the pesticidal and anti-oxidant activities of *Tithonia diversifolia* (Hemsl.) and *Tephrosia vogelii* (Hook f.). In the study, the composition of essential oils and non-volatile substances was evaluated by modern analytical tools. The essential oils were characterised using Gas chromatography (GC)-mass spectrometry/mass spectrometry (GC-MS/MS), GC and multivariate techniques. While phenolic compounds of *T. diversifolia* were comprehensively characterised using Ultra high performance liquid-phase chromatography coupled to a tandem mass spectrometer with electrospray ionisation (UHPLC-ESI-MS/MS). Both the essential oils and non-volatile components of the plant materials were evaluated for repellency, fumigant toxicity, contact toxicity and antifeedant potential in either a choice or no choice experiments. The non-volatile substances were evaluated for anti-oxidant properties. Using activity-guided isolation, three known compounds, a one pimarane diterpene type compound; sandaracopimaradiene-1α,9α-diol, a sesquiterpene lactone; deacetylviguiestenin and a caffeic acid derivative; 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid were obtained from *T. diversifolia*. The compounds were characterised by spectral methods; 1D and 2D nuclear magnetic resonance (NMR), Fourier transform Infra-red spectroscopy (FTIR) and mass spectrometry (MS). Characterisation of essential oils revealed three chemotypes that were found to grow at different places of eastern Uganda. Based on the profiles of the compounds of the farnesene family, results showed that farnesol was a major constituent in the oil of chemotype 1 while springene compounds (β-springene and α-springene) and the β-farnesene were widely expressed in chemotype 2. The third chemotype was a mixed hybrid of both farnesol and the springene chemotypes. In all the chemotypes, alkylbenzenes; o-xylene, m-xylene and ethylbenzene were in huge amounts as other significant components in the oil. 1,4-dihydroxy-p-menth-2-ene, 5,9-undecadien-2-one, 6,10-dimethyl, and 3-cyclohexen-1-carboxaldehyde,3,4-dimethyl were other most significant chemical constituents of the three chemotypes. The presence of chemical variation in the components of *T. vogelii* essential oil showed that the fumigant and repellency efficacy of this plant against *Sitophilus zeamais* Motschulsky were undermined. However, all the chemotypes of *T. vogelii* essential oil could show repellence and fumigant toxicity against *S. zeamais*. The comparison between insecticidal and repellent effects of the essential oils of chemotype 3 of *T. vogelii* with that of *T. diversifolia*

was done. Results indicated that the former could be promoted more for contact toxicity effect while *T. diversifolia* better suits fumigation effects against *S. zeamais*. Investigation of the feeding deterrence of both volatile and non-volatile substances of *T. diversifolia* leaves revealed that its essential oil did not show significant antifeedant activity at a dose of <0.29 µL/mg of flour disks. The crude extract, some fractions and the isolated compounds demonstrated feeding deterrence activity against *S. zeamais*. The feeding deterrence index of sandaracopimaradiene-1*α*,9α-diol was 81.19±5.94% at 0.1% w/w (1 mg/g food) compared to 97.45±0.43% for Neemazal extract (10% azadirachtin) (positive control) at 10%w/w (100 mg/g food). The EC_{50} of deacetylviguiestin, 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid and azadirachtin (commercial antifeedant) against *S. zeamais* were 22140.23± 9103.29, 3654.28± 2715.09 and 14.59±5.59 ppm respectively. Although these compounds showed less activity against *S. zeamais* than azadirachtin, their antifeedant activities were very significant and can be valuable alternatives.

UHPLC-DAD-ESI-MS/MS characterisation of fractions of *T. diversifolia* led to the identification of 7 novel hydroxycinnamates; 4-*O*-(1-*O*-caffeoylglucosyl)quinic acid, 4-*O*-caffeoyl-2-hydroxy-3-methylbutanoic acid, 4-*O*-caffeoyl-2-hydroxybutanoic acid, 3,4-dihydroxyphenyl-4-*O*-caffeoyl-2-hydroxybutanoate, 4-vinylphenyl-4-*O*-caffeoyl-2-hydroxy-3-methylbutanoate, 4-*O*-caffeoyl-caffeoyloxymethyl-2-hydroxy-3-methylbutanoate, 4-*O*-caffeoyl-2-(2-(3,4-dihydroxyphenyl)acetoyloxy)-3-methylbutanoic acid from *T. diversifolia* leaves that had not been previously reported in nature. Their structures have been assigned based on the unique MS/MS fragmentation patterns for each of the compounds in a switching negative or positive ionisation mode and deduction from 1D ^{1}H and ^{13}C NMR of similar compound. In total, 55 phenolic compounds from aqueous fractions of *T. diversifolia* have been identified including 4 hydroxybenzoic acids, 19 hydroxycinnamic acids, 22 flavonoids, 1 saponin triterpenoid and 9 coumarins and furocoumarins. The present study further showed that the DPPH radical scavenging effect and reducing power antioxidant activity of tested samples of *T. diversifolia* showed a moderate correlation with the phenolic content present in these samples. This implied that the antioxidant potential was just partly attributed to its phenolic content.

Keywords: Chemical variation, pesticidal activity, antioxidant potential, isolation, characterisation, bioactive compounds, *Tithonia diversifolia, Tephrosia vogelii*

Chapter One

Introduction and Literature review

1.1 Background

Food safety and security is an important aspect of the well-being of human beings. Food insecurity challenges have escalated with the rapid surge in the world population (Tubiello et al., 2007). Among many factors causing food insecurity are pests and diseases. It has been reported that the agricultural crop loss is about 35% and 14% due to pests and diseases on the field and in grain storage pests respectively every year (Okwute, 2012; Jitendra Kulkarni, 2009). A proper and sustainable pest control strategy therefore becomes an eminent solution to protect food crops from the destruction by pests. Many farmers use synthetic pesticides as a conventional approach to pest management. These pesticides although may be effective, they are less available in rural areas. They are also costly and are likely to be used with incorrect mixing or when expired. Furthermore, synthetic pesticides are toxic and pose a serious impact on food safety systems (Malhat et al., 2015) and human health (Mazid, 2011, Roca et al., 2014) amidst other long term effects. The World Health Organisation (WHO) estimates that two hundred thousand people are killed annually due to direct poisoning from pesticides (CAPE, 2009; Belmain et al., 2013). Moreover, pests are increasingly becoming more resistant to synthetic pesticides according to the United Nations Organisation (UNO). Additionally, they are known to be toxic to the environment and harm non-target species (Bolognesi and Merlo, 2011; Saxena, 2014).

Recent reports indicate that extracts, fractions and isolated compounds of traditional/local plants can effectively protectant crops against pests (Khater, 2012; Kareru et al., 2013). As a result of eco-toxicity effects due to the use of conventional pesticides, pesticidal plant use is gaining momentum in developing countries (Isman, 2008). Studies further show that pesticidal plants (botanicals) can provide farmers with a more friendly pest management alternative which is environmentally sustainable at a low cost (Dubey et al, 2008; Dubey, 2011; Sola et al, 2014; Grzywacz et al, 2014). Compounds of pesticidal plants rapidly decompose compared to those which are synthetic in nature (Grzywacz et al, 2014). They are naturally at low levels with a diverse suite of active ingredients (Mkenda et al, 2015a). Additionally, their mode of action as repellents or antifeedants leads to the preservation of the biodiversity (Mkenda et al, 2015a). As

a result, they possess less harm to non-target organisms, making them a good alternative to synthetic pesticides and thus a greener way to a sustainable food production.

1.2 Review on the effective use of botanicals as pesticides and antioxidants

Pesticidal plant species (Table 1.1) have been widely categorized into three groups according to (Belmain 2014):

1. Those that can be propagated easily and are fast-growing, invasive, and perennial weeds such as *Tithonia diversifolia* (Hemsl.)*, Azadirachta indica* A.Juss., *Tagetes minuta* L., *Melia azedarach* L., *and Melia volkensii* Gurke., *Tephrosia vogelii* (Hook f.), *Solanum incanum* L., *Dolichos kilimandscharicus* Harms ex Taub*, Lippia javanica*(Burm.f.) Spreng, *Neorautanenia mitis* (A. Rich.) Verdc, *Lantana camara* L.*, Dysphania ambrosioides* L.*, Tanacetum cinerariifolium* (Trev.) Sch, *Vernonia amygdalina* Delile.

2. Those that are difficult to propagate, woody, slow-growing and rare like; *Euphorbia candelabrum* Kotschy*, Zanha Africana* (Radlk.) Exell*, Bobgunnia madagascariensis* J.H.Kirkbr. & Wiersema, *Securidaca longepedunculata* Fresen.*, Euphorbia tirucalli* L.*, Aloe ferox* Mill., *Cissus quadrangularis* L. and,

3. Those that are consumed when planted as food or spice and can be waste products like; *Citrus sinensis* L.*, Capsicum annuum* L.*, Allium sativum* L.*, Piper guineense* Schumach., *Nicotiana tabacum* L.*, Citrus limon* L.*, Piper nigrum* L., *C. paradise, Cymbopogon* species*, Ocimum* species, and *Annona* species.

These pesticidal plant species if cultivated on farmland can provide a readily available supply of pesticides to smallhold farmers in the end raising their revenues. In East Africa, forexample, pyrethrum is grown as a cash crop by many farmers especially in Kenya (Khater, 2012). The neem tree, *Azadirachta indica* is a popular pesticidal plant used and commercialized in some African regions and South Asia (Anjarwalla et al, 2016). Phytochemicals from pesticidal plants have also brought major financial returns such as rotenoids from *Tephrosia* spp., *Derris* spp. and *Lonchocarpus* spp. Because they have been produced as organic pesticides (Jacobson, 1982). Essential oils often found in many herbs and spices, have also been commercialized in North America (Isman, 2000).

Table 1.1: Use of pesticidal plants

Pesticidal plant	Effective use and application period	Reference
Tephrosia vogelii	Mix dry powdered leaves of this material at 10% w/v in water having 1% liquid soap for a day (1% w/w). For application, the concentration is reduced to 1-2% by adding water five times and sprayed in the early evening. It can be used on stored legumes but less effective on storage-pests of maize. It may also be used for field applications	Reuben et al., 2006, Stevenson et al., 2010, Belmain, 2014
Dolichos kilimandscharicus and Neorautanenia mitis	Used in a similar way as *T. vogelii* leaves during pre-harvest and post-harvest and on livestock's ticks	Stevenson et al., 2010).
Tithonia diversifolia	Used as powdered material at one to five percent (w/w) at 10% solution on maize and legume pests for example those of tomatoes, beans and brassicas during field and stored pests offering general efficacy.	Stevenson et al., 2010, Adedire and Akinneye, 2004; Stevenson et al., 2010
Tagetes minuta (Mexican marigold, stinkweed, stinking roger and khaki bush)	Whole-plant extracts (1 to 5% w/v) show efficacy against many crop/soil pests during post-harvest storage	Stevenson et al., 2010
Tanacetum cinerariifolium	Used on crops, post-harvests' pest, ectoparasite of livestock and in domesticated/urban pest management	Anjarwalla et al, 2016
Solanum incanum (Bitter tomato, thorn apple)	Dry fruits of *S. incanum* are crashed, extracted in water and applied at 5% w/v on field pests or ticks	Madzimure et al, 2013
Dysphania ambrosioides	Whole plant or leaves and may time to time agitated to release volatile bioactive constituents, 1% w/w work well for bean weevil, 5% w/w is required against maize weevil and extracts at 10% w/v are effective against field pests.	Mkenda et al, 2015a, Stevenson et al., 2010
Securidaca longepedunculata (African violet tree)	Root bark powder admixed with stored grain shows efficacy when it is 1% w/w concentration. Optimization in water using soap to a usable solution of 1% w/v for field protection and post-harvest pests such as *Sitophilus granarius* L and *Prostephanus truncatus* Horn shows good efficacy	Belmain et al., 2001, Boeke et al., 2002, Stevenson et al., 2010

Bobgunnia madagascariensis (snake bean tree)	It is seed pods that are mainly used against pests, though the stem and root barks have shown antifeedant and contact toxicity effects on the pest of stored products, *Tribolium casteneum* as field pesticide but also vegetable pest during post-harvest protection	Adeyemi and Adebote, 2010, Nyirenda et al., 2011
Euphorbia candelabrum candelabra tree	Field pests, post-harvest protection	Shi et al., 2008
Euphorbia tirucalli (pencil tree or finger euphorb)	Field pests such as aphids and a mosquito insecticide Pre-harvest and post-harvest protection	Stevenson et al., 2010
Aloe ferox (bitter aloe)	Traditionally insect repellent when fence-planted all over the crop field. It's dried dead leaves burnt to ash can act as stored pest control at 2 to 5% w/w	Anjarwalla et al, 2016
Capsicum annuum (chilli pepper)	When dried and its fruit grounded can be used at 1% w/v as a post-harvest protectant. Effective use, levels of 10% are recommended. Fresh fruit extract (5% w/v) works against several field pests	Zibokere, 1994
Piper nigrum (black pepper)	Ground pepper can work well at 1% w/v against stored beans and cowpeas pests	Grainge et al 1984; Park, et al., 2002, Javier 1981
Nicotiana tabacum (tobacco)	Its dry leaf water extract 1 - 2% w/v shows considerable efficacy and at a higher amount, it demonstrates phytotoxicity potentials. Whole leaves admixed with beans/cowpeas are effective. with maize, the powder is effective at 5% w/v	Boeke, 2002
Citrus sinensis	Peel powder can protect against stored pests, effective at 1% w/v and a higher concentration of 5% w/v against maize weevils. Soak small pieces of rinds for 10 days, however, peels are heated for at least 5 minutes. Oils of citrus can work on aphids and caterpillars	Akunne et al., 2015, Kamaraj et al., 2008, SGA, 2019
Allium sativum (garlic)	Powdered extract at 2% (w/w) is used against wheat pest *T. granarium.* Pounded fresh materials of the water extract optimized with soap show that the solution at 10% w/v gives good results against *Brevicaryne brassicae* L. Lower concentrations with fresh material is more effective	Jood et al., 1993, Mudzingua et al., 2013
Ocimum gratissimum	Has aromatic volatiles and it works better as a whole	Silva de

	leaf/plant on stored-pest. The best solution to spray against pests from a dry or fresh material of the extract is 10%.	Vasconcelos, et al, 1999
Cymbopogon citratus, (DC. ex Nees) Stapf. *(*Lemon grasses), *C. nardus, C. schoenanthus* (citronella)	Used as layers in the granary, or as a powder admixed on post-harvest pests. As a water extract optimized with soap during production from either fresh or dry material, a 10% level would give better results	Saraswathi and Rao, 1987
Annona senegalensis Pers. (Custard apple), *Annona squamosa*; and *Annona squamosa* L. (soursop)	0.1% w/v inhibited F1 adult emergence of *C. chinensis*. Its ether extract applied against *Tribolium castaneum* exhibited moderate toxicity. The seeds of these plants are grounded to powder; extracted in water with soap optimization to a final concentration of 1% w/v and the plants are generally not recommended for post-harvest	Islam, 1987, Oliver-Bever, 1986

Note: Pesticidal plants can be effectively used to produce the desired efficacy. For better application, optimisation is required and improves efficiency and ease for adoption by farmers in the field and post-harvest storage. The form, part, concentration and pest target are key to the practical use of these plants.

So many spices and aromatic herbs such as those in category three above as described by Belmain (2014) contain chemical compounds exhibiting antioxidant properties (Madsen and Bertelsen, 1995). In developed countries, enough supply of food to satisfy hunger is not enough. Communities associate food with happiness and well-being. Consequently, health benefits have been an important role in food consumption (Carrillo et al. 2013). Some foods develop rancidity due to free radicals resulting in off-flavors and undesirable chemical compounds (Horton and Fairhurst, 1987). In addition to some harmful effects of free radicals such as degenerative diseases like cancer, inflammation and aging, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen have also participated in food deterioration (Shigrnaga and Hagen, 1993; Halliwell, Gutteridge and Cross, 1992; Larson, 1997; Chatterjee et al., 2007). Antioxidant compounds inhibit or delay oxidation of other molecules by terminating the initiation or propagation of oxidizing chain reactions (Chatterjee et al., 2007). Their presence neutralises free radicals and reduce the oxidative stress damage in the body. Synthetic antioxidants have been restricted in favour of natural antioxidants due to their carcinogenicity (Lindenschmidt et al. 1986). Screening studies for anti-oxidant properties of

botanicals have been so rampant in the recent past in an attempt to obtain natural anti-oxidants for the food industry. Unlike synthetic anti-oxidants, natural anti-oxidants reduce cases of carcinogenic nature (Lindenschmidt suhaj, 2006).

The anti-oxidant potential of plant extracts has been measured by several methods forexample via their ability to scavenge 1,10-diphenyl-2-picrylhydrazyl (DPPH) radical, inhibit lipid peroxidation etc in several in vitro assays (Singhand Singh, 2008; Chanda and Dave, 2009; Badarinath et al., 2010) (Table 1.2). The mechanisms of these in vitro antioxidant assays vary. Forexample, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method involves spectrophotometric determination of the stable free radical; 2,2-diphenyl-1-picryl hydrazil (DPPH) (Blois, 1958). Ferric Reducing Antioxidant Power (FRAP) assay involves reduction of Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) to form an intense blue Fe^{+2}-TPTZ (tris-pyridyl-triazine) complex and then measured spectrophotometrically (Benzie and Strain, 1996). FRAP and DPPH are presented as DPPH radical inhibition or FRAP value (IC_{50}) and the higher absorbance value, in a FRAP assay indicate higher antioxidant activity. The Trolox equivalent antioxidant capacity (TEAC) method involves discoloration of the amount of 2,2′Azinobis-3-ethylbenzthiazoline-6-sulphonic acid radical ($ABTS^{\bullet+}$) that are scavenged within a fixed time frame in relation to that of 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox) (Miller et al., 1993). That of ABTS assay measures the colored characteristic $ABTS^{\bullet+}$ radical cation spectrophototmetrically (Pelegrini et al., 1999). Oxygen radical absorbance (ORAC) method uses aperoxyl radical generated by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and the corresponding decrease in the fluorescence of a fluorescent probe which is then measured (Cao et al., 1993). In Superoxide anion scavenging activity method, there is generation of superoxide anions enzymatically in a hypoxanthinexanthine oxidase system coupled with nitroblue tetrazolium (NBT) reduction and the absorbance related to the reduction of nitro blue tetra- zolium is measurement at 540 nm (Beauchamp and Fridovich, 1971). Total phenol content (TPC) relies on the anti-oxidant ability of polyphenols arising from ability of their polyphenol derived radical to stabilize and delocalize the unpaired electron and from their potential to chelate metal ions (Rice-Evans et al., 1997). Total flavonoids content (TFC) results from the antioxidative properties of flavonoids in scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation (Benavente-Garcia, 1997).

Table 1.2: Data on anti-oxidant potential of common African plant species

Pesticidal plant	Part	method	IC_{50}	TPC	TFC	Reference
Tephrosia apollinea	MeOH leaf extract	GSH	2128.1 ± 119.8 (mmol g^{-1} Glutathione)	91.8 ± 1.8		Rizvi et al., 2018
Tithonia diversifolia	Acetone leaf extract	DPPH	41.05 µg/mL	251.63 GAEmg/100 g	98.21 QEmg/100 g	Ojo et al., 2018
Tagetes minuta	EO	DPPH	2.45 mg/mL	216.84 (mg GAE/100 g)		Igwaran et al., 2017; Katerere et al., 2012
	MeOH extract	DPPH/ABTS/ FRAP	1399.42/ 2.3/ 4.22 TEAC (mmol/100 g)			
Lippia javanica	MeOH extract	DPPH/ABTS/ FRAP	1462.54/ 1.5/ 2.38 TEAC (mmol/100 g)	221.31 (mg GAE/100 g)		Katerere et al., 2012
Bidens pilosa	MeOH extract	DPPH/ABTS/ FRAP	1210.05/ 5.77 / 6.12 TEAC (mmol/100 g)	333.56 (mg GAE/100 g)		Katerere et al., 2012
Solanum incanum	Fruit MeOH extract	DPPH	98.7[a] µg/mL	2.31±0.37 mg/g GAE	0.21±0.09 mg/g QE	Anwar, 2018 Ghosal, and Mandal, 2012
Dysphania ambrosioides	MeOH extract	DPPH	130.7±0.57 µg/mL			Zohra et al., 2019
Securidaca longepedunculata	Root bark	DPPH	5.5 µg/mL	9.86 mg/g GAE/DW	5.85 mg/g CE	Muanda et al., 2010
		superoxide	350.11±0.42 µg/mL	-	-	Abonyi et al., 2014
Euphorbia tirucalli	MeOH extract	DPPH	46.16 µg/mL	77.33mg/g GAE/DW	28.65± 6.45 (mg RE/g)	Munro et al., 2015
Aloe ferox	Whole leaf MeOH extract	DPPH/ ABTS	0.086 mg/mL / 0.02 mg/mL	25.01±0.07 mg/gTAE	5.22±0.05 mg/g QE	Wintola, et al, 2011
Capsicum annuum		DPPH	25.0±0.47[a]	2.20±0.22 mg/g	-	Gacch et al, 2010
Piper nigrum	Aqueous leaf extract	ABTS	68.96±0.03[a]	1.89±0.08 mg/g GAE/DW	1.04±0.06 mg/g QE	Shanmugapriya et al., 2012
	Ethanol extract	DPPH	14.15±0.02 µg/mg	62.3±0.08(µg/g)		Nahak et al., 2011
Nicotiana tabacum	MeOH extract	superoxide	3.263±0.227 µmoles of H_2O2 consumed/min/mg		838mg QE/g	Sharma et al., 2016
Allium sativum	80% MeOH gallic bulb	DPPH	1.03 to 6.01 mg/mL	0.05–0.98 mg GAE/gDE	4.16–6.99 lg QE/g FA	Bozin et al., 2008,
Ocimum gratissimum	leaf extract	DPPH	12.3± 1.95 µg/mL	0.839± 0.097GAE	39.12± 2.43 mg REF	Awah, 2010.

	MeOH leaf extract	DPPH	84.6[a] at 250 µg/mL GAE.	5.68 ± 0.06 mg/g GAE.		Akinmoladun et al., 2007
Cymbopogon nardus	MeOH extract	Acetylcholinesterase inhibitory activity	16.4 ± 6.8 to 26.4± 6.8 mg/mL	66.3 ± 1.7 µg PPG/DW	765± 31 µgRE/ Dw	Khadri et al., 2010
Annona senegalensis		DPPH	1.5-167.00 µg/mL			Kengni Yande et al., 2017

TPC = total phenol content, TFC = total flavonoid content, GAE=gallic acid equivalents phenolic content, REF= rutin equivalents flavonoid content, QE/g FA= quercetin equivalents/g of dry extract of flavonoid aglycones, PAG= phenolic acid glycosides,* is EC_{50}, TAE=tannic acid equivalent, µg PPG = micrograms pyrogallol per gram (DW/dry weight) extract, [a]percentage scavenging, ABTS= 2,2′Azinobis-3-ethylbenzthiazoline-6-sulphonic acid; DPPH= 1,1-diphenyl-2-picrylhydrazyl; FRAP= ferric reducing antioxidant potential, TEAC =Trolox equivalence antioxidant capacity, GSH= glutathione, CE = catechin equivalents, MEOH = methanol, EO= essential oil. IC_{50} means the concentration of the plant extract that is able to scavenge half of the DPPH free radical present in test solution. The lower this value the higher is the antioxidant activity of the extract.

1.3 Botanical and biological description of *T. diversifolia* and *T. vogelii*

Tithonia diversifolia (figure 1.1) is a pesticidal and medicinal flowering plant found among the plants of the Asteraceae family (Ayeni et al., 1997). It is a traditional medicinal herb that is believed to have its roots in the South and Central American regions (Carter, 1978; Zhai et al., 2010). It is also known for its insecticide properties in the field of agriculture (Casta-o-Quintana et al., 2013). The plant has an invasive character, producing flowers yearly and their seeds disperse by erosion and animals (Ayeni et al., 1997; Chagas-Paula et al., 2012). Figure 1.2 shows the distribution of *T. diversifolia* in Africa. In East Africa, *T. diversifolia* is commonly found in regions with an altitude of about 550-1950 m above sea level especially in disturbed places (Kandungu et al., 2015).

Figure 1.1: *Tithonia* spp

Source: World agroforestry center: Species database. 2015

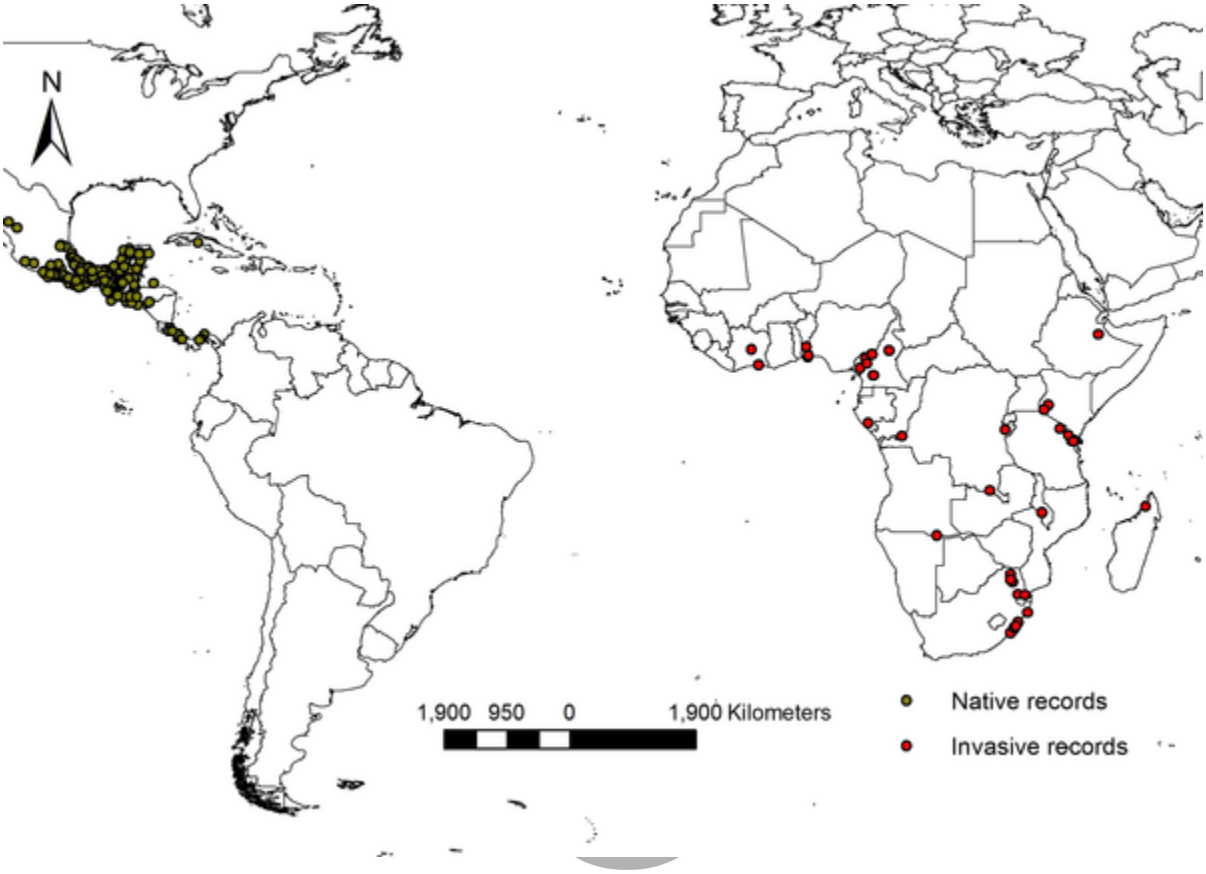


Figure 1.2: Geographic distribution of *T. diversifolia* in Africa

Source: Obiakara and Fourcade, 2018

It is also known to grow in grassland vegetation that receives a mean annual rainfall of 850-2000 mm and an annual range of 15-31^{0}C (Kandungu et al., 2015). In Uganda, *T. diversifolia* is found in almost all regions of the country especially in disturbed places. Although its use is not well adopted for agricultural practices, farmers in Uganda use *T. diversifolia* to control post-harvest and preharvest pests (Mwine et al., 2011; Kerebba et al., 2019). Stevenson et al., (2010) reported its general efficacy on the pests of vegetable crops in Malawi, Kenya and Zimbabwe.

Figure 1.3: *Tephrosia* spp

(World agroforestry center: Species database. 2015)

Tephrosia vogelii (Hook f.) (Figure 1.3) is a pesticidal plant native to tropical Africa (Matovu and Olila, 2007) that belongs to the family Leguminosae. It is also commonly referred to as fish poison bean because of its toxic effect on fish (Neuwinger, 2004). Its habitant widely varies from savanna vegetation through grass, forest, shrub to fallow areas of Africa (Stevenson et al., 2012). It is distributed in climate zones whose annual rainfall pattern ranges between 850mm to about 2700 mm with an average temperature between 12 ^{o}C and 26^{o}C annually (Mwaura et al., 2015). It also occurs at an altitude that stretches up to 2100 m above sea level (Mwaura et al., 2015). The flowers are bisexual; enabling it of self-pollination although carpenter bees are considered as the principal pollinators. Its herb is soft woody, 0.5-4.0 m tall and the branches have got dense leaves (Mwaura et al., 2015). Promotion of *T. vogelii* for field and storage applications have been done in eastern and southern Africa by various researchers with aim of continental wider farm use as a principle traditional pesticide, fish poison and soil enricher (Stevenson et al., 2010, Kamanula et al., 2011; Nyirenda et al., 2011).

1.4 Review on the pesticidal activity of *T. diversifolia* and *T. vogelii*

A detailed review of this section including the phytochemistry of the two plants has been published (Kerebba et al., 2019). This review aimed at evaluating the status of the current

chemistry of the pesticidal activity of *T. vogelii* and *T. diversifolia*. The review critically summarized the traditional use, phytochemistry of *T. diversifolia* and *T. vogelii* as generational botanical pesticides, evidence of their use in Africa and the current gaps in the effective use of the two plants. *Tithonia diversifolia* has been used against aphids and beetles (Mkenda et al., 2015b), red spider mite *Oligonychus coffeae* (Radhakrishnan and Prabhakaran, 2014), Aphis sp., *Crocidolomia binotalis*, *Ophiomyia phaseoli* and a borer species (Moreno, 1991), *S. zeamais* adults in corn grains (Tavares, et al., 2014) and on cowpea seed bruchid (Adedire and Akinneye, 2004). *Tephrosia vogelii* has been used in controlling bruchids in beans and cowpeas (Adebayo et al., 2007), *Megalurothrips sjostedti* and *Apion varium* on cowpeas (Alao et al., 2012), aphids (Brevicoryne brassicae L.) (Mudzingua et al., 2013), ticks and worms (Matovu, H. and Olila, 2007). The review noted that a huge amount of research work on *T. diversifolia* pesticide applications published in the last decade was done using its crude extract form rather than pure isolated compounds. It also highlighted the inadequacy of comprehensive study on the mechanisms of action of these extracts thus the gap on its pesticidal activity. The pesticidal application of *T. vogelii* is affected by the occurrence of different chemotypes within the same plant species. The chemical variability within its species compromises its efficacy as a source of botanical pesticide (Stevenson et al., 2012). However, the extent of chemical variation in *T. vogelii* was still unknown i.e. whether chemical variation would occur in the essential oils of this plant just like it occurred in the non-volatile substances. Therefore the review raised a question on whether there could be distinct chemotypes based on different components of the essential oils (Kerebba et al., 2019).

1.5 Review on the antioxidant activity of *T. diversifolia* and *T. vogelii*

Different extracts of *T. diversifolia* (aqueous methanolic and dichloromethane leaf extracts) have scavenged free radicals due to its phenolic content (Giacomo et al., 2015). A study by Giacomo et al., (2015) indicated that aqueous extract scavenged free radicals most (equivalent 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging = 94.89±2.69 mmol Trolox), followed by methanolic and then dichloromethane. The authors further noted that the scavenging activity can be compared to that of 80 mU of superoxide dismutase at 0.04 µg/mL. Similarly, Juang et al., (2014) reported that ethanolic leaf extract scavenged DPPH free radicals with IC_{50} = 0.93±0.20

µg/mL compared to that of the positive standard, ascorbic acid IC_{50} = 0.48±0.10 µg/mL. In a related report, free radical scavenging potential reached 241.04±11.93 mmol Trolox of dry extraction weight and the difference was attributed to variation in the phenolic content from *T. diversifolia* extract (Hiransai et al., 2016). The equivalent ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-radical scavenging activity of the ethanolic extract was determined to be 93.09±37.91 µM TEAC (Trolox equivalent antioxidant capacity) (Thongsom et al., 2013). These findings reveal that extracts of the aqueous extract of *T. diversifolia* could lead to the formation of lipid hydroperoxide in a free cell system in a dose-dependent manner leading to prevention of peroxidative damage of the plasma lipids (Giacomo et al., 2015). The essential oils of *T. diversifolia* scavenged DPPH (IC_{50} = 108.8 µg/mL) and ABTS (IC_{50} = 41.7 µg/mL) free radicals (Orsomando et al., 2016). Reports on the anti-oxidant potential of *T. vogelii* are extremely scarce. However, Li et al. (2010), reported free radical scavenging potential of the ether extract of the *T. vogelii* seeds.

As noted above, different values of the anti-oxidant potential of *T. diversifolia* have been linked to differences in the amount of phenolic compounds with antioxidant potential as will be discussed later. Phenolic acids and caffeic derivatives in the butanol extract from *T. diversifolia*; 4,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid ((E)-3-(((3-(3,4-dihydroxyphenyl)acryloyl)oxy)methyl)-2-methyloxyrane-2-carboxylic acid) showed strong DPPH radical-scavenging and a moderate ferric reducing antioxidant power (FRAP) (Pulido et al., 2017). This conclusion was consistent with the notion that total phenol content is in some cases is responsible for the antioxidant potential of the extract (Pulido et al., 2017). This implies that the antioxidant potential was currently attributed to its phenolic content. Orsomando et al., (2016) however has indicated that the essential oil did show some scavenging activity against DPPH radicals. The contents of the essential oil leading to this antioxidant potential have not yet been evaluated.

Researchers have observed the need to understand the phytochemical composition of plants so that their uses in biological activities are well established (Bisht and Kamal, 1994). Some phytochemicals are known for insecticidal and antioxidant actions. Insecticidal actions of phytochemicals are due to the possession of toxic principles like flavonoids and isoflavonoids. Phytochemicals also impose behavioral responses to insect pests like repellence, feeding

deterrence while some have growth-regulating potential and oviposition deterrence (Klocke et al., 1989; Hassanali and Lwande, 1989; Wheeler and Isman, 2001; Torres et al., 2003). Some phytochemicals have reactive oxygen species (ROS) detoxifying capacities e.g. flavonoids and sesquiterpene lactones, giving them antioxidant potentials.

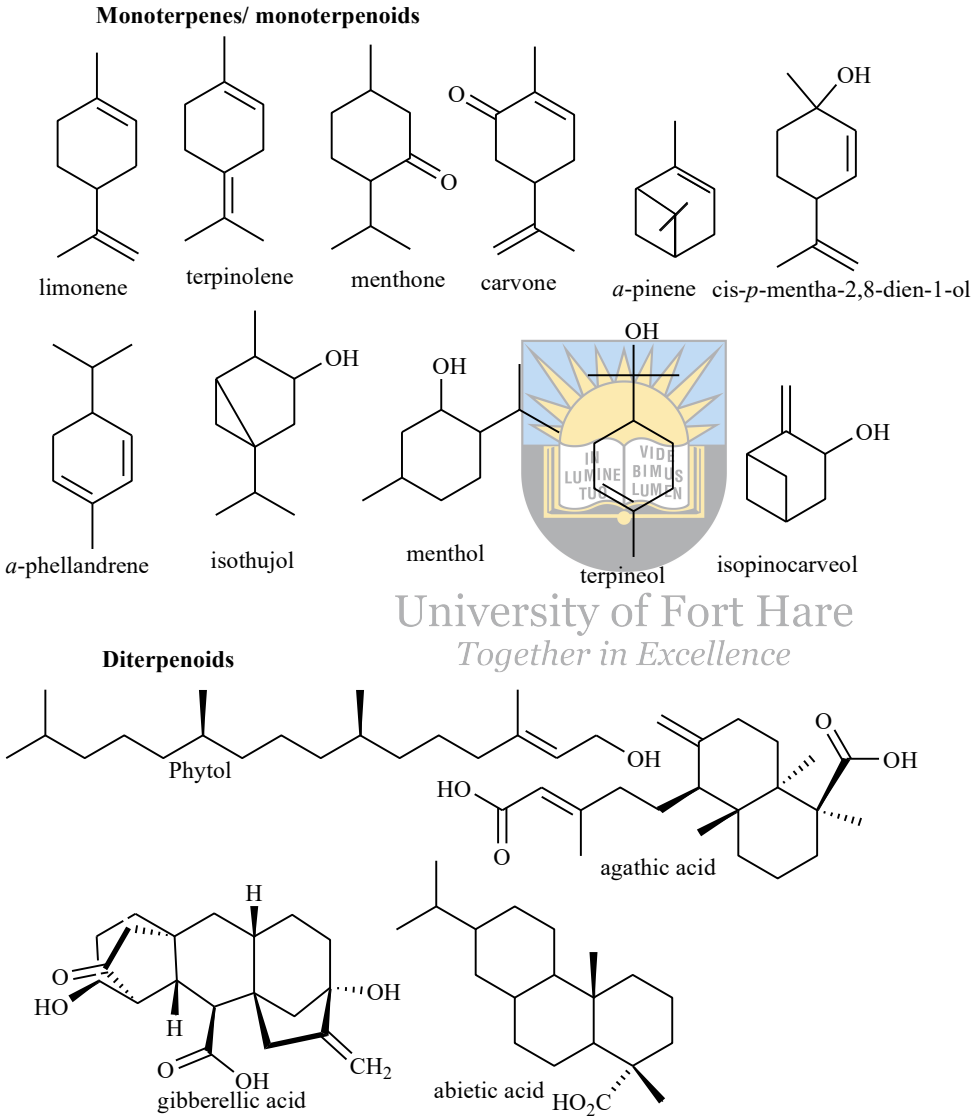
1.6 Classes of organic compounds from plants with promising pesticidal and anti-oxidant activities

Many phytocompounds such as flavonoids, terpenes, rotenoids, alkaloids, terpenoids, steroids, saponins and tannins, glycosides, essential oils and phenolics are linked to pesticidal and anti-oxidant activities (Abayomi, 1993; Bruneton, 2001; Paruch et al., 2001; Simmonds (2001, 2003); Kotkar et al., 2002; Ahmad et al., 2006; Stevenson et al, 2012; Belmain et al., 2012). Some of these phytochemicals possess toxic, antifeedant, repellent and growth-regulating effects against a wide range of insect pests (Klocke et al, 1989; Hassanali and Lwande, 1989). Terpenoids, the largest and most diverse family of natural products, are responsible for plant direct defense, or as signals in indirect defense responses that involve herbivores and their natural enemies (McCaskill et al., 1998). Less volatile but strongly bitter-tasting or toxic terpenes act as antifeedants, to protect some plants from being eaten by animals (antifeedants) (Degen-hardt et al., 2003). Terpenes play a role as signal plants and plant growth regulators (phytohormones) (Saxena, 2013). Insect antifeedants have been performed on neem triterpenoids, clerodane diterpenes from the *Lamiaceae* family (Klein Gebbinck et al., 2002) and sesquiterpene lactones from the *Asteraceae* family (Gonzalez- Coloma et al., 2002).Terpenoid of extracts of *Lantana. Camara* against American ball worm, *Helicoverpa armigera* larvae, are possible antifeedants (Paul and Choudhury, 2016). Monoterpenoids (under terpenoid group), are the major components of the aromas of plants and are volatile natural products considered to have insecticidal constituents of many essential oils (Coats et al. 1991; Konstantopoulou et al. 1992; Regnault-Roger and Hamraoui 1995; Kim et al. 2003). Several monoterpenes isolated and evaluated for toxicity to different insects include; α-pinene, β-pinene, 3-carene, limonene, myrcene, α-terpinene and camphene (Viegas junior, 2003). Monoterpenoids have been reported to inhibit reproduction of stored insect's cycle: inhibition in oviposition, ovicidal effects, a larvicidal effect on neonate larvae before the penetration of the seeds or a larvicidal effect on larvae settled within the seed, thus inhibiting the emergence of adults (Rice and Coats 1994a, b)

(Table 1.4). This mode of toxicity for monoterpenoids is believed to be via competitive inhibition of acetylcholinesterase (Ryan and Byrne 1988). Essential oils act as contact insecticides, repellents antifeedants and fumigants (Ibrahim et al, 2001, Isman, 2000, Lee et al., 2004). A large number of essential oils and their constituents have shown toxicity against stored-product insects of the *Sitophilus* genus as *S. zeamais* (Betancur et al., 2010), *Sitophilus oryzae* L. (Lee et al., 2001), and *Sitophilus granarius* L. (Aslan et al., 2004). Triterpenes or steroids which occur mainly as glycosides possess a common property of bitter principles. Chemically, the bitter principles contain the lactone group that may be diterpene lactones (e.g. *andrographolide*) or triterpenoids (e.g. *amarogentin*). Glucoside containing plants are capable of being used as counter-irritants (Heinrich et al., 2004), antifeedant (Kaufman et al., 1999).

Phenolics are another group of secondary metabolites of medical, biological, agricultural and chemical applications (Saxena, 2013). Across the plant kingdom, nearly 10,000 structures were identified like phenylpropanoids, coumarins, flavonoids, and tannins (Harborne, 1993). Tannin-containing plant extracts have shown antioxidant, anti-inflammatory, antiseptic, and hemostatic properties (Dolara et al., 2005). Flavonoids are one of the chemicals released by plants for protection against natural predators by regulating the ovipositing and feeding behaviour of the predator/ insects e.g Naringenin, hesperetin-7-*O*-rutinoside and quercetin-3-*O*-rutinoside, induce oviposition in citrus feeding swallowtail butterfly *Papilio Xuthus* (Ohsugi, et al., 1985) which have exhibited strong antioxidant potentials. Simmonds (2001, 2003) studied the bioactivity of different flavonoids and concluded that these compounds could modulate the feeding and oviposition behavior of insects. Also, flavonoids inhibited the mycelial growth of a crop pathogen, *Verticillium albo-atrum* (Picman et al., 1995). The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Pietta, 2000; Saxena, 2013). Flavonoids from leaves of *Annona squamosa* (Kotkar et al., 2002) and *Ricinus communis* (Upasani et al., 2003) were found to arrest the population growth of adzuki bean weevil, *Callosobruchus chinensis* L in green gram (*Vigna radiate* L.) during storage. Salnuke et al., (2005) suggested that flavonoids can act as potential grain protectants of legumes via contact, oviposition, deterrent and ovicidal action on bruchid eggs. Different flavonoids are found to alter moulting in insects, causing death (Stamp and Yang, 1996).

Some alkaloids have a stimulant property like caffeine and nicotine which some are used as antimalarial e.g. morphine (Saxena, 2013). People have been using alkaloids in the form of plant extracts for poisons, narcotics, stimulants, and medicines for several years. The repellent activity of the nor-diterpenoid and diterpenoid alkaloids were successively tested against *Tribolium castaneum* (red flour beetle) (Ulubelen et al., 2001). Of the 29 tested alkaloids, 21 compounds showed promising insect repellent activity.



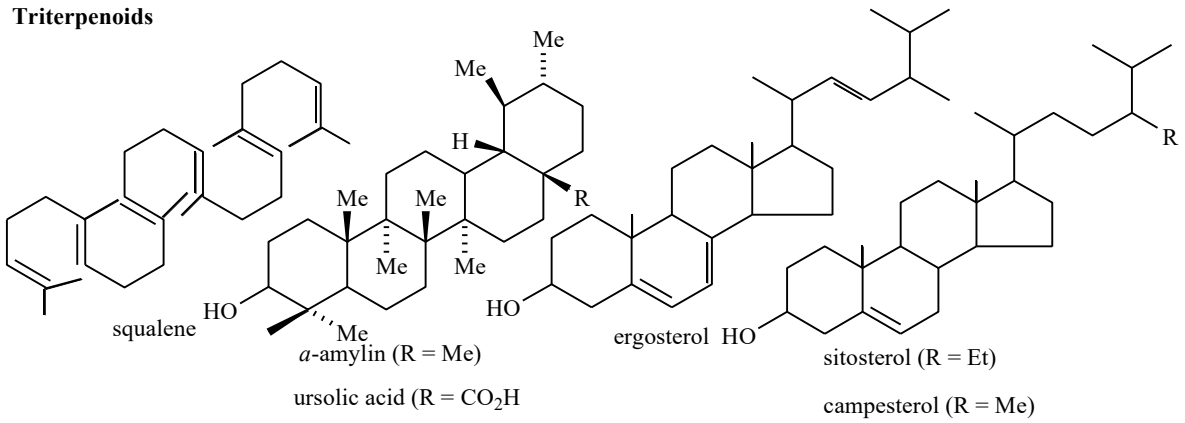


Figure 1.4: Some terpenes and terpenoid compounds

1.7 Structure, pesticidal and antioxidant properties of phenolics and sesquiterpene lactones

Most of the biological activities of plants are due to the possession of phenolic and sesquiterpene lactone compounds.

1.7.1 Structure, pesticidal and antioxidant activity of phenolic compounds

Phenolics are a group of secondary metabolites of medical, biological, agricultural and chemical applications (Saxena, 2013) (Figure 1.5). They are ubiquitously found across the plant kingdom with nearly 10,000 structures identified like phenylpropanoids, coumarins, flavonoids, and tannins (Harborne, 1993). Many phenolic acids and their derivatives have been studied in many medicinal plants including their biological activities such as antimicrobial, antiulcer, anti-inflammatory, antioxidant, cytotoxic, antitumor, antispasmodic, anti-oxidant and antidepressant activities (Ghasemzadeh, 2010). (Adlercreutz, 2002; Rietveld and Wiseman, 2003; Zhao et al., 2012; Pulido et al., 2017 Silva-Beltrán et al., 2017).

Flavonoids are a class of phenolics having two benzene rings separated by a propane unit. Different classes of flavonoids are distinguished by additional oxygen-containing heterocyclic rings and hydroxyl groups which include the chalcones, flavonols, flavones, flavanones, anthocyanins, and isoflavones (William and Grayer, 2004).

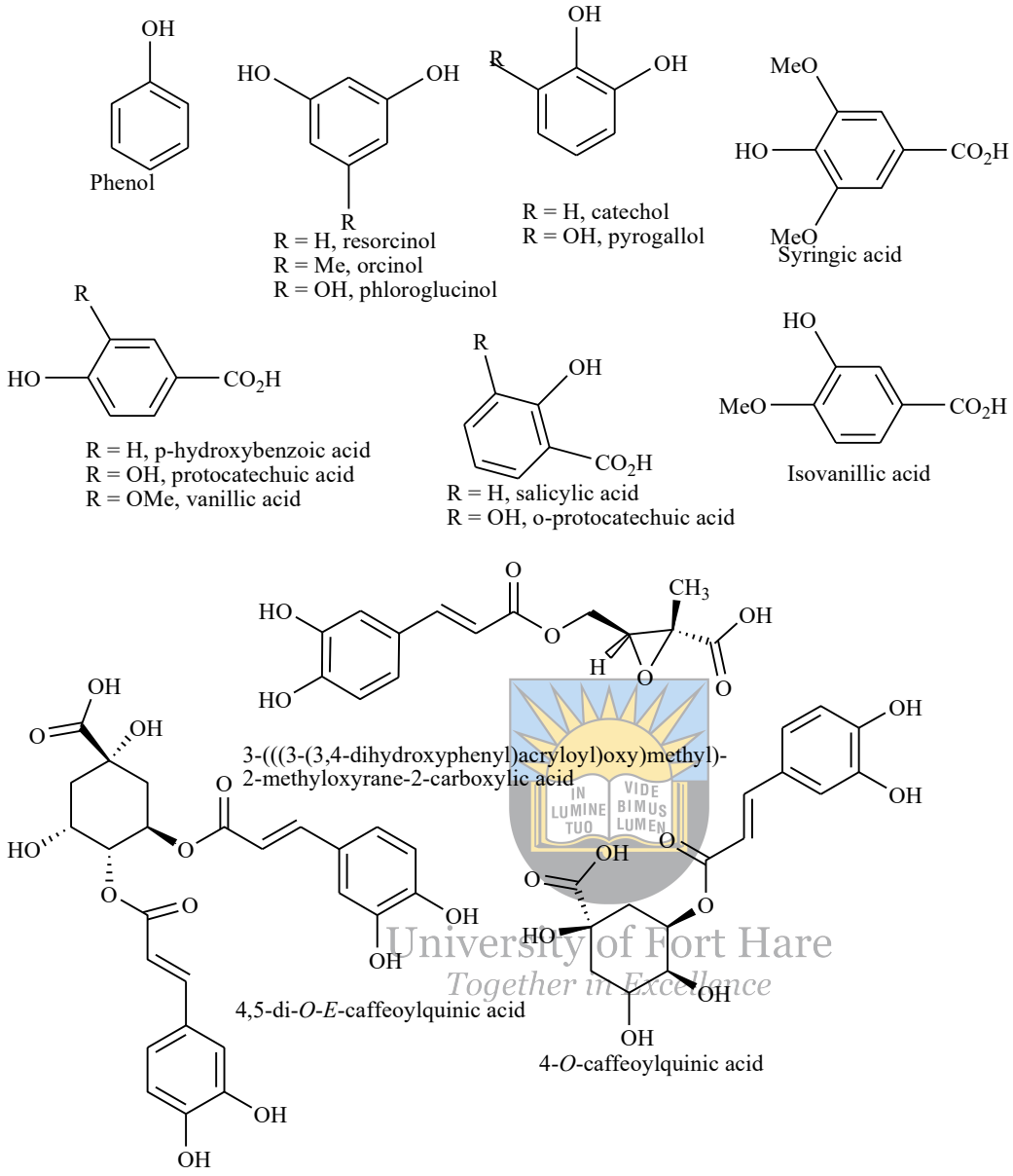


Figure 1.5: Structures of some phenols, phenolic acids and phenolic acid derivatives

Flavones, flavonol, flavanone and genistein belong to a group of flavonoids called flavonoid aglycones. Most flavonoids are built upon C6–C3–C6 flavone skeleton (Figure 1.6 and 1.7).

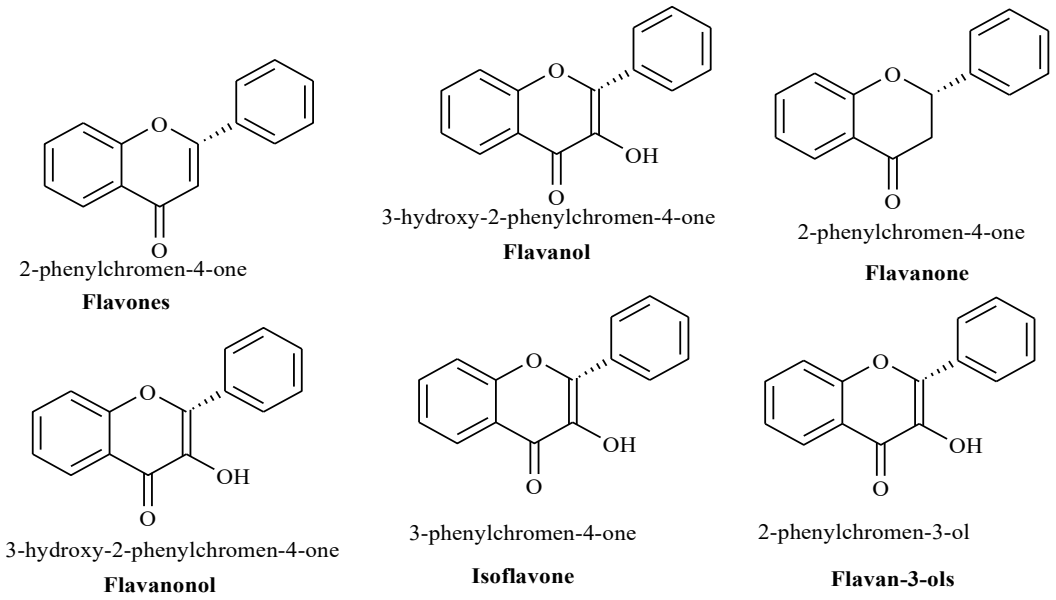


Figure 1.6: Structures of some flavonoid aglycones

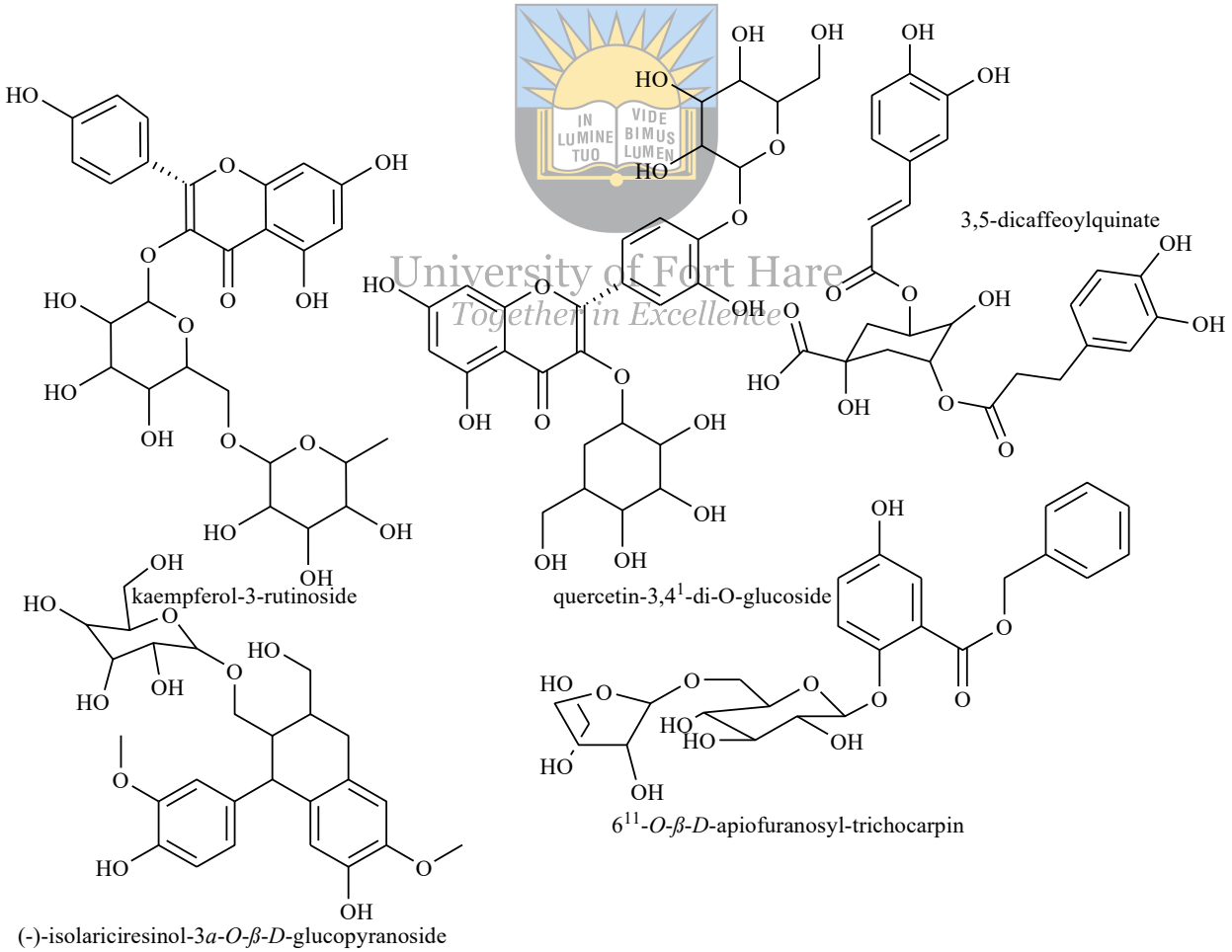


Figure 1.7: Phenolics with sugar moeities

Some flavonoids or phenols that contain sugar component are called flavonoid glucosides or phenolic glucosides. They containing glucose (or a different sugar) and other part comprises a non-sugar molecule such as flavonoid aglycone or a phenolic compound. Zhao et al., (2012) identified phenolic glucosides 6^{11}-*O*-*β*-*D*-apiofuranosyl-trichocarpin, (-)-isolariciresinol-3α- *O*-*β*-*D*-glucopyranoside, 3,5-dicaffeoyl quinate and many others from *T .diversifolia*. Stevenson et al, (2012) isolated several flavonoid glycosides in addition to flavonoid aglycones from methanol extract of *T. vogelii*. These were: quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, isorhamnetin 3-*O*-galactoside, isorhamnetin 3-*O*-glucoside, isorhamnetin 3-*O*-pentoside, quercetin 3-*O*-robinobioside, quercetin 3-*O*-rutinoside. In this study, the quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, and a quercetin 3-*O*-pentoside were the most abundant flavonoid glycosides (Stevenson et al., 2012).

Flavonoid aglycones such as rotenoids: deguelin and tephrosin were identified as major components with sarcolobine, rotenone, and α-toxicarol being minor from methanolic extract of *T. vogelii* (Stevenson et al., 2012). Stevenson et al. (2012) further isolated the known prenylated flavanone compounds: obovatin 5-methyl ether and *Z*-tephrostachin and the previously unknown flavonoid aglycones. The isolation study of flavonoid aglycones by Stevenson et al., (2012) revealed two chemical varieties of *T. vogelii*: one containing rotenoids as the main type of flavonoid aglycones (chemotype 1) which possessed pesticidal properties and the other containing flavanones as the main aglycone type (chemotype 2) (Figure 1.8) which was nonpesticidal. More of these discussions have been published (Kerebba et al., 2019)

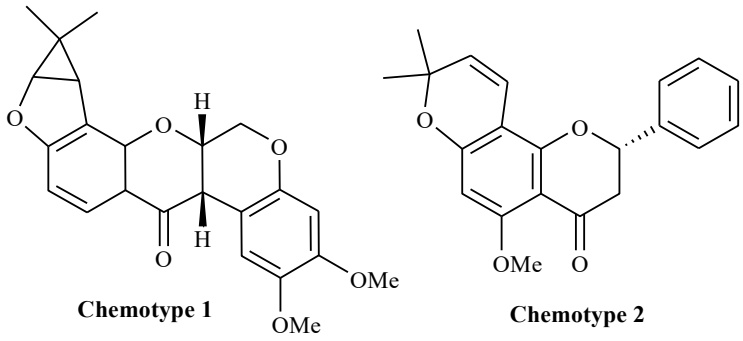


Figure 1.8: Structures of chemotype1 and chemotype 2 of non-volatile components of *T. vogelii*

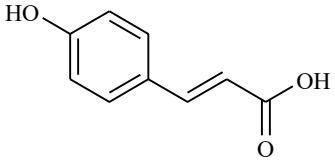
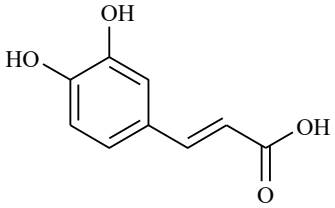
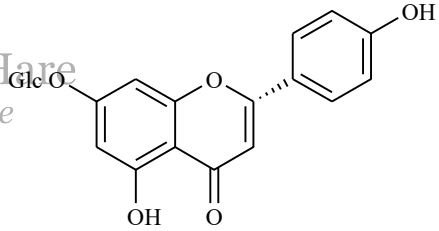
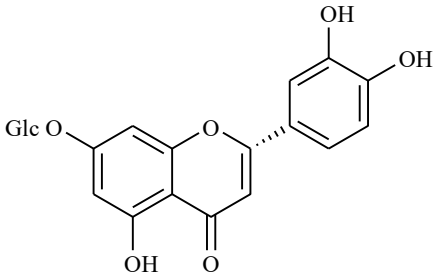
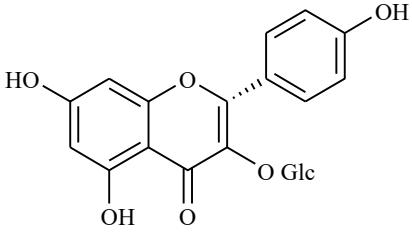
Many other flavonoids have been found to possess pesticidal properties. For example, Naringenin, hesperetin-7-*O*-rutinoside and quercetin-3-*O*-rutinoside could induce oviposition in

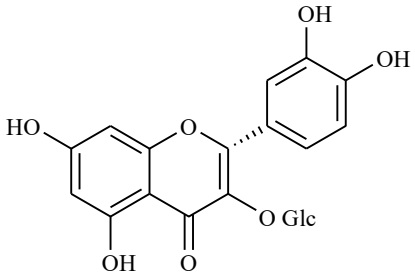
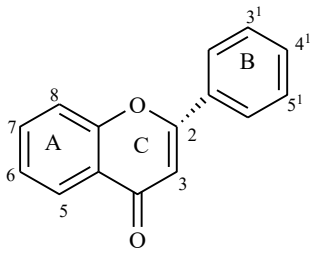
citrus feeding swallowtail butterfly *Papilio Xuthus* L. (Ohsugi, et al., 1985). The presence of the C-glycosyl flavone, maysin (2"-*O*-a-L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulosyl) luteolin), and the phenylpropanoid, chlorogenic acid enable maize to respond to corn earthworm *Helicoverpa zea* Boddie. Some flavonoids support oviposition such as luteolin 7-*O*-(6"-malonyl glucoside) and isorhamnetin glucoside (Feeny et al., 1988) while quercetin-3-*O*-rutinoside inhibits and deters ovipositing (Tabashnik, 1987). Flavones such as 5-hydroxyisoderricin, 7-methoxy-8-(3-methylbutadienyl)-flavanone and 5-methoxyisoronchocarpin, and isoflavonoids (judaicin, judaicin-7-*O*-glucoside, 2-methoxyjudaicin) deter feeding behavioural responses and altering growth of various insects (Simmonds, 2003). In addition kaempferol, quercetin and myricetin (flavonols) act as deterrents (Wuyts et al., 2006). Some flavonoids have been found to cause mortality and inhibit development such as rutin and quercetin glucosides (Gould and Lister, 2006). 6-Methoxyapigenin from *T. diversifolia* has been identified as insect feeding deterrents (Challand and Willcox, 2009).

Phenolic compounds such as flavonoids, tannins, hydroxycinnamate esters and lignin possess antioxidant properties (Cook and Samman, 1996; Czeczot, 2000). They (including vitamin C) follow under water soluble natural anti-oxidants. There also lipid soluble antioxidants i.e. vitamin E and carotenoids. Vitamin C (ascorbic acid and dehydroascorbic acid- oxidation product) has a biological function as enzyme cofactor, a radical scavenger, and as a donor/acceptor in electron transport at the plasma membrane. Ascorbic acid can scavenge hydroxyl radicals, superoxide, and regenerate α-tocopherol (Davey et al., 2000). The antioxidant capacity of phenolics is due to their ability to scavenge reactive oxygen species (ROS) as a result of their electron-donating properties and reaction as hydrogen donors (Rice-Evans and Miller, 1996). Phenolic compounds scavenge free radicals because of the lower one-electron reduction potentials of phenolic (phenoxyl) radicals compared to those of oxygen radicals such as $O_2^{-\bullet}$, $ROO^{\bullet}$, $RO^{\bullet}$ and $HO^{\bullet}$ radicals. This makes it possible for oxygen radicals to oxidize phenolics readily to respective phenoxyl radicals. The donation of electron of the 'acidic' phenolic hydroxyl group thus makes phenolic compounds excellent antioxidants. Additionally, good structural chemistry that supports free radical scavenging activity. Phenolic compounds with the ortho-dihydroxy structure are considerably more active as hydrogen-donating antioxidants than monohydroxy phenolics (Rice-Evans et al., 1996) (Table 1.3). This is because an additional hydroxyl group in the ortho position lowers the one-electron reduction potential of the phenolic

group by approximately 300–400 mV and increases the stability of the corresponding phenoxyl (semiquinone) radical (Pietta, 2000). Consequently, phenolics containing the catechol structure are more easily oxidized, and, therefore, are much better radical scavengers than monohydroxy phenolics.

Table 1.3: Scavenger activities (IC_{50}) against the synthetic free radical 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and the superoxide anion (O_2^{-}) of mono- and di-hydroxy cinnamic acids and flavonoid derivatives.

Flavonoid	**IC_{50} (DPPH)/ µM**	**IC_{50} (O_2^{-})/µM**	**Structure**
p-Coumaric acid	>500	>500	
Caffeic acid	83.4	52.7	
Apigenin 7-*O*-glucoside	>500	>500	
Luteolin 7-*O*-glucoside	54.5	45.4	
Kaempferol 3-*O*-glucoside	>500	>500	

Quercetin 3-*O*-glucoside	46.0	39.3	
Flavan			

Source: Agati et al., (2011), Tattini et al., (2004). Glc denotes glucose sugar

Among flavonoids, antioxidant activity is further enhanced by the presence of a 2, 3 double bond on the C ring, a free hydroxyl group at the 3 position on the C ring and the presence of hydroxyl groups in the 7 and 5 positions on the A ring (Rice-Evans et al., 1996a,b; Pietta, 2000). For example, flavonoids having adjacent hydroxyl groups on their C phenyl ring have greater anti-oxidant potential (Cao et al., 1997). Arora et al., (1998) attributes the antioxidative characteristic of phenolics to the potential of flavonoids to change peroxidation kinetics through lipid packing order modification and on reducing the membrane fluid. Such changes potentially pose a steric hindrance to the diffusion of free radicals and restrict peroxidative reactions. In vitro studies have shown that phenolic compounds demonstrate higher antioxidant activity than antioxidant vitamins and carotenoids i.e the tocopherols and ascorbate (Vinson et al., 1995). Carotenes and xanthophylls constitute the carotenoids, another group of antioxidants. They are yellow, orange, and red pigments often found in fruits and vegetables. Most of these carotenoids are precursors of vitamin A (i.e. *β*-carotene, *γ*-carotene, and *β*-cryptoxanthin). The presence of conjugated double bonds in carotenoids enables them to have both radical scavenging activities and quenching or suppressing effect on the singlet oxygen. Another lipid-soluble antioxidant is Vitamin E whose biological activity is exhibited by tocopherols and tocotrienols, especially α-tocopherol. The tocopherol antioxidant activity reaction involves a hydrogen atom donation, leading to the formation of tocopheroxyl radical (Lampi et al., 2002).

Rice-Evans and Miller, (1996) further note that the capacity of polyphenol-derived radical to stabilise and offer chain-breaking role, their ability to chelate transition metals, in addition to the number and location of hydroxyl groups determines the high anti-oxidant potential. Polyphenol-derived radicals include those that may come from tannins and saponins. Tannins are divided into hydrolyzable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolyzable tannins are called gallotannins or egallitannins. Common example of hydrolyzable tannin is theaflavin (from tea), and that of condensed tannin is proanthocyanidin (Figure 1.9).

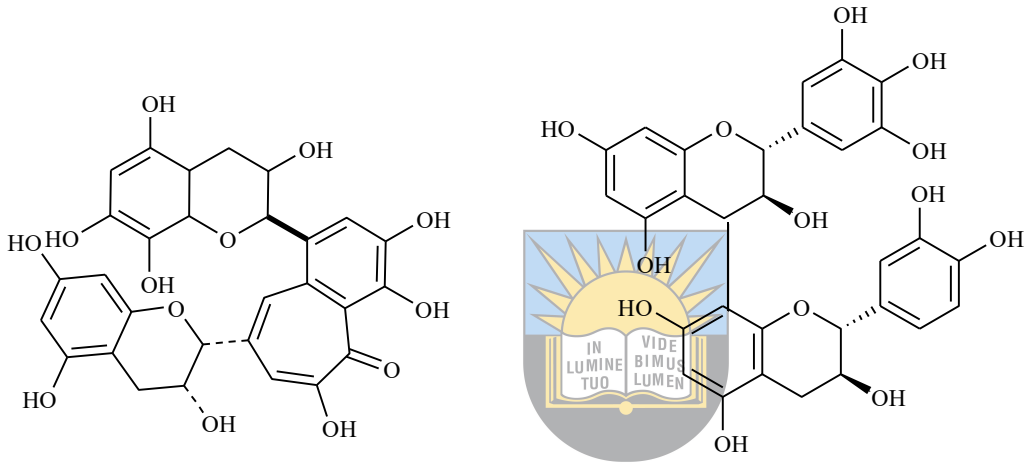


Figure 1.9: Structures of tannins

Saponins are high molecular weight triterpene glycosides containing a sugar group attached to either a sterol (steroid aglycone) or other triterpenes. Thus like glycosides, on hydrolysis, they give aglycones which are called sapogenin. There are therefore two major groups of saponins and these include steroid saponins and triterpene saponins. In the two types of sapogenin: steroidal and triterpenoidal, usually, the sugar is attached at C-3 in saponins, because in most sapogenins there is a hydroxyl group at C-3. The two major types of steroidal sapogenin are diosgenin and hecogenin.

1.7.2 Structure, pesticidal and antioxidant activity of sesquiterpene lactones compounds

Sesquiterpene lactones (STLs), an extensively diverse group of bioactive compounds of the terpenoid class, are found mainly in the Asteraceae family with thousands of reported structures

(Modzelewska et al., 2005). STL result from the condensation of 3 isoprene units which subsequently cyclize and oxidise to transform to a cis or trans-fused lactone (Figure 1.10)

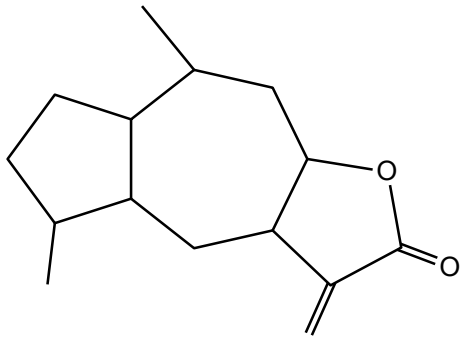
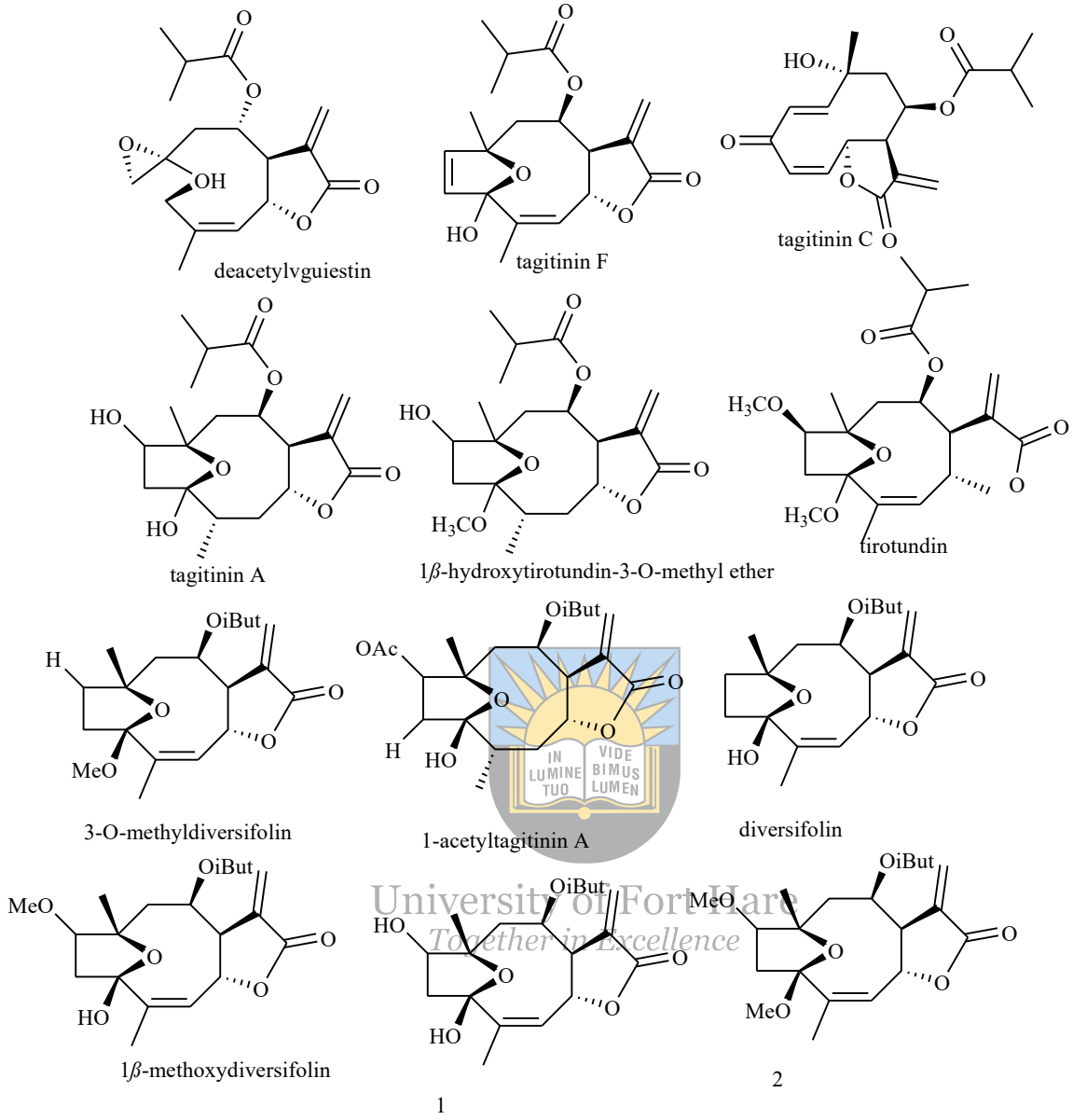


Figure 1.10: General structure of STLs

They are generally grouped based on the carbocyclic skeleton into germacronolides, eudesmanolides, guaianolides, pseudoguaianolides, heliangolides and hypocretenolides (Figure 1.11) (Chaturvedi, 2011). They also have a very important feature; γ-lactone ring (closes towards C-6 or C-8 carbon skeleton) and very often an α-methylene. Many pharmacological and biological activities have been demonstrated by STLs including the antimalarial activity of artemisinin (Klayman, 1985), anti-microbial activities, serve as antifeedants (Mullin et al., 1991; Mori and Matsushima, 1993) anti-inflammatory (Heinrich et al., 1998) and anti-oxidant (Shoaib et al., 2017). Tagitinins A, B, C, and F, diversifolin, tirotundin, tithonine and sulphurein chemotypes from *T. diversifolia* has been identified as insect feeding deterrents (Challand and Willcox, 2009)



3*β*,10*β*-Epoxy-1,3-dihydroxy-8-[(2-methylpropanoyl)oxy]germacra-4,11(13)-dien-12,6a-olide (**1**), 3*β*,10*β*-Epoxy-1,3-dimethoxy-8-[(2-methylpropanoyl)oxy]germacra-4,11(13)-dien-12,6a-olide (**2**) ((Pal et al., 1976; Baruah et al., 1979; Goffin et al., 2002; Kuronde et al., 2002; Gu et al., 2002; Ambrósio et al., 2008; Zhao et al., 2012; De Toledo, 2014; Miranda et al., 2015; Green et al., 2017).

Figure 1.11: Examples of some STL compounds and bioactive compounds identified in *T. diversifolia*

1.8 Study gaps and scope

The previous sections of this report have summarized plants and their bioactive compounds from different families that have been used as pesticides and antioxidants. Some of these botanicals, unfortunately, show a large variation in toxicity, making it hard to transfer specific uses from one place to another (Stevenson et al., 2010). The plants like *Tephrosia vogelii*, *Solanum incanum, and Lippia javanica* can exhibit an extreme variation of their bioactive principles from the same species in same and different environments (Stevenson et al., 2010; 2012, Kamanula et al., 2017) thus become a limiting factor in their effectiveness. Plant materials therefore should be used while considering the source with a phytochemical and pesticidal composition. *Tephrosia* species obtained from 13 locations from central and northern Malawi which were planted for use as an insecticide showed nearly 25% of the *T. vogelii* material non-pesticidal (Nyirenda et al., 2013) i.e. Chemotype 2 according to Stevenson and coworkers, (Stevenson et al. 2012), and so were unsuitable for this application (Sarasan et al., 2011). A blanket recommendation to farmers will not work since the material being used by farmers is lower in the bioactive component (Nyirenda et al., 2013). This means that the chemistry related to biological activities is very important (Stevenson et al., 2014). There is a need to fully comprehend chemically the biological activity by identifying chemical compounds responsible for the effect (Sarasan et al., 2011; Stevenson et al., 2012).

This study focuses on two botanical plants from Uganda *T. diversifolia* and *T. Vogelii*. Farmers use *T. diversifolia* in Uganda against the field and post-harvest pests (Mwine et al., 2011), although it is not widely promoted. The biological activities of mostly extracts of *T. diversifolia* have been discussed in the previous sections. i.e. its anti-oxidant and pesticide activities. The search for phytochemical specificity for biological activities such as pesticides or antioxidant is required (Bisht and Kamal, 1994). This study, therefore, aims to establish specific compounds in *T. diversifolia* that are against a specific pest. It also seeks to evaluate the phenolic compounds and sesquiterpene lactones from the fractions of the crude extract that exhibited promising anti-oxidant activities, correlate the phytochemical content such as flavonoid and total phenolic content with the antioxidant potential of *T. diversifolia*. For *T. vogelii*, the existence of chemical varieties from different places means that its use is not universal with farmers reporting no pesticidal results. The study on *T. vogelii*, therefore, aims at investigating chemical varieties in

the volatile component which can compromise the efficacy of a plant and finally assess the potential and implications of *T. vogelii* essential oils on pest control.

1.9 Research problem

Chemical variability of *T. vogelii* affects its efficacy as a traditional pesticide. Lack of specific information about compounds responsible for some pesticidal activity on a wide range of pests and antioxidant effects of *T. diversifolia* limits its adoption for use since most of its bioassays have been conducted using its crude extracts and a few of its fractions. This does not fully demonstrate the effectiveness and thus little progress in developing new products. Thus the bioactive compounds of *T. diversifolia* against specific pests or its antioxidant activity need to be investigated.

1.10 Justification and morale

Chemical variability research can assess the potential of plants in pest control and explain why efficacy is lost or varies for example absence of deguelin and rotenone among 25% of sampled *T. Vogelii* materials from 13 different locations in Malawi resulted in two distinct chemotypes being proposed i.e. pesticidal and non-pesticidal (Stevenson et al., 2012) which likely explained the experience some farmers face with the report of no pesticidal effect. In *T. diversifolia*, most of the studies published in the last 10 years have been carried out with plant extracts and a few of their fractions, whereas fewer studies have been conducted with isolated compounds thus the chemical basis of biological activity is less accounted for and lastly, it is necessary to find out whether biological activities are due to multiple components in plant extracts. The study will enable the exploitation of plants in controlling insect pests. The study of pesticide and antioxidant activities of *T. diversifolia* is possible because of the effect of some phytochemicals such as flavonoids participating in plants' interaction with animals (insects). Additionally, the study could further open discussions on the safety of pesticidal bioactive compounds from the crude extracts.

1.11 Aim

To carry out a chemical evaluation of the pesticidal and antioxidant applications of *T. diversifolia* and *T. vogelii* collected from some parts of Uganda.

1.11.1 Specific objectives

i) Investigation of chemical variation in the components and composition of *T. vogelii* essential oils and its pesticidal implication against *S. zeamais*

ii) To evaluate and compare the pesticidal potential of essential oils of *T.diversifolia* and *T.vogelii* against *S. zeamais*.

iii) Chemical evaluation of feeding deterrence activity of *T. diversifolia* non-volatile and volatile substances against *S. zeamais*

iv) Characterisation of phenolic compounds from *T. diversifolia* and evaluation of antioxidant properties

1.11.2 Hypotheses

1) *T. diversifolia* extract, fractions, essential oils and isolated compounds would deter feeding and cause mortality to maize weevil.

2) *T. diversifolia* extract, fractions and isolated compounds possess antioxidant potentials.

3) Essential oils and extracts of *T. diversifolia* are good antifeedants against maize weevil

4) Chemical variation would occur in the *T. vogelii* essential oils from leaf materials which would significantly affect the pesticidal activity.

5) Essential oils of *T. diversifolia* and *T. vogelii* would repel, and cause mortality to *S. zeamais* in a significantly different manner.

6) Antioxidant activities of *T. diversifolia* would correlate with its total phenol and flavonoid content and some compounds could exhibit anti-oxidant potential.

1.12 Structure of the thesis presentation

The thesis is presented in chapters. Chapter one gives a general overview of the concept of the study and a detailed review of the phytochemical study and biological activities. The other chapters, 2 to 6 are written in general format. Chapter two is the experimental section. Chapter three is the results and discussion section for the chemical evaluation of the pesticidal and antioxidant activities of the two plants. Chapter four is the results and discussion section from evaluation of the pesticidal and antioxidant activities of the two plants. Chapter five summarises the findings and future work concerning the chemical and biological potential of *T. diversifolia* and *T. vogelii*.

Chapter Two

Experimental

2.1 Botanical identification and collection of plant materials

The leaf materials used are the ones collected from eastern Uganda under the voucher specimen details deposited at Makerere University Herbarium and the voucher numbers are Kerebba N. No 1- *Tephrosia vogelii* Hook. f. (Leguminosae) (Access No. MHU 50735), Kerebba N. No 2- *Tithonia diversifolia*. (Hems) (Access No. MHU 50733). The plant species were authenticated by a senior Botanist Rwaburindori Protase at the Department of Botany, Makerere University.

2.2 Investigation of chemical variation and implications on pesticidal activity *T. vogelii* essential oils against *S. zeamais*

2.2.1 Plant collection and study sites

The collection of plant materials took place in Butaleja district, eastern Uganda. Two plant collection sites were considered for the study; Mazimasa sub-county, Nampologoma Parish, Muyago village and Kachonga sub-county, Kyadongo parish, and Kyadongo B village. Kachonga sub-county surrounds the Doho Wetland found in Mazimasa sub-county and the area is ten kilometers from Mbale Town (33° 55′ to 34° 05′ E and 0° 50′ to 1° 00′ N). The coordinates of the district are 00 56’ N, 33 57’ E. The district (Butaleja District) area is approximately 653.1km^2. The altitude of the district ranges from 1050 m to 1100 m above sea level. Several tropical climate conditions with average temperatures between 16 ^{0}C and 29 ^{0}C occur due to different altitudes. The mean annual rainfall varies between 1500 mm to 1,750 mm and received within four months (Government of Uganda, 1967). The bimodal rainfall peaks are; March-May and August-September (Oonyu, 2011). The soil is sandy with low organic content although some clay soils transferred from a neighbouring volcanic mountain in Mbale district form along rivers (Government of Uganda, 1967).

2.2.2 Plant materials

Different plant leaf materials of *T. vogelii* plant species were collected from Muyago and Kyadongo villages, Butaleja District, eastern Uganda. Leafy materials were collected from

branches, air-dried and stored. To determine the effect of geographical and seasonal variations in the existence of *T. vogelii* chemotypes, collection of plant materials was done within two major seasonal rainfall patterns in the district: rainy season (March-May, and August-September) and dry season (January and June-July) (Table 2.1) from two different villages. The distance between Muyago and Kyadongo villages is about 7 km.

Table 2.1: Location of the *T. vogelii* samples

Village (sample)	Flower color	Location Latitude North	Longitude East	Sampling date	Altitude
Muyago (TV1 muya)	White	0°84′20″	34°03′15″	14/05/2017	1080m
KyadongoB (TV1 kya)	White	0°90′14″	34°08′30″	1/06/2017	1098m
Kyadongo B (TV1 kyb)	White	0°90′30″	34°09′45″	1/06/2017	1098m
Kyadongo B (TV1 kyc)	White	0°80′10″	34°08′40″	1/06/2017	1098m
Muyago (TV2 muya)	White	0°84′14.9″	34°03′10″	15/08/2017	1080m
Kyadongo B (TV2 kya)	White	0°70′30″	34°07′35″	15/08/2017	1098m
Kyadongo B (TV2 kyb)	White	0°90′14″	34°08′30″	15/08/2017	1098m
Kyadongo B (TV2 kyc)	White	0°90′20″	34°09′40″	15/08/2017	1098m
Kyadongo B (TV2 kyd)	White	0°90′35″	34°08′45″	15/08/2017	1098m
Muyago (TV3 muya)	White	0°84′00″	34°02′45″	01/03/2018	1090m
Muyago (TV3 muyb)	White	0°84′14″	34°03′09″	01/03/2018	1090m
Muyago (TV3 muyc)	White	0°84′14.9″	34°03′10″	01/03/2018	1090m
Kyadongo B (TV3 kya)	White	0°90′14″	34°08′30″	01/03/2018	1098m
Kyadongo B (TV4 kya)	White	0°70′30″	34°07′35″	10/01/2019	1098m
Kyadongo B (TV4 kyb)	White	0°90′14″	34°08′30″	10/01/2019	1098m
Kyadongo B (TV4 kyc)	White	0°90′20″	34°09′40″	10/01/2019	1098m
Muyago (TV4 muya)	White	0°84′00″	34°02′45″	10/01/2019	1090m
Muyago (TV4 muyb)	White	0°84′14″	34°03′09″	10/01/2019	1090m
Muyago (TV4 muyc)	White	0°84′14.9″	34°03′10″	10/01/2019	1090m

Rain season sampling was between March, May and August. Dry season sampling was between January and June,

2.2.3 Extraction and analysis of essential oils

2.2.3.1 Extraction

Each sample of plant leaf materials (20.0 g) was hydro-distilled for 4hrs using a Clevenger apparatus set up as prescribed by British pharmacopoeia for essential oils (Cartwright and Anthony, 2014). The oils were collected using a Pasteur pipette and dried using anhydrous sodium sulphate. The dry oil was then put in a small weighed dark brown bottle (5 mL) and refrigerated at 4 ^{0}C for analysis. For pesticidal evaluation, more masses of the sample were hydro-distilled.

2.2.3.2 Identification and quantification of volatile and non-volatile constituents

2.2.3.2.1 Synthetic chemicals

Ethylbenzene (> 99.8%), (±)-linalool (> 95.0%), 2-undecanone (95%), *o*-xylene(≥ 99.0%), p-cymene (> 95.0%) and R-(+)-limonene (> 98.0%), undecanoic acid (> 99.0%) were purchased from SigmaAldrich (Gillingham, Dorset, UK). n-decane (> 99.0%) was bought from BDH chemicals. *α*-Terpineol (98.0%) was purchased from Fisher Scientific, (Loughborough, Leicestershire, UK). A mixture of xylene isomers (*o*-xylene and *m*-xylene) was purchased from pronalys while *α*-pinene was purchased from B.C. Treatt &co. Ltd. (*E*)-*β*-farnesene (≥ 98%) and Trans (2*E*,6*E*)-farnesol (98.0%) standards were purchased from career Henan chemical co. China. Neemazal (10% azadirachtin) was purchased from Sigma–Aldrich (Gillingham, Dorset, UK). Catechin (>99%), caffeic acid (>99%), epicatechin (>99%), ferulic acid (>99%), rutin (>99%), hesperidin (>99%), *p*-coumaric acid (>99%) and phloridzin (98%) were purchased from Acros Organics. Chloroform(98%), methanol (99.9%), ethyl acetate (99%), dichloromethane(98%) petroleum ether (99%) and ethanol (95%) used in extraction and isolation of *T. diversifolia* leaves was supplied by Sains Agencies Co., Ltd, Port Elizabeth, South Africa. Deuterated chloroform ($CDCl_3$) or water (D_2O) or/ and methanol (CD_3OD) were obtained from Sigma Chemicals Co., USA. HPLC-grade acetonitrile (MeCN) was purchased from Merck (Darmstadt, Germany), glacial acetic acid (99.8%) (Hengxing Chemical Reagent Co., Ltd., Tianjin, China), formic acid (98%) and ultrapure water (Milli-Q, Waters) used as mobile phase was bought from Merck (Darmstadt, Germany).

2.2.3.2.2 Identification of compounds with Gas Chromatography (GC)

GC analysis was done using Brunker 300 Gas Chromatograph equipped with FID detector and ZB-5 column (30 m in length × 0.25 mm i.d × 0.25 µm film thickness). The carrier gas was hydrogen at a flow rate of 1.0 mL/min and inlet pressure 52.6 Kpa. The column oven temperature was programmed to 50-250 ^{o}C at the rate of 3.0 ^{o}C/min. injector and detector temperature were set at 250 ^{0}C, volume injector 1.0 µL of the oil; split ratio 1:5. Peaks were measured by electronic integration. n-alkanes of C_8 to C_{30} were run under the same condition for Kovats indices determination (Wannes et al., 2009).

2.2.3.2.3 Identification and quantification of volatile constituents by Gas Chromatography-Quadruple Mass spectrometry (GC/MS/MS)

The essential oil was analyzed by a Bruker 300-MS along with the 431-GC and CP-8400 Autosampler (quadrupole mass spectrometer) equipped with a ZB-5 capillary column (30 m length × 0.25 mm i.d × 0.25 µm film thickness). The oven temperature was programmed from 50 ^{o}C – 250 ^{o}C at the rate of 3.0 ^{o}C/min, electron ionization was at 70 eV. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Injector and detector temperature were set at 280 ^{o}C, split ratio 1:5. 1.0 µL of the diluted oil in hexane was injected into the GC/MS. Compounds in the essential oil were identified by matching their Kovats indices and mass spectra with the ones recorded in WILEY NIST 11 library and by comparing them with literature values (Babushok et al., 2011), where possible authentic compounds were co-injected. To quantify the constituents in the oil, standard solutions of 5, 10, 20, 40 and 70≤ Y≤ 100 ppb, (Y, was based on the equivalent 5 or 10 µL stock of compound used due to different densities) for the linear regression curves were prepared from synthetic reference materials. These were run on the same day of the sample analysis and regression equations obtained by plotting peak areas against the concentration levels. For compounds whose standards were available, quantification was done. For unavailable standards, compounds were grouped into chemical classes (hydrocarbons, alkylbenzenes, aldehydes, alcohols, etc.) and subclasses (monoterpenes, sesquiterpenes, oxygenated monoterpenes, etc.) and a semi-quantification approach was carried out using one (or more) reference standard per group. The compound composition was then expressed as percentage peak area i.e.

Constituent percentage peak area= (Xs)*100/(1000 x R)

where Xs is the constituent concentration with respect to its peak area (ppb/µg mL^{-1}) relative to peak area in the injection volume (1 µL =1000 ppb), and R is the recovery (R was taken as 100% since average recovery on spiking was 93.1±9.8, n=11).

2.2.4 Evaluation for chemical variation in the oils of *T. vogelii* leaf samples

Principal component analysis (PCA) and Agglomerative hierarchical clustering (AHC) were performed on the data to group components and samples into clusters using statistical software SPSS for Windows version 25. PCA is a statistical tool that aims to represent the variation

present in the data. It allows similarities and differences between data to be seen easily. During PCA, values in the loadings matrix were obtained through the transformation of data from correlated to new uncorrelated variables called principal components (Johnson et al., 2007). PCA was performed on a combined set of data from the two locations giving 19 samples × 23 variables for PCA (see appendix 10). Analysis followed the standardization of data using Varimax rotation. Factor loadings generated indicate the correlations of each chemical constituent with its corresponding component. Loading scores which were greater than 5% of the variance of a given variable were considered however only loadings higher than an absolute value of 0.23 were considered meaningful throughout the analysis.

AHC is an algorithm that brings together related objects into clusters. The clustering makes it easier to see the correlations. The endpoint is having clusters that are distinctive of other clusters and the objects within a cluster are very similar. AHC based on the Euclidean distance was used to analyse the seasonal and geographical influence on the yield and composition of the samples of *T. vogelii*. Finally, the classification of samples was done based on the composition and chemical constituents.

2.2.5 Pesticidal evaluation of different chemotypes

The repellency and fumigant toxicity for the different varieties were evaluated for selected samples (Tv1kyb, Tv4kyc and Tv4muyc). The detailed set up of the experiment was done as described in chapter 4. Plastic containers were used to bleed colonies of the *S. zeamais*. Initial stocks of *weevils* were obtained from infected maize from a market in Mthatha, Eastern Cape province, South Africa. Rearing occurred at 25-29 °C, 60 ±5% relative humidity and a photoperiod of 12:12 dark: light.

2.3 Comparative toxicity and repellent effects of the essential oils of *T. vogelii* (Hook f.) and *T. diversifolia* (Hemsl.) A. Gray against *S. zeamais* Motschulsky

2.3.1 Extraction of essential oils

4.5 kg of plant leaf materials (500 g per run) were hydro distilled using Clevenger apparatus set-up as prescribed by British pharmacopoeia for essential oils (Cartwright and Anthony, 2014).

The essential oils were then collected into hexane and transferred to a dark brown vial and stored in the refrigerator at 4 ^{0}C for insecticidal evaluation and further analysis.

2.3.2 Pesticidal evaluation of essential oils

2.3.2.1 Rearing of insects

The insects used in the bioassays were obtained from infected maize from a market in Mthatha, Eastern Cape Province, South Africa. Weevils were identified by Dr. SK Kuria an entomologist, of the Department of Biological and Environment Sciences, Walter Sisulu University. Plastic containers were used to bleed colonies of the maize weevils. In each container containing maize (*Zea mays* L.), was added 150 adult maize weevil to lay eggs at 30 ± 1 °C, 60 ± 5% RH and a photoperiod of 12:12 dark: light. The prior infestation was avoided when the grain was washed and dried in a stove at 25 °C for 12 h and then frozen at 4.0 ± 1 °C for 48 h before it was used. The old adult insects were removed from the flasks after 5 days which allowed the emergence of the new generation. After 7 days, the first generation of weevils of the same age was considered for the bioassay. Adult *S. zeamais* used in all the experiments were about 7-14 days old. The study was approved by the University of Fort Hare, Animal Research Ethics Committee (UAREC) with certificate reference number: OYE011SKER01.

2.3.2.2 Repellence bioassay set up against *S. zeamais*

Repellence assay was set up according to the area preference method earlier described by Tapondjou et al., (2005). Here Petri dishes of diameter 9.0 cm and height 1.2 cm and discs of filter paper half (31.8 cm^2) were used. Different test solutions of concentrations 1, 5 and 10 µL/mL of essential oils corresponding to 0.03, 0.16 and 0.31 µL of oil per cm^2 respectively were used to check for the repellent potential of essential oils. Whatman filter papers were cut into two halves. To one half was applied the oil treatment uniformly using a micropipette. To the other half was a control treatment of 1.0 mL of hexane and the data was compared with 15% formulation of IR3535 (ethyl 3-(N-acetyl-N-butylamino)- proportionate) as a positive control. Both treated halves were allowed to dry so that the solvent could evaporate completely. The halves were then cellophane taped to the dish bottom in a manner that would avoid the seep through of the test samples from one disc to another. 30 adult *S. zeamais* were released at the disc center and the Petri dish was covered and kept in the dark at 25 to 29.5^{0}C. Three replicates

for each level were taken. The numbers of weevils in both the treatment and control paper disc were counted after 1, 2, 12, 24 and 48 hours.

Percentage repellency (PR) was calculated using the formula in Equation (1):

$$PR(\%) = \frac{C - T}{C + T} \times 100 \quad (1)$$

C = insect number found on untreated half,
T = insect found on treated half.
Preference index (PI) was obtained using the formula in Equation (2):

$$PI = \frac{A - B}{A + B} \quad (2)$$

A= percentage of insects in treated halves,
B = percentage of insects in untreated halves.

The experiments were successively repeated twice, with three and two replicates each time, and separate controls were set in all the replicates in a completely randomized design and non-random design, respectively. Data were treated as a mean percentage repellency ± standard error of the mean (SEM).

2.3.2.3 Evaluation of contact toxicity activity of essential oils against maize weevil

The contact toxicity mortality of the adult *S. zeamais* due to the essential oil of *T. diversifolia* and *T. vogelii* leaves was investigated according to the methodological protocol by Obeng-Ofori and Reichmuth (1997) with minor modifications. Well dried and mixed maize grains with oils of 0, 0.25, 0.5 and 1.0 µL/g in 500 ml were set up (Figure 2.2). The control was made of grains treated with 1 mL of hexane corresponding to 0.0 µL/g while Deltamethrin 25 g a.i./L of the formulation was used for comparison as a positive control. The grains were then infested with thirty adult *S. zeamais* (2 weeks old) in each jar. Jars were covered with cloth pieces using rubber bugs. Three replicates for each treatment level were considered. Weevil mortality was followed up to the 96th hour. The percentage of the dead weevils was recorded after 48 h. The data were expressed as a percentage of initial weevils introduced and then corrected through $\log_{10}$-transformation during ANOVA using XLSTAT 2018.3.51059. The lethal concentrations LC_{50} were then computed using probit analysis (Finney, 1971).

2.3.2.4 Fumigation bioassay for essential oils against *S.zeamais*

Fumigation toxicity was investigated according to the methodology of Pires et al. (2006). 1 mL of each 0, 20, 40, and 80 µL/L air concentration was separately applied to 7 mm discs of Whatman No.1 filter paper, dried for 10 min and placed at the bottom of 500 mL glass jars. Thirty adult weevils of 7-14 day old were then put on cotton cloths (21 x 29 mm) each with forty grams of maize grains. The clothes were hung at the center of the jars and tight tied so that there was no air entry using glass jar lids. Mortality counts were recorded every after 12 h based on preliminary counts of 6 and 12 h using n-hexane was used as negative control and results were compared to methyl bromide fumigant (positive control). The maximum possible amount of essential oil in the air (µL/L air) was used to express the fumigant toxicity.

2.3.4 Analysis and quantification of the components of the essential oils

Analysis and quantification of the oils of *T. diversifolia* and *T. vogelii* was done as described in section 2.2.3.2.3

2.4 Extraction, isolation and identification of bioactive compounds from *T. diversifolia*

2.4.1 Extraction and isolation

Air-dried *T. diversifolia* leaf materials (2338.0 g) were extracted in methanol after deffating in petroleum ether (PE). The petroleum ether fraction was redissolved in ethanol and added to the methanol extract to yield 393.1 g of the extracted black syrup (16.8%). The syrup was resuspended in different percentages of aqueous methanol to give five fractions redristributed in petroleum ether i.e 80%, 70%, 60%, 100%, 0% MeOH/H_2O for fractions F1 (161.1 g), F2 (26.8 g), F3 (9.3 g), F4 (19.8 g), F5(10.8 g) respectively and F6 (151.1g) was petroleum ether fraction that was redistributed in ethanol. The crude extract and all the fractions were subjected to feeding deterrency bioassay test. F4 was partitioned in a column over dichloromethane: methanol (5:0, 5:1, 5:2, 5:4, 4:6, 2:8, 1:9, 0:1) to give 139 sub fractions of average 150 mL each. Pure bioactive compound NK1F4 (4.9 g) was collected from sub fractions 77-139 which exhibited one major spot on the TLC. Sub fractions 7-53 from F4 showed a mixture of three compounds. They were combined, dried and later subjected to bioassay test and also to a secondary column using chloroform: ethyl acetate (EA) (100:0, 75:25, 55:45) to give bioactive compound NK3F4 (21.6

mg) upon recrystallisation in methanol and also other subfractions. F5 was partitioned in a column over dichloromethane: methanol (5:2) to give again compound NK1F4 (9.3 g) after filtering on whatman paper. F3 was chromatographed on silica gel (EA: PE, 7:3) to give mixtures of 2 and 3 compounds which were subjected to bioassay. The mixture of 3 compounds was purified on preparative TLC (Chloroform: MeOH, 9:1) to give bioactive compound NK1F5 (0.7 g). Fractions F1, F2 and F6 were not isolated although they were very active. The structural identification of pure compounds was done using FTIR, ^{1}H and ^{13}C NMR, LC-MS and using spectral data from literature. The samples were also tested for antioxidant properties.

Spectral data:

Deacetylviguiestenin (NK1F5): Isolated as a colourless crystalline solid. $[\alpha]_D^{24}$ +35.2 (c 0.41, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 202 (0.72), 237(0.22); IR (ν_{max} cm^{-1}): 3419 (OH), 1781 & 1766 (*α*-methylene *γ*-lactone), 1368 (ester C-O), 1232 (epoxide), 1173 (alkoxy C-O), 824 (unsaturation). ^{1}H NMR (400 MHz, $CDCl_3$, δ_H ppm) and ^{13}C NMR (151 MHz, $CDCl_3$, δ_C ppm) see Table 3.7. HRESIMS; *m/z* 352 ($[M+2H]^+$ molecular formula; $C_{19}H_{26}O_6$.

Sandaracopimaradiene-1α,9α-diol (NK3F4): Isolated as a powder. $[\alpha]_D^{25}$ + 64.7 (c 0.64) (data from Prawat et al., 1993). UV (MeOH) λ_{max} (log ε) 258 (0.32); IR (ν_{max} cm^{-1}): 3327 (OH), 3014, 1645& 801 (trisubstituted sp^2C-H), 2936 (sp^3 C-H), 1466 (weak sp^2 C-H), 1056(alkoxy C-O). ^{1}H NMR (400 MHz, $CDCl_3$, δ_H, ppm) and ^{13}C NMR (151 MHz, $CDCl_3$, δ_C ppm) see Table 3.8. HRESIMS: ESIMS; *m/z* 303 ($[M-H]^-$), *m/z* 289 $[M-CH_3]^+$, *m/z* 274 $[M-2CH_3]^+$, molecular formula; $C_{20}H_{32}O_2$

3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid or simply 4-O-caffeoyl-2-methyloxirane-2-carboxylic acid (NK1F4): Isolated as a pale yellow solid; $[\alpha]_D^{25}$-9.02 (c 3.66×10^{-5}, MeOH) (Data from Pulido et al., 2017); UV(MeOH) λ_{max}: 267, 300nm; IR (ν_{max} cm^{-1}): 3783-3364 (OH), 2392 (carboxylic acid O-H), 1763 (C=O), 1373, (sp^3 C-H), 1232(epoxide), 824 (para aromatic sp^2 C-H). ^{1}H NMR (400 MHz, D_2O/ CD_3OD, δ_H ppm) and ^{13}C NMR (100 MHz, D_2O/ CD_3OD, δ_C ppm) see Table 3.9. HRESIMS: ESIMS; *m/z* 296 ($[M+2H]^+$), 282 (M +2H-$CH_3]^+$, *m/z* 102 ($C_4H_6O_3^{\bullet+}$), ESIMS-MS; 137($C_8H_{10}O_2^{\bullet+}$), 109($C_6H_6O_2^{\bullet+}$), 193($C_{10}H_9O_4^{\bullet+}$), 179 ($C_9H_7O_4^{\bullet+}$), 236 ($C_{12}H_{12}O_5^{\bullet+}$), 265($C_{14}H_{17}O_5^{\bullet+}$), molecular formula; $C_{14}H_{14}O_7$.

2.4.2 Evaluation of feeding deterrence activity of *T. diversifolia* volatile substances and non-volatile substances

2.4.2 .1 Flour disk bioassay for volatile (essential oils) component

Disks were prepared following protocols set by Xie et al. (1996). A suspension of wheat flour was made by dissolving 10.0g in 50 mL of distilled water. Several 200 µL aliquots of a suspension were pipetted onto a Petri dish using a micropipette to give disks which were air-dried overnight at 25 ^{0}C. Before the deterrence setup (Figure 1), the disks were allowed to equilibrate at 30 ^{0}C and 60 ± 5% r.h. for 24 h. Flour disks weighed between 34 - 56 mg. The amount of moisture content was determined as 12.9%. Flour disks were treated with 0 (control), 5, 10 and 20 µL (0, 0.07, 0.15 and 0.29 µL/mg of flour disks) concentrations of *T. diversifolia* oil dissolved in 0.5 mL n-hexane and later, the hexane was allowed to evaporate. The disks were then placed in glass vials (diameter 2.5 cm, height 5.0 cm). Twenty-five group-weighed adult *S. zeamais* (1-2 weeks old) were added to each preweighed vial containing four flour disks for each concentration and control. Three replicates were prepared. The weights of the vials plus disks and live insects after 72h were again taken while recording dead insects (if any). Nutritional indices were determined as previously described[25, 26] with minor modifications:

Relative growth rate (RGR) = (X-Y)/(Y×t), X-Y = change in insect weight. Relative consumption rate (RCR) = D/(Y×t). Efficiency of conversion of ingested food (ECI) (%) = (RGR/RCR)* 100. Feeding deterrence index (FDI) = [(C-T)/C] * 100,[27]control and treated disks were placed in separate vials in no-choice tests, X = weight of live weevils after 72 h (mg)/no. of live weevils after 72 h, Y = original weight of insects (mg)/25; weight of consumed disk, D = biomass ingested (mg)/no. of live weevils after 72 h; C and T are control and treated disk weight consumed by the weevils respectively. t = feeding period (3days).

The data for the Treatment means were compared using analysis of variance (ANOVA) and separated by the Fisher LSD test at $P < 0.05$ (XLSTAT 2018.3.51059).

2.4.2.2 Flour disk bioassay for non-volatile substances

Disks were prepared following protocols set by Xie et al. (1996). 1.0g of flour was mixed with a solution of 100 mg of the test substance in 5 mL of solvent giving a concentration of 10% w/w (100 mg/g food or 20 mg/mL w/v) and mixed using a magnetic stirrer. Several other

concentrations were prepared to give 5%, 1% and 0.5% in which 50 mg, 10 mg and 5 mg per gram food of non-volatile test substances were respectively added to 1.0g of the flour. Flour without treatment acted as negative control while treatment with neemazal powder (10%w/w, azadirachtin) was used as a positive control representing 0.01, 0.005, 0.001 and 0.0005%w/w, azadirachtin (100 ppm, 50 ppm, 10 ppm and 5 ppm respectively). Representative concentrations of the isolated compounds (5, 3, 2 and 1 mg, dissolved in 250 µL of water and mixed with 50mg of flour) were prepared. Aliquots of 200 µL (100 µL for isolated compounds) of the suspension were pipetted onto a petri dish 90×15 mm using a micropipette to give disks that were then air-dried overnight at room temperature. Four flour disks were weighed on the next day and transferred into new Petri dishes with twenty-five weevils each which were previously starved for 24 h. The Petri dishes containing the weevils and disks were then sealed with parafilm paper and kept between 27-30 ^{0}C and 60 ± 5% r.h. for 72 h. The reverse method of weighing the dish with its contents was done after 3 days. The diet consumption by the weevil for the treated disks was compared to that of the control. The experiment was done in triplicates of two separate experiments.

2.4.3 Antioxidant Assays

2.4.3.1 2, 2- diphenyl-1-picrylhydrazyl (DPPH) Assay

DPPH assay was performed using a 96-well microplate reader format following the protocol by Sdiri et al., (2012) with slight modifications. The DPPH reagent (0.1mM) was prepared by dissolving 39.4 mg in 1000 mL of methanol. The 0.1mM DPPH was incubated in a brown bottle. Various concentrations of the samples and standards were respectively mixed with equivalent volumes of DPPH. The solution was incubated for 30 min at room temperature. After which 200 µL for (1, 0.5, and 0.25 mg/mL) and 100µL for 0.125 mg/mL were pipetted into a microplate reader and the point absorbances taken at 490 nm. The capacity to scavenge the free radical DPPH was monitored accordingly using synthetic antioxidant, ascorbic acid and 2,6-di-tert-butyl-4-methylphenol (BHT) as standard positive controls while a solution of DPPH (0.1mM) and methanol were used as a negative control. Five replicate determinations of total antioxidant were done per sample with lower absorbances indicating higher free radical scavenging activities (Barros et al., 2008). DPPH scavenging effect was calculated with respect to the control. DPPH scavenging ability (%) = $[(Ab_{control} – Ab_{samples})/Ab_{control}] \times 100$, where $Ab_{control}$ is the absorbance

of the control (freshly prepared DPPH solution in methanol). A graph of percentage scavenging activity versus log concentration was set up, from which IC_{50} value was calculated by linear regression (Blois, 1958). The IC_{50} refers to sample concentration providing 50% inhibition.

2.4.3.2 Reducing property assay

Protocol for reducing power was earlier described by Kajaria et al, 2012 with minor modifications. Briefly sodium phosphate buffer (0.2 mL, 0.2 M, pH 6.6), 1% potassium ferricyanide (0.2 mL) was added to different solutions (0.2 mL) of each test samples and standards (concentration: 1, 0.5, 0.25mg/mL) and the mixture was incubated at 50 °C for 20 min. After, 10% trichloroacetic acid (w/v) (0.2 mL) was added, and then the mixture was centrifuged at 10.0 rpm for 10 min. The supernatant (upper layer) (100 µL) obtained was immediately mixed with 0.1% of ferric chloride (20 µL) and distilled water (100 µL); followed by measurement of absorbance after 10 min at 750 nm. The test was carried out in triplicate using ascorbic acid and 2,6-di-tert-butyl-4-methylphenol (BHT) as positive controls and methanol was used as the negative control. All tests were done three times and the graph was plotted with the average of the three determinations. A higher absorbance value indicates higher reducing power and thus higher antioxidant power. The reducing property of the test sample was standardized against ascorbic acid expressed as µg/mL. The reducing ability was calculated as percentage inhibition.

2.4.3.3 Determination of total phenol

Folin Ciocalteu reagent was used to determine the amount of phenol in the extracts, fractions and isolated compounds according to the method of Slinkard and Singleton (1977) and Spabos et al., (1990) modified by crop research institute report. 2.5mL of 10% Folin-Ciocalteu reagent and 2ml of sodium carbonate (2%w/v) was added to 0.5mL of each test solution (1 mg/mL). The resulting mixture was incubated at 45^0C for 15 min. The absorbance of the test solution was measured at 765nm using iMark^{TM} microplate reader/spectrometer Bio-Rad (168-1135). The absorbance of the same reaction with ethanol instead of the extract or standard was subtracted from the absorbance of the reaction with the sample results and then expressed as milligrams of gallic acid dissolved in water. That is; TPC = (GAE×V×DF)/W, where GAE is Gallic acid equivalent concentration (mg/ml) in the sample based on the calibration curve, V is the volume of extract, DF is dilution factor, W is mass of sample used.

2.4.3.4 Estimation of total flavonoid

Aluminium chloride colorimetric method was used for flavonoid determination as earlier described by Chang et al., (2002). 1 mL of the test solution (1mg/mL) was mixed with 3mL of methanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1M potassium acetate and 5.6 mL of distilled water and then kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415nm using an iMark microplate reader. The content was determined by extrapolation of calibration curve made by preparing riboflavin standard solution 1, 0.5, 0.25, 0.125, 0.063 and 0.031 mg/mL in distilled water. The amount of the flavonoid was expressed in terms of mg/mL as riboflavin equivalent (RFE).

2.4.4 Identification of bioactive compounds from non-volatile samples

2.4.41 General instrumentation

Optical rotations were measured using a PerkinElmer polarimeter (Series 341/343, USA). UV spectra were recorded on an Agilent 8453 UV spectrophotometer (Agilent Co.). The Fourier transform infra-red (FT-IR) was recorded using Perkin Elimer Spectrum One (FT-IR) Spectrometer coupled to a universal attenuated total reflection (ATR) sampling accessory. The high-resolution electrospray ionisation mass spectrometry (HRESIMS) measurements were made using a Thermo LTQ-Orbitrap XL LC-MS/MS. The NMR spectra were acquired in either deuterated chloroform ($CDCl_3$)or water (D_2O) or/ and methanol (CD_3OD) at 300 C on a Bruker Avance 400 MHz or Avance 600 MHz instruments equipped with TCI-cryoprobes. Standard pulse sequences and parameters were used to obtain 1D and 2D ^{1}H and ^{13}C. Two dimensional (2D) experiments were used to assign signals and these included: ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC). Internal TMS at 0.00 ppm was used as a reference for taking chemical shifts. Column chromatography was performed using silica gel 60 (0.040-0.063 mm, Merck Co., Germany). Thin-layer chromatography (TLC) was performed using a pre-coated silica gel 60 F_{254} plate (Merck Co., Germany) with solvent systems from chloroform, dichloromethane, methanol, and water solvents. Spots were observed under UV radiation (254 nm) and spraying with anisaldehyde-H_2SO_4 followed by heating.

2.4.4.2 LC-MS analysis

Analysis was performed on an LC-MS/MS. For LC, a Dionex UHPLC using a Thermo Scientific Fisher, Sunnyvale, CA, USA) equipped with a Kinetex 5u XB C-18 column (150 × 4.6 mm, 100A; Phenomenex, USA) was used, at 30 °C, a flow rate of 0.3 mL/min at gradient elution. MS was performed on a Bruker Compact QToF mass spectrometer with electrospray ionization (ESI) probe (Bruker, Bremen, Germany) with Orbitrap™ technology equipped with a Thermo Scientific DAD detector. Acetonitrile (A) in H_2O and 0.1% formic acid (B) mixture were used as mobile phases. The gradient elution started with 5% of A for 5 minutes then 5%–100% of A/ 50 min and finally 100% of A for 10 min. 10 µL of test solution (1 mg of extract/compound/mL in water) was injected and the temperature was kept at 12 °C. The operation of the spectrometer was in polarity switching mode having the set up: Scan range 75–1200 *m/z*, fragmentation HCD gas off, high resolution, Microscan 1, lock mass positive, AGC target balanced, maximum inject time 250 ms. The ESI source set-up: Sheath gas flow rate 50, auxiliary gas flow rate 17, sweep gas flow rate 0, spray voltage (|kV|) 4.5, spray current (MA) 1.4, capillary temperature (°C) 320, capillary voltage (|V|) 30, tube lens voltage (|V|) 90, skimmer voltage (|V|) 20. To identify compounds in the fractions, MS and MS^2 were applied. For optimisation of LC-MS^2, an optimized chromatographic separation was achieved on acetonitrile-water containing 0.1% formic acid solvent system as mobile phases after formic acid addition trials (0.05% and 0.1%). Trials of flow rates (0.1, 0.2, and 0.3 mL min^{-1}) on gradient elution were used for satisfactory analytical method, chromatographic conditions, including mobile phases (methanol, acetonitrile and acetonitrile-water). For structural characterisation, all the factors related to MS performance, including both ionization modes (positive and negative), sheath gas flow rate, sweep gas flow rate, spray voltage of the ion source, spray current and collision energy were optimised. Data was collected and analyzed with Xcalibur 2.2 (Thermo Scientific) software. The manufacturer's guide was used for the calibration. Mass calibration was extended to each polarity before sample analysis and adjustment of the mass range was extended from 0 to 1304 Da to cover small molecular weight metabolites by inclusion of low-mass contaminants with the standard Thermo calmix masses.

The MS raw data with the retention time for the samples were sliced to positive and negative datasets using the MassConvert tool from ProteoWizard (Kessneret al., 2008) and then converted to the mzML format prior to importing to MZmine 2.51 for data treatment.

2.4.5 Statistics and data analysis

Each test solution was performed in triplicate of independent experiments and all results were reported as mean value (± SEM). Analysis of variance (ANOVA) was used to distinguish data for the treatment means and separation was done using paired t-test for two entities and Fisher LSD test at $P < 0.05$, after being $\log_{10}$ transformed using statistical software (XLSTAT 2018.3.51059) for heterogeneity correction. The EC_{50} (the concentration needed to inhibit insect feeding by 50% relative to the negative control), median repellency dose (RD_{50}), lethal dose / concentration LD_{50}/ LC_{50} (the dose/concentration needed to repel or kill 50% of insects upon treatment) were determined by probit regression. Concentration providing 50% inhibition (IC_{50}) was determined from a graph of percentage scavenging activity versus concentration in the three replicates. Correlations between the total phenolic and flavonoid content versus total antioxidant capacities were made using the Pearson procedure ($p < 0.01$). The percentage dead for 0 and 100 were corrected before the determination of probits as under: Corrected % formula for 0 and 100% mortality or repellence: For 0% dead: 100(0.25/n), for 100% dead: 100(n-0.25/n) according to Ghosh, (1984), n = mortality. For the standard error of the RD_{50} or LC_{50} or EC_{50} were approximated using the equation given by Miller and Tainter, (1944) below (for example for EC_{50}):

$$Approx.S.E = \frac{Log(EC84) - Log(EC16)}{\sqrt{2N}}$$

, where N is the number of insects in each group, the $logEC_{84}$ and $logEC_{16}$ values for the probits of 84 and 16 obtained from the probit table were obtained from the probit regression line.

Chapter Three

Chemical studies on *T. vogelii* and *T. diversifolia*

In this chapter, the results of chemical investigations carried out on the extracts of plant materials of *T. vogelii* and *T. diversifolia* collected from different places in Butaleja district, eastern Uganda will be presented and discussed.

3.1 Analysis of essential oils of *T. vogelii* and *T. diversifolia*

Essential oils were obtained from the leaves of *T. vogelii* and *T. diversifolia* by hydro-distillation. The percentage yield of the yellow distillate of *T. vogelii* was 0.2 ± 0.0% (w/w, N=4) dry weight. For *T. diversifolia*, the yield of its light yellow distillate was also 0.2 ± 0.0% (w/w, N=4). Quantification/semi-quantification for the constituents in the oils of these two plants was done using reference materials. The compositions were expressed as mean peak areas (% ± SEM) of the major compounds quantified from sample sites of the two study areas. The results of the compositions of the essential oils are summarized in Tables 3.1 and 3.2. Twenty-five volatile constituents were identified from *T. diversifolia* (Table 3.1) which represented 54.6 ± 5.2% composition of total constituents identified in the fresh leaves essential oils. *D*-(+)-*α*-pinene was the main constituent (31.1 ± 4.7%) in this study, followed by *β*-pinene (6.8 ± 0.7%) then *α*-phellandrene (2.4 ± 0.2%), *α*-thujene (2.4 ± 0.1%) and D-limonene which contributed an average peak area of 2.2 ± 0.1%. Some of these major components are the same as those reported from leaf oils of the same plant by Lawal et al (2012); *α*-pinene (60.9 %), *β*-pinene (10.7%) and limonene (4.3 %) from the uMhlathuze district, KwaZulu-Natal Province, South Africa. Additionally, Moronkola et al. (2007) from Nigeria reported that leaf oils of *T. diversifolia* contained *α*-pinene (32.9 %), *β*-caryophyllene (20.8 %), germacrene *D* (12.6 %), *β*-pinene (9.5 %) and 1,3-cineole (9.1 %) as principal components. In all these studies, *α*-pinene and *β*-pinene are seen to be the predominant components of essential oils of *T. diversifolia*. Forty-nine compounds were identified from *T. vogelii* oil sample (Table 3.2). These compounds accounted for 86.8 ± 1.5% of total oil constituents identified in the leaves essential oils. The main components were o-xylene (25.6±2.3%), *m*-xylene (22.3 ± 2.1%), 3-cyclo-1-carboxaldehyde,3,4-dimethyl (4.1 ± 2.1%), Cis-*p*-Mentha-1(7),8-dien-2-ol (4.0 ± 0.0%), farnesol (3.2 ± 1.6%) and ethylbenzene (3.2 ± 0.4%). Compared to the report by Bendera, (2007) who detected nerolidol

(7.73%), *β*-caryophyllene (6.89%), caryophyllene oxide (6.22%) and germacrene *D* (6.20%) as major components in the *T. vogelii* essential oil, in the present study the highest composition representation of *T. vogelii* leaf essential oils came from the alkylbenzenes; ethylbenzene, *o*-xylene, *p*-xylene and *m*-xylene. In total, forty-nine compounds were identified from *T. vogelii* oil sample (Table 3.2). These compounds accounted for 86.8 ± 1.5% of total oil constituents identified in the dried leaves essential oils. The main components were o-xylene (25.6 ± 2.3%), *m*-xylene (22.3 ± 2.1%), 3-cyclo-1-carboxaldehyde-3,4-dimethyl (4.1 ± 2.1%), Cis-p-Mentha-1(7),8-dien-2-ol (4.0 ± 0.0%), farnesol (3.2 ± 1.6%) and ethylbenzene (3.2 ± 0.4%). Compared to the report by (Bendera, 2007); nerolidol (7.73%), *β*-caryophyllene (6.89%), caryophyllene oxide (6.22%) and germacrene *D* (6.20%) were the major components in the *T. vogelii* essential oil unlike the present study, although they were detected.

Table 3.1: Chemical composition of essential oils from fresh leaves of *T. diversifolia*

Compound	RT	KI[1]	Peak area (%) ±SEM	Identification method[2]	Quantification /semi-quantification standard
3-Hexen-1-ol	4.514	857	0.8±0.5	M/KI	(±)-linalool
Ethyl benzene	4.690	878	0.3±0.0	M/KI/CI	Ethyl benzene
o-Xylene	4.885	886	1.4±0.4	M/KI/CI	*o*-Xylene
2-butoxyl ethanol	5.696	896	0.7±0.0	M/KI	(±)-linalool
D-(+)-*α*-Pinene	6.743	931	31.1±4.7	M/KI/CI	*α*-Pinene
α-Phellandrene	8.093	1000	2.4±0.2	M/KI	*α*-Pinene
β-Pinene	8.292	1011	6.8±0.7	M/KI	*α*-Pinene
Myrcentyl acetate	9.295	1031	1.0±0.1	M/KI	*α*-Terpineol
D-limonene	10.362	1031	2.2±0.1	M/KI	R-(+)-limonene
α-Thujene	10.633	1035	2.4±0.1	M/KI	*α*-Pinene
Butanoic acid, 2-methyl-3-methylbutylate ester	13.763	1084	0.7±0.1	M/KI	*α*-Terpineol
2,4-dimethyl-2-pentene	18.643	1124	0.3±0.0	M/KI	Decane
1,4-cadinadiene	25.859	1324	0.3±0.0	M/KI	*β*-farnesene
(-)-Isocaryophyllene	27.695	1407	0.8±0.1	M/KI	*β*-farnesene
Cis *α*-Bergamotene	30.708	1434	0.3±0.0	M/KI	*β*-farnesene
Trans α-Bergamotene	30.819	1693	0.4±0.1	M/KI	*β*-farnesene
α-Farnesene	33.438	1489	0.3±0.0	M/KI	*β*-farnesene
(*E,E,E*)-*α*-Springene	33.446	1536	0.3±0.0	M/KI	*β*-farnesene
(-)-Spathulenol	33.959	1583	0.3±0.1	M/KI	Trans farnesol
Isoaromadendrene epoxide	34.143	1579	0.3±0.0	M/KI	Trans farnesol

Ledene oxide	34.242	1670	0.3±0.0	M/KI	Trans farnesol
Tetracyclo[6.3.2(25).0(1,8)tridecan-9-ol 4,4-dimethyl	36.236	1631	0.2±0.0	M/KI	Trans farnesol
Thunbergol	37.005	1899	0.2±0.0	M/KI	Trans farnesol
(*E*)-nerolidol	38.493	2036	0.2±0.0	M/KI	Trans farnesol
Heptadecanal	52.076	2235	0.5±0.0	M/KI	Decane
Monoterpene hydrocarbons			44.9±5.6		
Oxygenated monoterpenes			1.7±0.2		
Sesquiterpene hydrocarbons			2.4±0.2		
Oxygenated sesquiterpenes			1.4±0.1		
Oxygenated diterpene			0.2±0.1		
Other hydrocarbons			4.1±0.0		

[1] KI = Kovats index relative to ZB-5 column, [2] M= mass spectrum matching with NIST library. CI= co-injection with standard, RT= Retention time, and compared with Babushok et al., (2011) (90%RI) and Data from NIST (Wannes et al., 2009).

Table 3.2: Chemical composition of essential oils from a fresh leaf sample of *T. vogelii*

Compound	RT	KI[1]	Peak area (%) ±SEM	Identification method[2]	Quantification /semi-quantification standard
3,5-dimethylheptane	4.179	823	0.2±0.0	M/KI	Decane
Ethyl benzene	4.753	878	3.2±0.4	M/KI/CI	Ethylbenzene
o-Xylene	4.962	886	25.6±2.3	M/KI/CI	*o*-Xylene
m-Xylene	5.539	896	22.3±1.5	M/KI/CI	*m*-Xylene
2,4,6-trimethyldecane	5.714	899	0.5±0.0	M/KI	Decane
2-butoxy ethanol	5.776	895	2.8±0.2	M/KI	(±)-Linalool
2-Ethyltoluene	6.445	949	0.3±0.0	M/KI	Ethylbenzene
D-(+)-*α*-Pinene	6.811	931	1.8±0.1	M/KI/CI	*α*-Pinene
β-pinene	8.377	1011	0.5±0.0	M/KI	*α*-Pinene
Decane	9.244	1015	0.2±0.0	M/KI/CI	Decane
D-limonene	10.713	1031	0.2±0.0	M/KI/CI	(±)-Limonene
Linalool	13.514	1098	1.8±0.0	M/KI/CI	(±)-Linalool
2,6,8-trimethyl decane	13.601	1121	0.2±0.0	M/KI	Decane
α-Campholenal	14.691	1125	0.8±0.0	M/KI	*α*-Terpineol
Cis-verbenol	15.402	1142	1.0±0.0	M/KI	*α*-Terpineol
Cis-p-Mentha-1(7),8-dien-2-ol	15.561	1203	4.0±0.0	M/KI	*α*-Terpineol
p-Mentha-1,5-dien-8-ol	16.737	1170	0.9±0.0	M/KI	*α*-Terpineol
Terpineol	17.820	1135	1.6±0.0	M/KI/CI	*α*-Terpineol
D- verbenone	18.345	1218	0.5±0.2	M/KI	Undecanone
trans-Carveol	18.909	1229	1.0±0.4	M/KI	*α*-Terpineol
(-)- Dihydroedulani(II)	22.258	1349	0.8±0.0	M/KI	*α*-Terpineol
Isochavibetol	24.780	1039	0.7±0.0	M/KI	*α*-Terpineol

Cis-*α*- santalol	26.466	1678	0.4±0.2	M/KI	Trans farnesol
Tridecane	27.054	1518	0.3±0.1	M/KI	Decane
(*Z,Z*)-α-Farnesene	27.674	1489	0.2±0.0	M/KI	*β*-farnesene
Geranyl acetone	28.883	1453	0.9±0.2	M/KI	(±)-Linalool
Cis-*α*-Bisabolene	29.156	980	0.2±0.0	M/KI	*β*-farnesene
GermacreneD	30.208	1491	0.2±0.0	M/KI	*β*-farnesene
BicyclogermacreneD	30.528	1494	0.3±0.1	M/KI	*β*-farnesene
6,11-dimethyl-2,6,10-dodecatrien-1-ol	31.664	1695	0.2±0.0	M/KI	Trans farnesol
(-)-Isolongifolene	31.743	1402	0.2±0.0	M/KI	*β*-farnesene
Muurola-4,10(14)-dien-1-*β*-ol	32.632	1450	0.2±0.0	M/KI	Trans farnesol
Aromadendrene oxide	32.959	1549	0.2±0.0	M/KI	Trans farnesol
(*E*)-Nerolidol	33.432	1564	0.2±0.0	M/KI	Trans farnesol
(-)- Spathulenol	33.959	1576	0.3±0.1	M/KI	Trans farnesol
Caryophyllene oxide	34.158	1581	0.8±0.3	M/KI	Trans farnesol
Hexadecane	35.058	1600	0.3±0.1	M/KI	Decane
3-cyclo-1-carboxaldehyde,3,4-dimethyl	35.205	1492	4.1±2.1	M/KI	*α*-Terpineol
α-Acorenol	35.929	1631	0.2±0.0	M/KI	Trans farnesol
(+)-Spathulenol	36.830	1576	0.2±0.0	M/KI	Trans farnesol
1H-Cyclopentalen-7-ol,decahydro-3,3,4,7a-tetramethyl	37.006	1565	0.2±0.0	M/KI	Trans farnesol
Thunbergol	37.477	1899	0.2±0.1	M/KI	Trans farnesol
Farnesol	39.254	1705	3.2±1.6	M/KI/CI	Trans farnesol
β-Springene	39.410	1918	0.2±0.0	M/KI	*β*-farnesene
Farnesol Isomer a	39.916	1443	0.9±0.7	M/KI	Trans farnesol
Nookatone	40.118	1834	0.2±0.0	M/KI	Trans farnesol
7-epi-trans-sesquisabinene hydrate	40.827	1575	0.3±0.1	M/KI	Trans farnesol
Heptadecane	42.289	1700	0.6±0.1	M/KI	Decane
n- hexadecanoic acid	47.545	1711	1.3±0.0	M/KI	Undecanoic acid
Monoterpene hydrocarbons			2.4±0.2		
Oxygenated monoterpenes			13.1±0.2		
Sesquiterpene hydrocarbons			1.4±0.1		
Oxygenated sesquiterpenes			7.0±1.5		
Oxygenated diterpene			0.2±0.0		
Other hydrocarbons			62.9±1.5		

[1] KI = Kovats index relative to ZB-5 column, [2] M= mass spectrum matching with NIST library. CI= co-injection with standard, RT= Retention time, and compared with Babushok et al., (2011) (90%RI) and Data from NIST (Wannes et al., 2009).

The monoterpenoids (46.5±5.4%) were the predominant constituents identified in all the *T. diversifolia* oil out of which monoterpene hydrocarbons were the most represented; 44.9 ± 5.6% while oxygenated monoterpenes only constituted 1.7±0.2%. Sesquiterpenoids were found to be

low (3.8 ± 0.3%). However, other hydrocarbons (mostly aromatic hydrocarbons) were the most presented components (62.9 ± 1.5%). The monoterpenoids contributed only 15.5 ± 0.8% while sesquiterpenoids contributed 8.4 ± 1.6% in *T. vogelii* oils. In both plants though, the diterpenoids were in low levels; *T. vogelii* (0.2 ± 0.0%) than in *T. diversifolia* oil (0.2 ± 0.0%). Monoterpenoids, sesquiterpenes, and alcohols have been proven to contribute to pesticidal properties for example repellent properties of the essential oils (Sathantriphop et al. 2015). It should be noted that some of the chemical components have already been individually tested against *Sitophilus* species (most often *S. oryzae*) for repellency, mortality, or both in other studies (Lee et al., 2001); for farnesol, α-pinene in (Chaubey et al., 2012); while ethylbenzene has been reported to be released from *Sitophilus*-infested grain. Some studies of *T. vogelii* essential oil composition have also been carried out previously (Bendera, 2007; Noudogbessi et al., 2012). Due to the existence of chemical variation in *T. vogelii* bioactive principles as earlier reported (Stevenson et al., 2012), the quality of these studies may be questionable. *T. vogelii* materials from East Africa (Kenya and Tanzania) and Malawi have been reported to be of three different chemotypes using the phytochemical analysis approach (Mkindi et al., 2019). The essential oils of some plants such as *Lippia Javanica* have also shown variation in their bioactive compounds (Kamanula et al., 2017). The novelty in this investigation therefore comes largely from the fact that the essential oil of some plants exhibit chemical variation in their bioactive component. This variation needs to be characterised to establish if it may have serious implications on the biological activity.

3.2 Characterisation of the variation in the composition and components of the essential oils of *T. vogelii* leaf

3.2.1 Characterisation of the composition of essential oils

To characterize the variation in the chemical composition of *T. vogelii* essential oil, different samples from different locations in Butaleja district, eastern Uganda were taken over the course of about three years. The results show that percentage yields of the Yellow oils were between 0.18 ± 0.01% to 0.22 ± 0.01% (w/w) dry weight for samples from Kyadongho B (Table 3.3) and 0.16 ± 0.00% to 0.22 ± 0.01% (w/w) dry weight for Muyago samples (Table 3.4).

Table 3.3: Mean peak area (% ± SEM) composition of major chemical constituents identified using GC-MS in the essential oils of *T. vogelii* dry leaf samples from Kyadogho B

Compounds (RT, KI) [1]	TV1 kya	TV1 kyb	TV1 kyc	TV2 kya	TV2 kyb	TV2 Kyc	TV2 kyd	TV3 kya	TV4 kya	TV4 kyb	TV4 kyc	Identification Method [2]
Ethylbenzene (4.753, 878)	0.2 (0.0)	4.0 (0.4)	0.6 (0.3)	0.4 (0.1)	1.4 (0.4)	0.2 (0.0)	0.5 (0.0)	0.7 (0.1)	nd	nd	nd	MS/KI/ CI
o-Xylene (4.962, 886)	1.1 (0.0)	29.4 (1.6)	3.2 (2.1)	2.1 (0.3)	9.0 (1.7)	1.1 (0.1)	3.4 (0.6)	6.7 (2.3)	nd	nd	nd	MS/KI/ CI
p-Xylene (5.448, 887)	0.9 (0.0)	nd	1.6 (0.6)	nd	nd	nd	1.5 (0.1)	nd	nd	nd	nd	MS/KI
m-Xylene (5.539, 896)	0.3 (0.0)	25.0 (1.2)	3.6 (1.6)	1.0 (0.0)	5.1 (0.8)	0.3 (0.0)	2.1 (0.1)	3.3 (0.6)	nd	nd	nd	MS/KI/ CI
2-Butoxyethanol (5.776, 896)	0.3 (0.0)	2.7 (0.1)	3.4 (0.9)	0.6 (0.2)	1.0 (0.2)	0.2 (0.0)	2.9 (0.3)	1.1 (0.2)	nd	nd	nd	MS/KI
D-(+)-*α*-pinene (6.811, 931)	0.7 (0.1)	1.7 (0.1)	0.7 (0.1)	0.5 (0.1)	1.0 (0.2)	nd	0.5 (0.1)	nd	0.4 (0.1)	0.9 (0.2)	nd	MS/KI/ CI
D-Limonene (10.419, 1031)	nd	0.2 (0.0)	0.2 (0.0)	0.8 (0.4)	0.2 (0.0)	nd	0.1 (0.0)	nd	nd	0.1 (0.0)	nd	MS/KI/ CI
Linalool (13.514, 1102)	nd	1.8 (0.0)	nd	nd	nd	nd	nd	nd	nd	nd	nd	MS/KI/ CI
(*E*,*E*)-Cosmene (15.875, 1132)	nd	nd	nd	nd	nd	nd	nd	nd	0.2 (0.1)	0.6 (0.1)	0.5 (0.0)	MS/KI
6,10-Dimethyl-5,9-undecadien-2-one (28.883, 1453)	nd	0.8 (0.2)	0.9 (0.2)	1.2 (0.4)	1.3 (0.0)	0.3 (0.0)	nd	nd	0.2 (0.0)	0.5 (0.0)	1.1 (0.1)	MS/KI
Isocaryophyllene (30.511, 1409)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2 (0.0)	MS/KI
(*E*)-Nerolidol (33.432, 1564)	nd	0.2 (0.0)	nd	nd	0.2 (0.0)	0.2 (0.0)	nd	nd		nd		MS/KI
(-)-Spathulenol (33.959, 1566)	nd	0.2 (0.0)	0.2 (0.0)	0.3 (0.0)	0.3 (0.0)	0.2 (0.0)	nd	nd	0.2 (0.0)	nd	0.2 (0.0)	MS/KI
3,4-Dimethyl-3-cyclohexen-1-carboxaldehyde (34.520, 1492)	nd	nd	nd	nd	2.9 (0.6)	nd	nd	nd	nd	nd	nd	MS/KI
Cis-p-metha-1(7)-8-dien-2-ol (35.208, 1233)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.2 (0.0)	MS/KI
Isoaromadendrene epoxide (37.465, 1577)	nd	0.2 (0.0)	nd	nd	nd	nd	nd	0.2 (0.0)	0.3 (0.0)	0.3 (0.0)	0.7 (0.3)	MS/KI
1,4-Dihydroxy-p-menth-2-ene (37.767, 1243)	nd	0.2 (0.0)	0.4 (0.0)	nd	1.4 (0.2)	nd	nd	nd	nd	1.1 (0.2)	0.9 (0.1)	MS/KI
β-Farnesene (39.188, 1456)	0.3 (0.0)	nd	nd	nd	0.3 (0.0)	0.8 (0.3)	nd	nd	nd	nd	nd	MS/KI
Farnesol(*E*)-methylether (39.186, 1682)	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.0 (0.0)	nd	MS/KI

Z, Nerolidol (39.204, 2036)	nd	nd	nd	nd	nd	nd	nd	nd	0.9 (0.1)	nd	nd	MS/KI
Farnesol	nd	1.8 (0.2)	2.2 (0.7)	nd	nd	nd	0.3 (0.0)	0.4 (0.1)	nd	nd	2.9 (0.2)	MS/KI/ CI
β-Springene (39.410, 1918)	nd	nd	nd	0.6 (0.1)	5.7 (2.9)	nd	nd	0.3 (0.1)	nd	nd		MS/KI
α-Springene (40.793, 1731)	nd	0.2 (0.0)	0.2 (0.0)	nd	nd	nd	nd	nd	nd	nd	0.2 (0.0)	MS/KI
Hexadecane (42.274, 1818)	0.2 (0.0)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	MS/KI
Density (g/mL)	1.014 0.014	1.001 0.024	1.026 0.003	0.986 0.014	0.964 0.036	0.979 (0.043)	0.959 (0.013)	0.987 (0.013)	1.087 (0.016)	0.974 (0.002)	1.076 (0.049)	
Yield (% w/w*)*	0.19 (0.01)	0.21 (0.00)	0.20 0.01	0.18 0.01	0.20 0.01	0.19 0.04	0.18 (0.00)	0.19 (0.01)	0.22 (0.01)	0.19 (0.02)	0.21 (0.02)	
Total mean %	**4.0 (0.1)**	**68.4 (3.8)**	**17.2 (6.5)**	**7.5 (1.6)**	**29.8 (7.0)**	**3.4 (0.4)**	**11.3 (1.2)**	**12.7 (3.4)**	**2.2 (0.3)**	**4.5 (0.5)**	**7.9 (0.7)**	

Data presented as mean (SEM)./desity (SEM). [1] RT= Retention time and KI = Kovats index relative to ZB-5 column KI is compared with Babushok et al., (2011) (90%KI). [2]M= mass spectrum matching with NIST library (Wannes et al., 2009), KI= kovat index and CI= co-injection with the standard. nd= none detected

Table 3.4: Mean peak area (%±SEM) composition of major chemical constituents identified in the essential oils of *T. vogelii* dry leaf samples from Muyago village

Compounds (RT, KI) [1]	**TV1 muya**	**TV2 muya**	**TV3 muya**	**TV3 muyb**	**TV3 muyc**	**TV4 muya**	**TV4 muyb**	**Tv4 muyc**	**Identification Method** [2]
Ethylbenzene (4.753, 878)	2.7 (0.2)	2.4 (0.4)	nd	1.7 (0.3)	2.3 (1.6)	nd	nd	nd	MS/KI/CI
o-Xylene (4.962, 886)	17.6 (0.6)	22.1 (1.2)	0.9 (0.0)	8.6 (0.8)	23.4 (17.3)	nd	nd	nd	MS/KI/CI
p-Xylene (5.448, 887)	nd	Nd	0.9 (0.0)	3.1 (2.0)	5.2 (3.4)	nd	nd	nd	MS/KI
m-Xylene (5.539, 896)	14.4 (0.2)	18.1 (2.7)	nd	5.3 (0.6)	6.7 (5.1)	nd	nd	nd	MS/KI/CI
2-Butoxyethanol (5.776, 896)	nd	2.2 (0.7)	0.3 (0.1)	3.7 (0.9)	2.7 (0.1)	nd	nd	nd	MS/KI

D-(+)-*α*-pinene (6.811, 931)	1.2 (0.0)	1.6 (0.3)	nd	1.5 (0.2)	1.8 (1.2)	0.4 (0.0)	0.6 (0.0)	nd	MS/KI/CI
D-Limonene (10.419, 1031)	0.2 (0.0)	0.3 (0.0)	nd	0.2 (0.0)	0.1 (0.0)	nd	nd	nd	MS/KI/CI
Linalool (13.514, 1102)	nd	1.5 (0.3)	nd	nd	nd	1.0 (0.2)	nd	nd	MS/KI/CI
(*E,E*)-Cosmene (15.875, 1132)	nd	Nd	nd	nd	nd	0.4 (0.2)	0.6 (0.2)	nd	MS/KI
6,10-Dimethyl-5,9-undecadien-2-one (28.883, 1453)	0.7 (0.0)	0.6 (0.4)	nd	0.7 (0.0)	nd	0.7 (0.4)	1.0 (0.2)	0.6 (0.2)	MS/KI
Isocaryophyllene (30.511, 1409)	nd	Nd	nd	nd	nd	0.3 (0.0)	0.2 (0.0)	0.4 (0.0)	MS/KI
(*E*)-Nerolidol (33.432, 1564)	nd	0.3 (0.0)	nd	0.2 (0.0)	nd	nd	nd	0.2 (0.0)	MS/KI
(-)-Spathulenol (33.959, 1583)	0.2 (0.0)	0.3 (0.0)	nd	0.2 (0.0)	0.3 (0.0)	nd	0.2 (0.0)	nd	MS/KI
3,4-Dimethyl-3-cyclohexen-1-carboxaldehyde (34.520, 1492)	nd	Nd	nd	nd	3.4 (1.6)	nd	nd	nd	
Cis-p-metha-1(7)-8-dien-2-ol (35.208, 1233)	nd	Nd	nd	nd	nd	nd	nd	1.1 (0.0)	MS/KI
Isoaromadendrene epoxide (37.465, 1579)	nd	Nd	nd	0.8 (0.2)	nd	0.3 (0.0)	nd	nd	MS/KI
1,4-Dihydroxy-p-menth-2-ene (37.767, 1243)	1.1 (0.0)	2.3 (2.1)	nd	nd	nd	nd	1.3 (0.4)	nd	MS/KI
β-Farnesene (39.188, 1456)	nd	Nd	nd	nd	nd	nd	nd	0.8 (0.5)	MS/KI
Farnesol(*E*)-methylether	0.8	nd	nd	nd	nd	nd	nd	0.2	MS/KI

(39.186, 1682)	(0.0)							(0.0)	
Farnesol	nd	6.3 (1.3)	nd	4.5 (2.1)	5.9 (1.7)	nd	1.2 (0.8)	0.2 (0.0)	MS/KI/CI
β-Springene (39.410, 1918)	nd	0.2 (0.0)	0.2 (0.1)	0.2 (0.0)		2.0 (0.3)	0.9 (0.1)	2.0 (0.1)	MS/KI
α-Springene (40.793, 1731)	nd	Nd	nd	nd	0.2 (0.0)	nd	nd	0.2 (0.0)	MS/KI
Hexadecane (42.274, 1818)	0.2 (0.0)	0.5 (0.1)	nd	0.3 (0.1)	nd	nd	nd	nd	
Density	0.988 (0.038)	0.988 (0.012)	0.942 (0.001)	0.973 (0.000)	0.973 (0.027)	1.030 (0.030)	1.000 (0.000)	1.017 (0.017)	
Yield (% w/w)	0.20 (0.01)	0.22 (0.01)	0.16 (0.00)	0.18 (0.00)	0.18 (0.00)	0.18 (0.00)	0.17 (0.01)	0.18 (0.01)	
Total mean %	**39.1 (1.0)**	**58.7 (9.5)**	**2.3 (0.2)**	**31.0 (7.2)**	**52.0 (32.0)**	**5.1 (1.1)**	**6.0 (1.7)**	**5.7 (0.8)**	

Data presented as mean (SEM)./desity (SEM). [1] RT= Retention time and KI = Kovats index relative to ZB-5 column KI is compared with Babushok et al., (2011) (90%KI). [2] M= mass spectrum matching with NIST library (Wannes et al., 2009), KI= kovat index and CI= co-injection with the standard. nd= none detected

The densities of these oils were between 0.942 ± 0.001 g/mL and 1.030 ± 0.030 g/mL. The analysis showed that the composition varied from non-detectable levels (nd) to 29.4 ± 1.6%. *o*-Xylene was the highest in amount (nd - 29.4 ± 1.6%), from Kyadong B village samples and it varied between n.d- 23.4 ± 17.3% for Muyago village. This was closely followed by *m*-xylene with nd- 25.0±1.2% (Kyadongho B village) and nd- 18.1 ± 2.7% (Muyago village) range and finally the ethylbenzene; nd – 4.0 ± 0.4% (Kyadong B) and nd to 2.7 ± 0.2% (Muyago) in this category. There was a significant amount of farnesol, varying between nd to 6.3 ± 1.3% (Muyango) and nd to 2.9 ± 0.2% (Kyadongho B). *β*-Springene was another major compound with the composition of between nd to 2.0 ± 0.3% (Muyago samples) and nd to 5.7 ± 2.9% (Kyadongho B samples).

To establish the existence of chemotypes, two techniques were used: principle component analysis (PCA) and hierarchical clustering (HC). Upon analysis, PCA led to a total of 8 factors and their loading extracted (i.e. whose eigenvalues were greater than unity) represented in Table 3.5. These components could explain about 88% of the total variance. Multiple linear regression (MLR) of the elements in the factor score matrix was carried out against the total composition for the data to estimate the contribution of each major component in the chemotype on the total component composition of the samples. Significance of the regression coefficients (R^2 = 0.95, observations; N = 19) was at 95% confidence level ($p < 0.05$). The regression results based on 8 factor scores showed that components: 4 (p = 0.35), 5 (p = 0.63), 6 (p = 0.25), 7 (p = 0.96) and 8 (p = 0.33) did not significantly influence the component composition. Therefore, the components were reduced to 3 factors, which explained 55% of the total variance. Principle component one (PC1) could describe 30% while PC2 about 14% and finally PC3, 11% of the total variance. The MLR equation (R^2 = 0.95, ANOVA significance $f < 23.43$, $p < 0.05$) was as follows: Total composition = 19.39SC1 + 7.70SC2+1.92SC3+19.4, where SC1, SC2 and SC4 are factor scores for samples on component 1, 2 and 3 respectively.

Table 3.5: Loading score of extracted principal components

	Principal Components							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Ethylbenzene(EB)	.893	.277						
o-Xylene(oX)	.889	.348						
p-Xylene(pX)		.923						
m-Xylene(mX)	.974							
D-(+)-*α*-pinene(αP)	.720	.554						
D-limonene(Dl)						.932		
Ethanol, 2-butoxy-(E2B)	.430	.537				.248	.282	

Linalool(Linl)	.917							
Isocaryophyllene(Icrpn)				.791				
5,9-undecadien-2-one,6,10-dimethyl (UD)			.632	.368		.446		.252
(*E*)-Nerolidol(EN)							.233	
β-Farnesene(*β*F)			.311					
(-)-Spathulenol(SP)	.418	.267	.357			.509	.246	
Cis-p-metha-1(7)-8-dien-2-ol(zMD)					.961			
1,4-dihydroxy-p-menth-2-ene (Dm)	.431		.440	.470				
(*E,E*)-Cosmene (Do)				.823				
3-cyclohexen-1-carboxaldehyde,3,4-dimethyl(ccd)		.754	.561					
Hexadecane(Hxd)	.938							
Isoaromadendrene epoxide(Isamdn)				.307	.811			
β-Springene(*β*S)			.851					
Farnesol(Fnso)	.518	.602			.344		.229	
α-Springene(*α*Sp)	.248	.230			.239		.291	.635
Farnesol(*E*)-methylether(FnsoEm)								

Analysis of the composition through the PCA classification reveals three chemical groupings based on the compounds of the farnesene family. Figure 3.1 shows the farnesene compounds that formed different chemotypes. Farnesol is an oxygenated sesquiterpene of various isomers; (*E,Z*)-farnesol, (*Z,E*)-farnesol and (*E,E*)-farnesol. The most common isomer is (*E,E*)-farnesol that could represent 95% of farnesol and was the most identified. Springene is an isoprenoid hydrocarbon of diterpene nature. The two springenes: *β*-springene (7,11,15-trimethyl-3-methylene-1,6,10,14-hexadecatetraene) and α-springene (*E,E,E*-3,7,11,15-tetramethyl-1,3,6,10,14-hexadecapentaene) were identified. (*E,E*)-*β*-springene is a diterpene homolog of (*E*)-*β*-farnesene (4) (which formed part of the second grouping, see later).

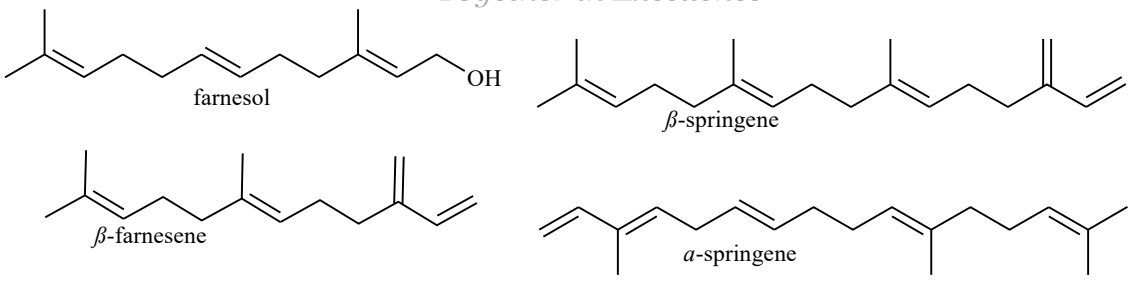


Figure 3.1: Compounds of chemotaxonomic significance identified from *T.vogelii*

The graphical representation of the three chemical groupings is shown by principal components (Fig. 3.2). The first category shows that some samples showed farnesol (Fnso) type (Chemotype 1). Farnesol was detected in the following samples: Tv1kyb, Tv1kyc, Tv1 muya, Tv2muya, Tv2kyd, Tv3muyc, Tv4kya, Tv4kyb, Tv4kyc, where in some cases it was one of the major components. Among other compounds in this grouping were: Ethanol-2-butoxy (E2B) and D-(+)-*α*-pinene (αP). Not all these other compounds could be detected in each of these samples as was the case for farnesol. The second grouping was majorly

springene compounds (*β*-springene (*β*S) and *α*-springene (*α*Sp)) and the *β*-farnesene (βF) which was referred to as Chemotype 2. There is a positive correlation between β-springene and *β*-farnesene (Pearson correlation, r=0.3), *α*-springene and *β*-farnesene (Pearson correlation r=0.2) (see Appendix 10). However, there is no positive correlation between either *β*-springene or *β*-farnesene with farnesol signifying a different chemical grouping. *β*-Springene, *α*-springene and *β*-farnesene were detected in the rest of the samples other than those detected in farnesol above (Tv3muyb, Tv3muya, Tv2kyb, Tv2kya Tv4muya and Tv4muyc, Tv2kyc and Tv1kya). The other compounds in this Chemotype 2 were: (*E*)-nerolidol (EN), cis-p-metha-1(7)-8-dien-2-ol (zMD), D-limonene(Dl), 1,4-dihydroxy-p-menth-2-ene (Dm) 5,9-undecadien-2-one,6,10-dimethyl (UD), and farnesol (*E*)-methylether (FnsoEm). *β*-springene was the most represented in this category since it was encountered in six samples out of a total of 19 samples (Tv3muyb, Tv3muya, Tv2kyb, Tv2kya Tv4muya and Tv4muyc). *β*-farnesene was detected in 2 samples (Tv1kya and Tv2kyc). All samples, however, were dominated by the alkylbenzenes; ethylbenzenes and xylene isomers for either in abundance or trace amount (treated as non-detectable). There was a minimum composition of the mixture of farnesene compounds (farnesol, *β*-springene and *α*-springene and *β*-farnesene) with a huge amount of alkylbenzenes which formed the mixed chemotype for example in samples like Tv4muyb and Tv3kya. There is a large correlation between alkylbenzenes and farnesol (Pearson correlation, r >0.4, p<0.05, for all alkylbenzene) and also a correlation between *α*-springene and farnesol (Pearson correlation, r >0.4, p<0.05).

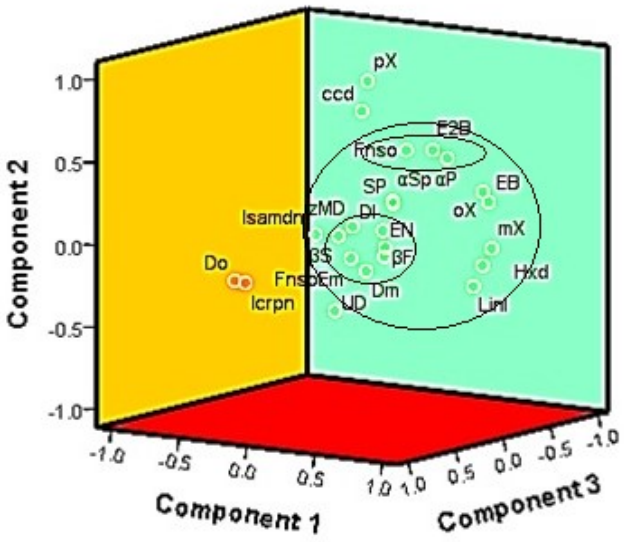


Figure 3.2: Three dimensional scatter plot of different correlations of chemical components using PCA

Other acronyms: *p*-Xylene (pX), Linalool (Linl), Isocaryophyllene (Icrpn), (-)-Spathulenol (SP), (*E,E*)-Cosmene (Do), 3,4-dimethyl-3-cyclohexen-1-carboxaldehyde (ccd), Hexadecane(Hxd), Isoaromadendrene epoxide (Isamdn)

To further amplify on the significance of this PCA analysis, agglomerative hierarchical clustering (AHC) was performed on the samples. Figure 3.3 shows the major hierarchical clustering classification of the major components in the oil. Farnesol, *β*-springene, *α*-springene, *β*-farnesene could form separate clusters thus affirming the above observations. A cluster of ethylbenzene, *o*-xylene and *m*-xylene was formed. The hydrocarbon cluster was the first in the dendrogram. The compounds within each cluster are broadly similar to each other.

The above three groupings reveal a very significant chemotaxonomic importance where in this part of the world, *T. vogelii* is of three chemical varieties: Farnesol variety and springene type, and the mixed variety. *β*-Springene has been previously found in the essential oils of *Heracleum persicum* Desf. ex Fischer leaves from Kandavan, northern Tehran in Iran (Mojab et al., 2002) and *Sigesbeckia jorullensis* Kunth (Asteraceae) from North-East of Hamburg, German (Heinrich et al., 2002). It was also detected in *Lagochilus cabulicus* Benth (Lamiaceae) (19.4%) from the Wakhan Corridor in Afghanistan (Jeppesen et al., 2012). Additionally, it was found in small quantities from *Salvia sclarea* Clary (Lamiaceae) (1.1%) from France (Laville et al. 2012) and *Salvia reuterana* Boiss (Lamiaceae) (0.3%) from Iran (Karamian et al., 2013). *α*-Springene was detected as a major component in the essential oil of *Murraya exotica* L.(Rutaceae) flowers, got from India; (23.8%) (Raina et al., 2006) and in that of *Teucrium marum* L. (Lamiaceae) from Corsica; (1.1 – 17.8%) (Djabou et al., 2013). It is therefore noteworthy to report the presence of *α*- and *β*-springene as major constituents of the Uganda *T. vogelii* essential oils given that the alkyl benzenes were not consistently found although were detected in large amounts at certain times.

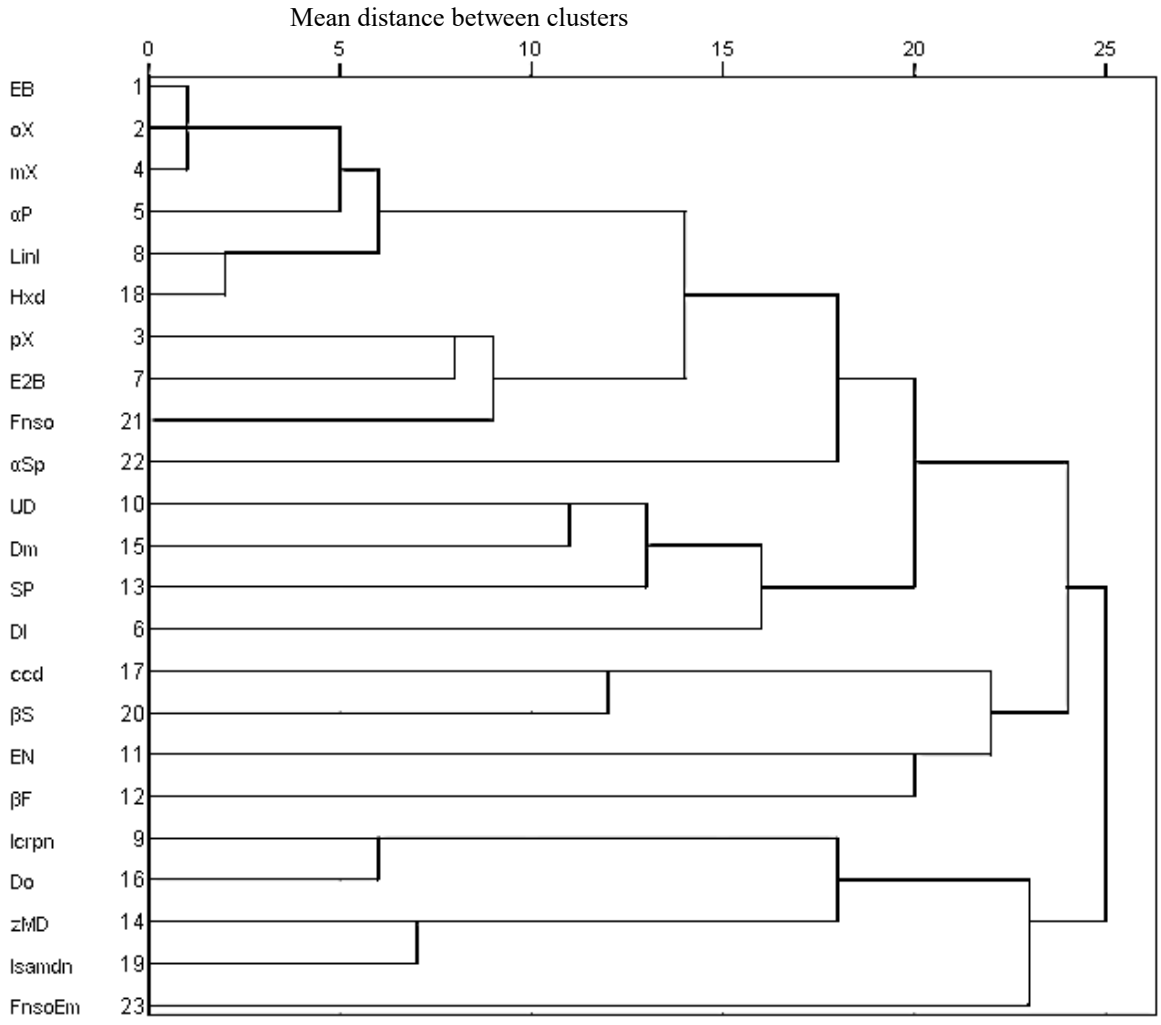


Figure 3.3: Dendrogram of major components obtained based on the classification of samples of *T. vogelii*

3.2.2 Effect of season variation on the percentage yield and major composition of the oils

Two seasonal variations were considered in this study: rain season and dry season represented with the green pattern and red pattern respectively in Figure 3.4. The rainfall bimodal peaks in the district occur between March to May and August- September. Samples obtained during this time were: Tv1muya, Tv2muya, Tv3muya, Tv3muyb, Tv3muyc, Tv2kya, Tv2kyb, Tv2kyc, Tv2kyd and Tv3kya. During the dry season, samples were samples in January and June and were Tv1Kya, Tv1kyb, Tv1kyc, Tv4kya, Tv4kyb, Tv4kyc, Tv4muya, Tv4muyb and Tv4muyc. Considering this, there was no significant difference between the percentage oil yield between the two seasons and from the two sampled areas.

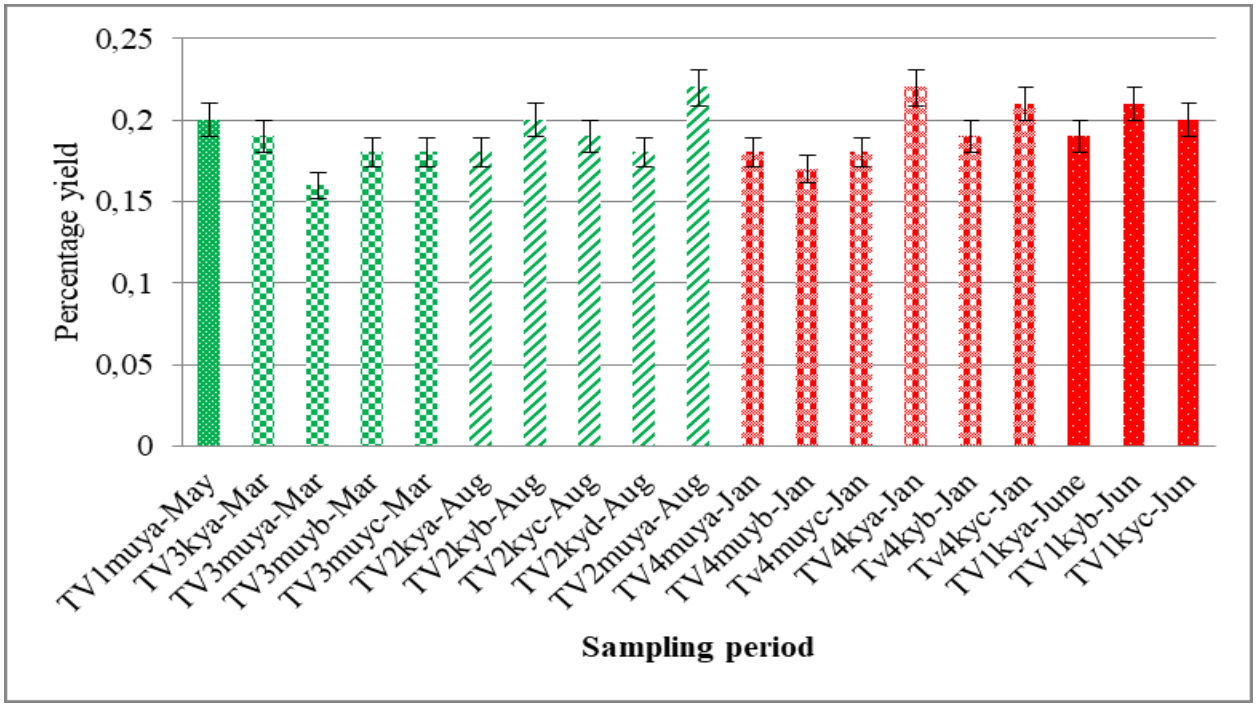


Figure 3.4: A continuous histogram depicting the percentage yield of the oils from samples of *T. vogelii*.

The effect of seasonal variation on the constituents and composition of the samples was done using cluster analysis of the constituent composition in the samples and several clusters were formed (Figure 3.5). Cluster 1 was majorly for the composition of samples taken during the rainy season; March-May and August (Tv1muya, Tv2muya, Tv3kya) and one sample of the dry season, June (Tv1kyb). Cluster 2 and 4 were compositions for the samples during rainy season sampling i.e cluster 2-Tv3muyb and Tv3muyc) were for March sampling, Cluster 4-Tv2kya and Tv2kyb for august sampling. Besides, Tv3muya that formed Cluster 6 was sampled in March thus during the rainy season. Cluster 3 and 5 were for major compounds of the samples from dry and rain season (Tv1kyc and Tv2kyd for cluster 3 and Tv2kyc and Tv1kya for cluster 5). And finally, samples of cluster 7 (Tv4muya and Tv4muyc), cluster 8 (Tv4kyc and Tv4muyb), cluster 9 (Tv4kyb) and cluster 10 (Tv4kya) were obtained during the dry season (January). These correlations indicate major seasonal effects on the composition of the major constituents. However compounds like ethylbenzene, *o*-xylene and *m*-xylene could not be detected in the samples that were picked in January but were observed in samples for June. The compounds were however found in trace amount and therefore were not quantified. This clustering certainly had serious implications on the pesticidal potential of the *T. vogelii* leaf material. It is therefore clear that there exists variation in the components of

the essential oils of *T vogelii* and this variation affects the pesticidal properties of this plant. More of these discussions have been published (Kerebba et al., 2020).

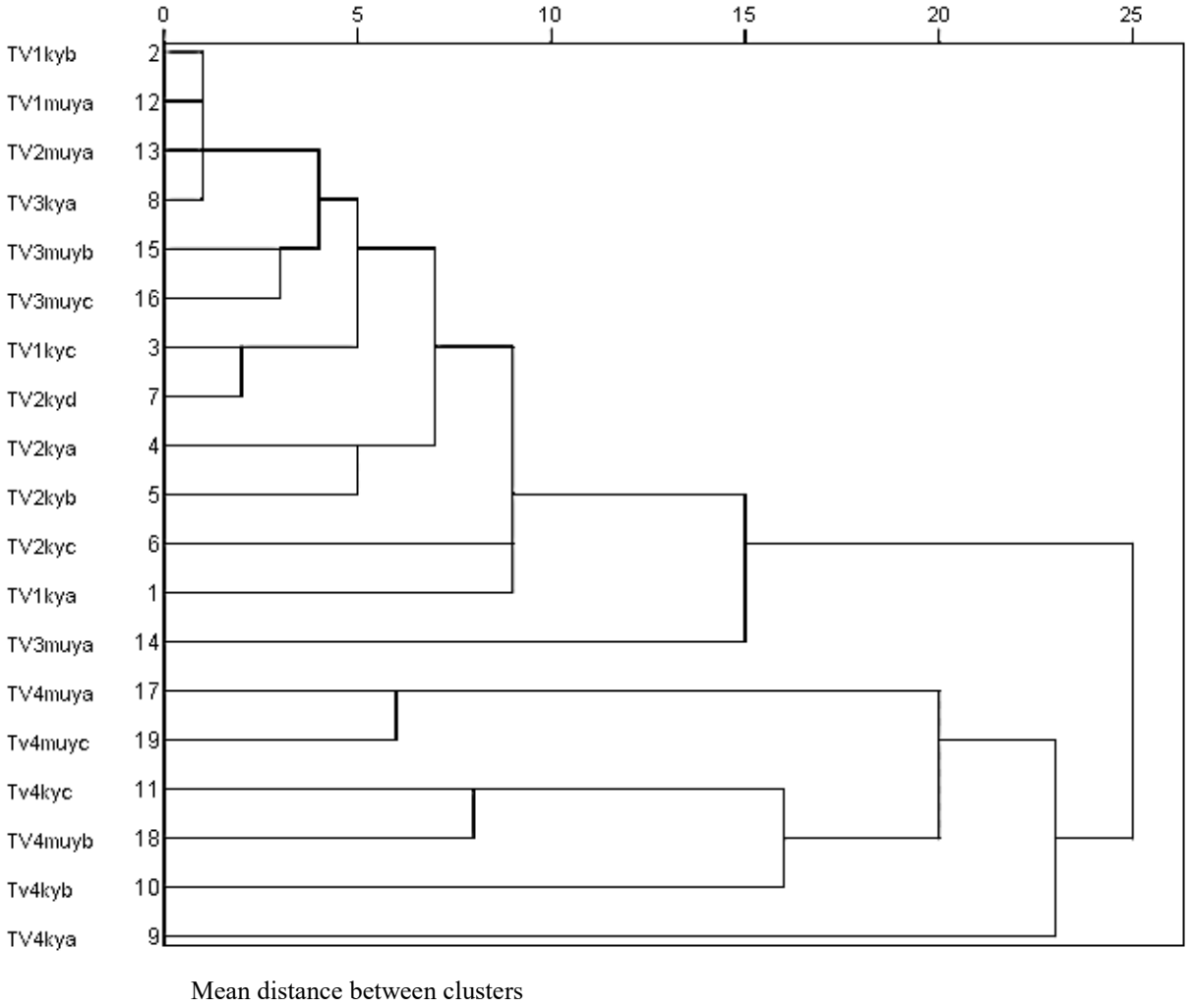


Figure 3.5: Dendrogram of samples due to classification based on their major composition

3.4 Chemical evaluation of the anti-oxidant potential of *T. diversifolia* non-volatile substances

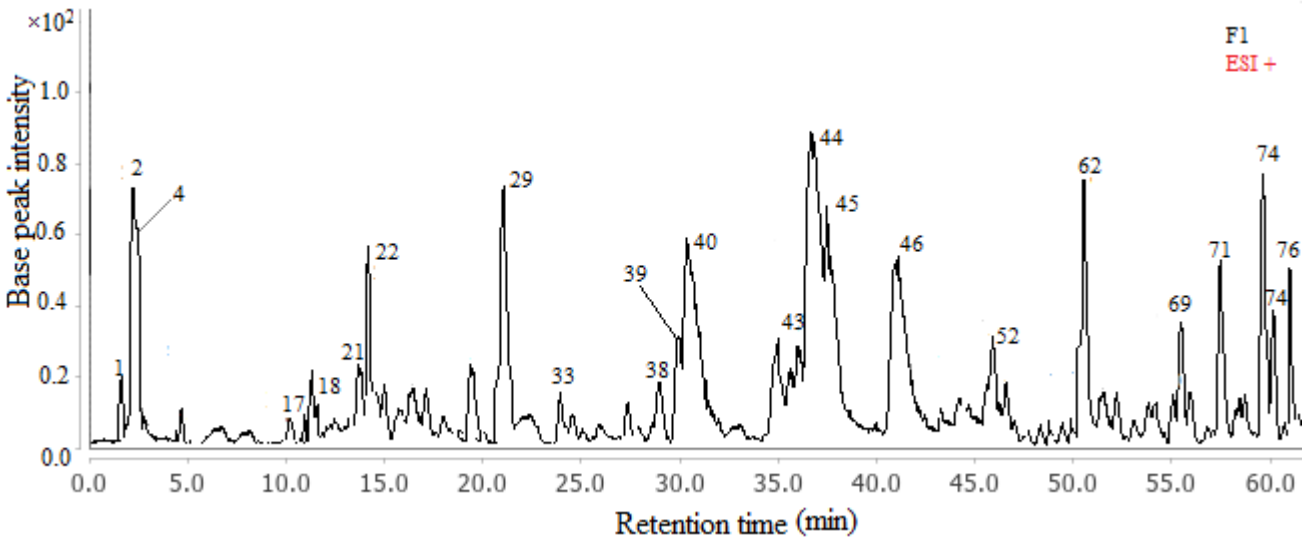
Previous reports on *T. diversifolia* have indicated that its anti-oxidant potential is directly related to its total phenol and flavonoid contents in the crude extracts (Ojo et al., 2018, Pantoja Pulido et al., 2017; Orsomando et al., 2016 and Giacomo et al., 2015). These studies have focused more on the quantitative determination of total phenol and flavonoid contents in the extracts and to a little extent identify a few bioactive compounds via the conventional classic bioassay-guided isolation. For example Ojo et al., (2018) obtained the total phenol content of 251.63 mg g^{-1} gallic acid equivalent (GAE) and total flavonoid content of 98.21 mg g^{-1} QUE (quercetin equivalent). The authors further reported high amounts of chlorogenic acid (7.95 ± 0.02 mg g^{-1}), gallic acid (1.28 ± 0.01 mg g^{-1}), p-coumaric acid (0.53 ± 0.01 mg g^{-1}), apigenin (5.09 ± 0.02 mg g^{-1}) and caffeic acid (5.18 ± 0.02 mg g^{-1}) in *T. diversifolia* (Ojo et al., 2018). Thus the existing information does not satisfactorily evaluate the chemistry of antioxidant properties of *T. diversifolia* i.e a detailed phytochemical analysis that reveals numerous phenolic compounds and other compounds believed to possess anti-oxidant potential (polyphenolic compounds) has been missing. This study has attempted to identify individual or group of phenolic compounds tentatively using the highly sensitive UPLC-ESI-QTOF-MS/MS that will enhance further phytochemical and biological studies including pesticidal activity. It has also evaluated the dependence of antioxidant potential on total phenol and flavonoid contents as reported in earlier reports.

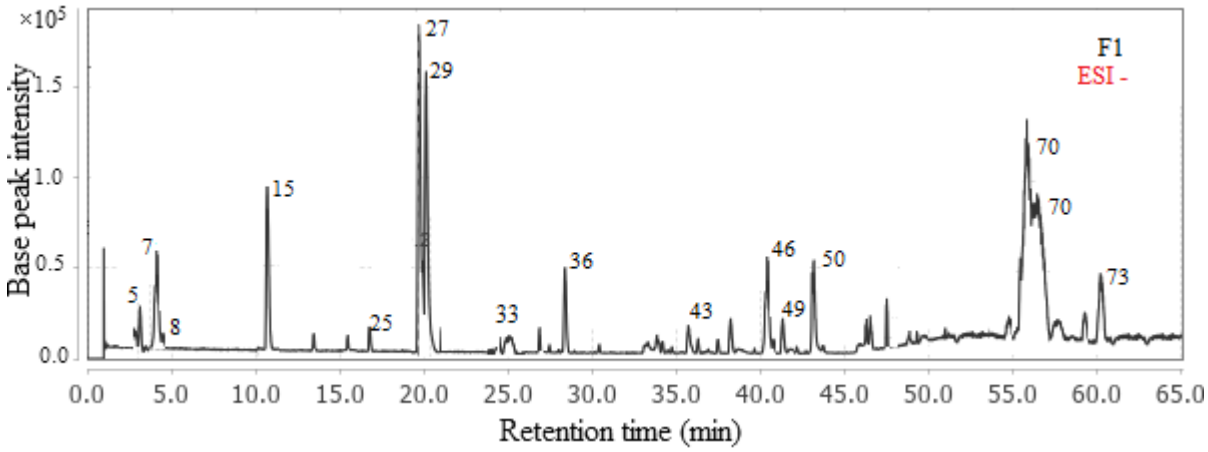
3.4.1 Structural characterisation using UPLC-ESI- QTOF- MS/MS

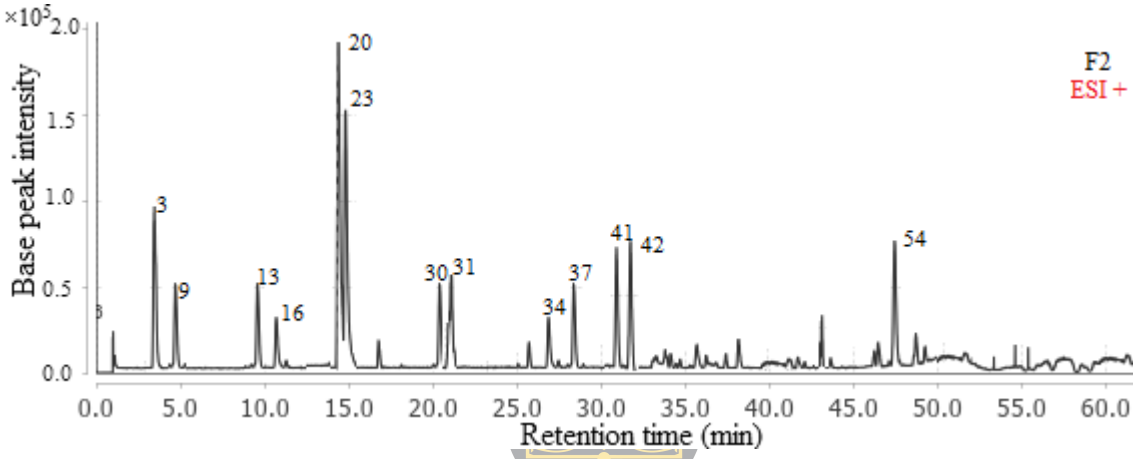
Compounds were identified and assigned tentative names of identity on the basis of three criteria i.e.; an accurate mass match which is automatically searched, using METLIN (http://metlin.scripps.edu/metabo_search.php) and other libraries including PubChem (http://pubchem.ncbi.nlm.nih.gov/), Massbank, NIST etc. with all compounds whose accurate mass error (AME) > 5 ppm, were considered unidentified. Secondly, mass fragmentation patterns searched in the above databases, if available. A few phenolic compound standards were spiked under similar LC/MS/MS conditions and fragmentation patterns were compared to identify a given compound (Fig. 3.7) based on retention time, mass fragmentation and ionisation modes i.e. (by corresponding ions of neutral, protonated and deprotonated moieties). Since many compounds could be identified by UPLC-ESI-MS/MS, it was not possible to obtain all standards thus other similar compounds' MS and MS^2 fragment ions in

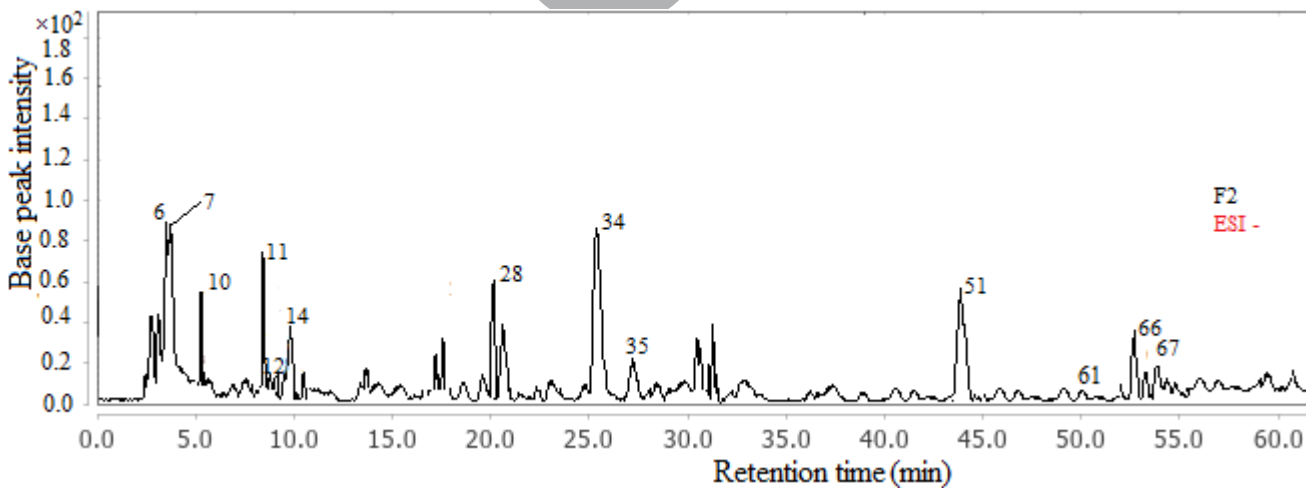
literature and data bases were used for annotation of compounds. MS-MS of the compounds in the sample fragmented to corresponding product ion mass spectra. And lastly, the number of carbon atoms in the peak was calculated if isotope abundances were available. The predicted number of carbon atoms in the putatively identified compound was used to reduce false annotations.

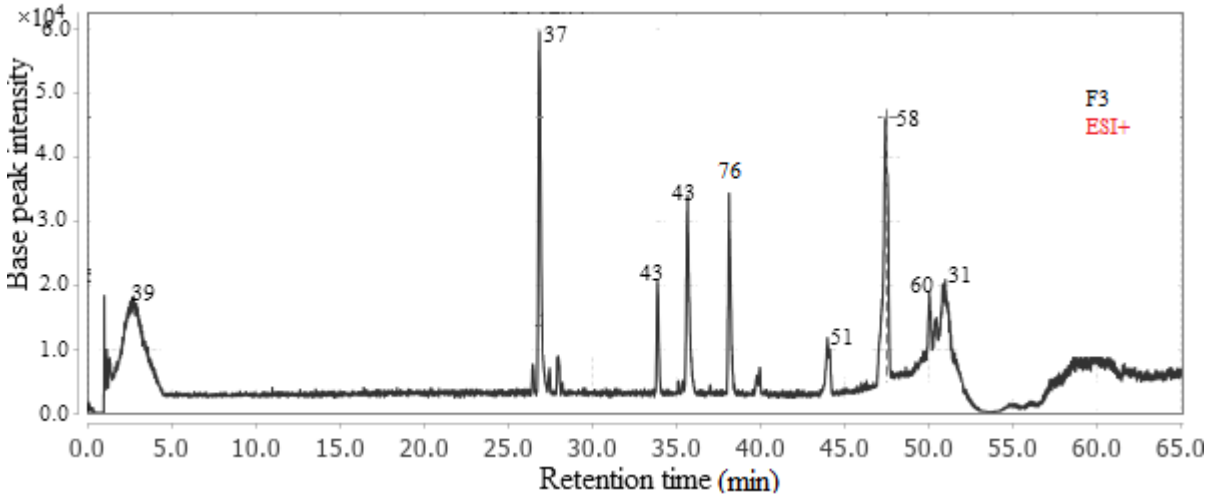
The phenolic screening of aqueous methanolic fractions F1, F2, F3, F4 and F5 was performed. Representative base peak chromatograms of the aqueous methanolic fractions F1, F2, F3, F4, and F5 for which phenolic compounds were screened are shown in Fig. 3.6. A total of 76 peaks were detected by LC-MS/MS among which 55 phenolic compounds could be annotated (Table 3.6). These included 4 hydroxybenzoic acids, 19 well characterised hydroxycinnamic acids including known peaks and 1 phenyl propane glycerol, five unknown hydroxycinnamic acid derivatives, 12 flavonols, 5 flavones, 2 flavanones, 4 flavanols, 2 unknown flavonoids, eight coumarins and furocoumarins, 1 saponin triterpeneoid, 4 other hydroxyl compounds that included quinic acids, glucaric acids and 1 fatty acid compound were characterised.

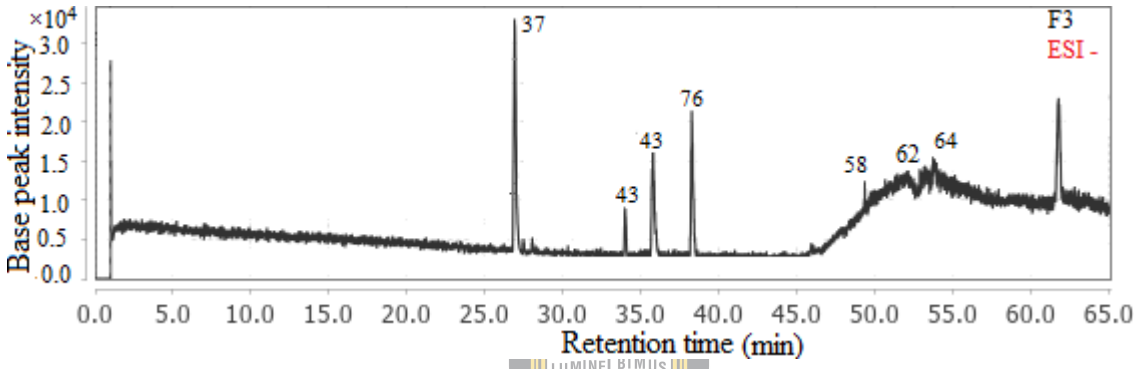


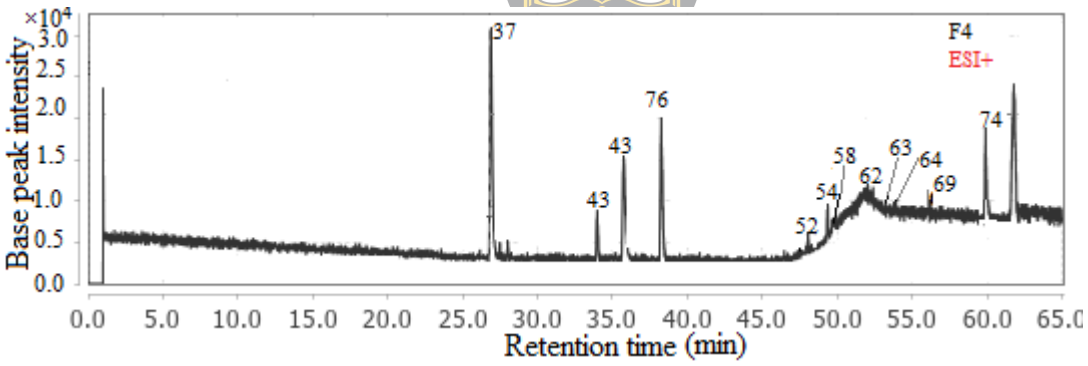

×10^5
Base peak intensity
F1
ESI -
Retention time (min)

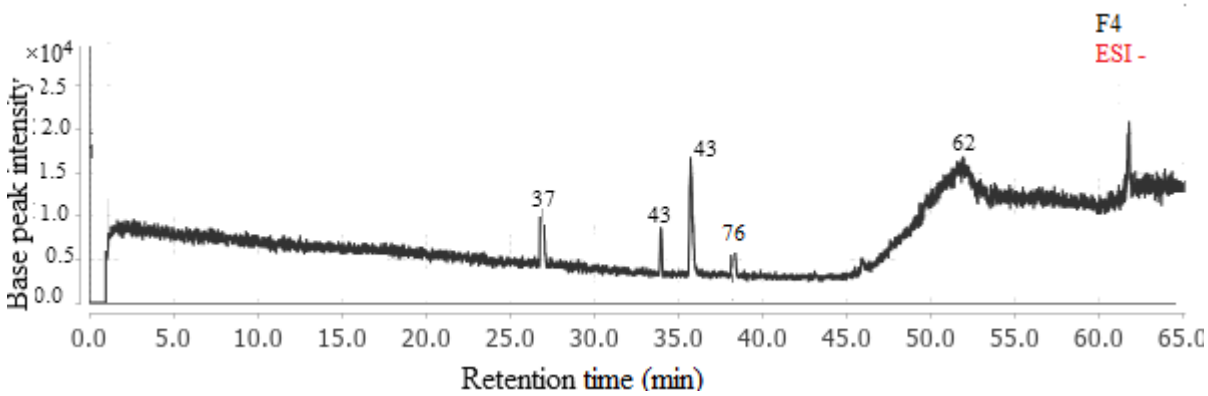

×10^5
Base peak intensity
F2
ESI +
Retention time (min)


×10^2
Base peak intensity
F2
ESI -
Retention time (min)


×10^4
Base peak intensity
F3
ESI+
37
58
76
43
43
39
60
31
51
Retention time (min)


×10^4
Base peak intensity
F3
ESI -
37
76
43
43
58
62
64
Retention time (min)


×10^4
Base peak intensity
F4
ESI+
37
76
43
43
74
58
63
64
62
54
69
52
Retention time (min)


×10^4
Base peak intensity
F4
ESI -
43
62
37
43
76
Retention time (min)

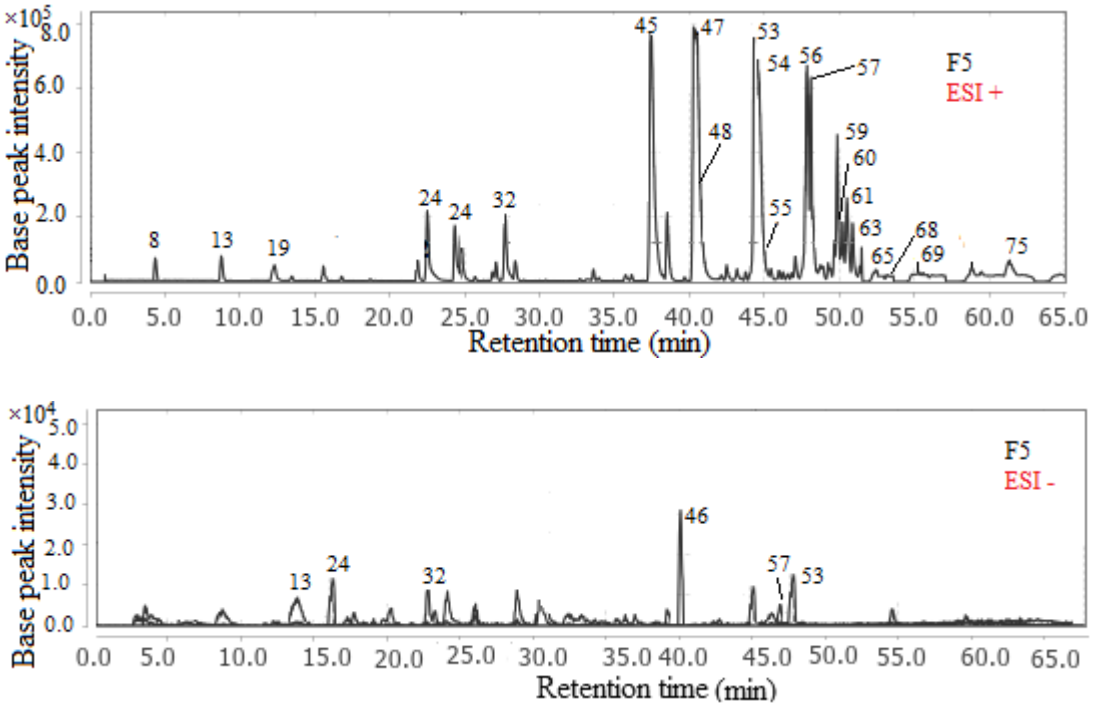
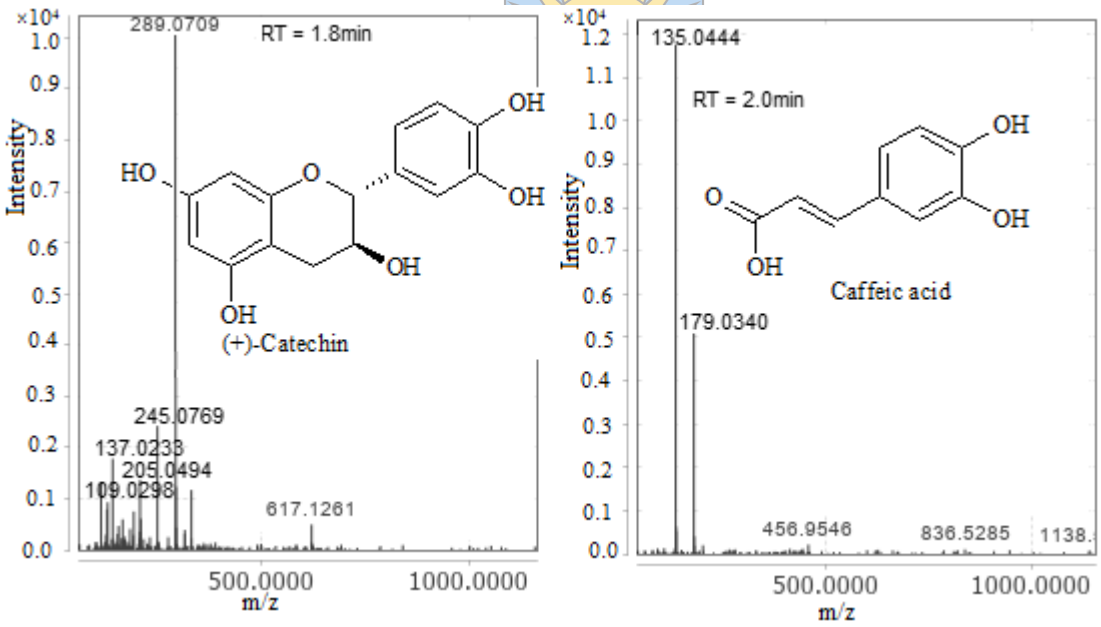
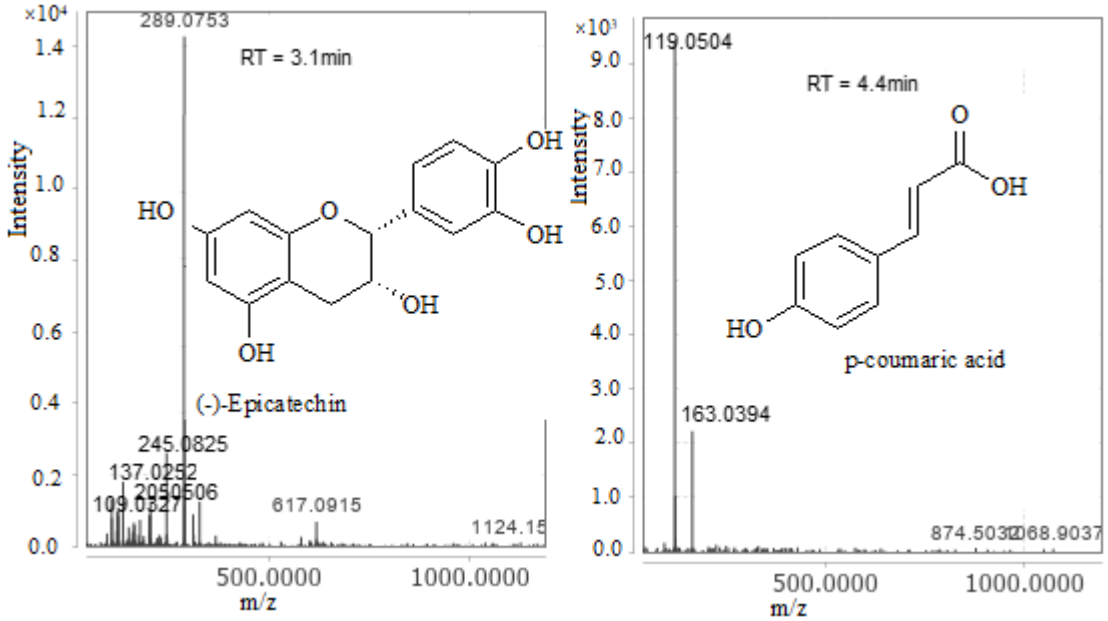
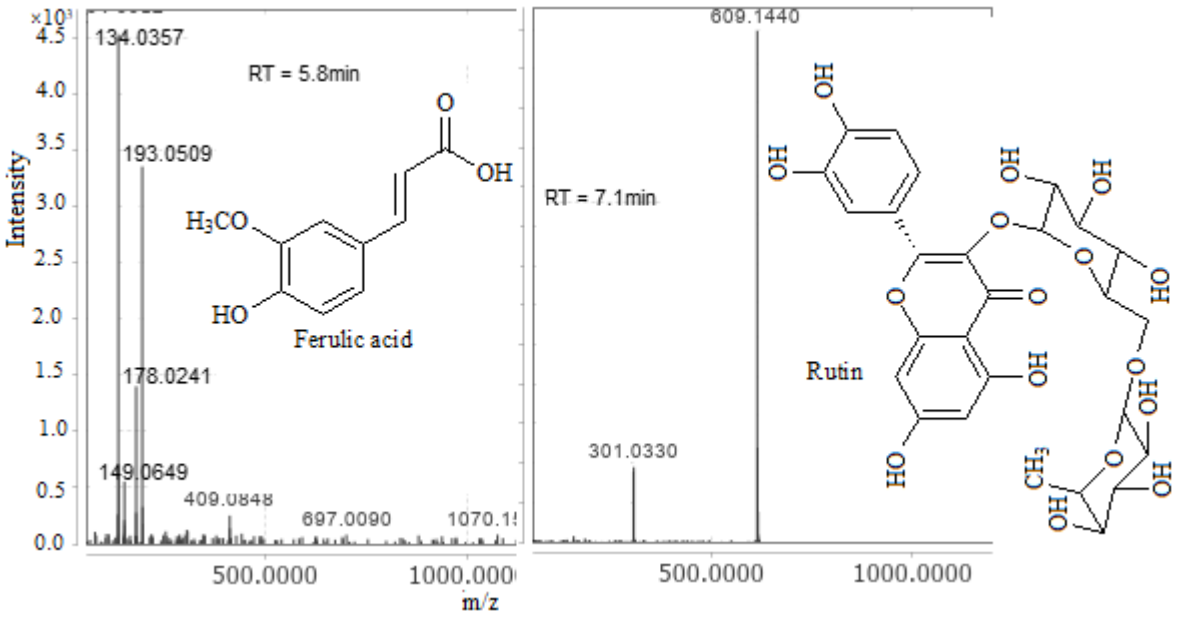


Figure 3.6: HPLC-ESI-MS base peak chromatograms for fraction F1, F2, F3, F4, and F5




×10⁴
289.0753
RT = 3.1min
Intensity
HO
OH
OH
OH
OH
(-)-Epicatechin
245.0825
137.0252
109.0527
617.0915
1124.15
500.0000
1000.0000
m/z
×10³
119.0504
RT = 4.4min
Intensity
O
OH
HO
p-coumaric acid
163.0394
874.5032
1068.9037
500.0000
1000.0000
m/z


×10³
134.0357
RT = 5.8min
Intensity
193.0509
O
OH
H₃CO
HO
Ferulic acid
178.0241
149.0649
409.0848
697.0090
1070.15
500.0000
1000.000
m/z
609.1440
RT = 7.1min
OH
OH
OH
OH
O
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O
OH
CH₃
OH
OH
500.0000
1000.0000

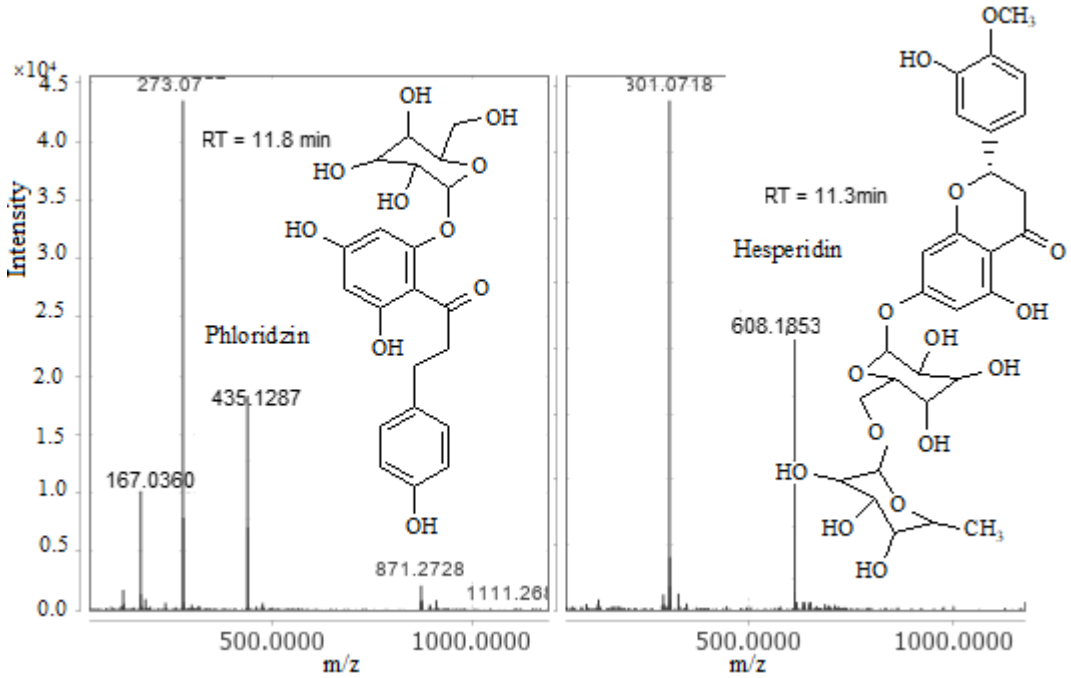


Figure 3.7: MS-MS spectra for standard compounds in negative ion mode

3.4.2 Identification of anti-oxidant phenolic compounds

Zhao et al, (2012) showed that polar extracts of *T. diversifolia* have phenolic compounds. In this study, the phenolic screening of aqueous fractions F1, F2, F3, F4 and F5 was performed (Table 3.6). A group of compounds was tentatively identified. These include mono- and di-hydroxycinnamic acids and free flavonoids, flavonoid derivatives and other polyphenols.

3.4.2.1 Identification of free phenolic acids and their derivatives

3.4.2.1.1 Characterisation of hydroxybenzoic acid derivatives

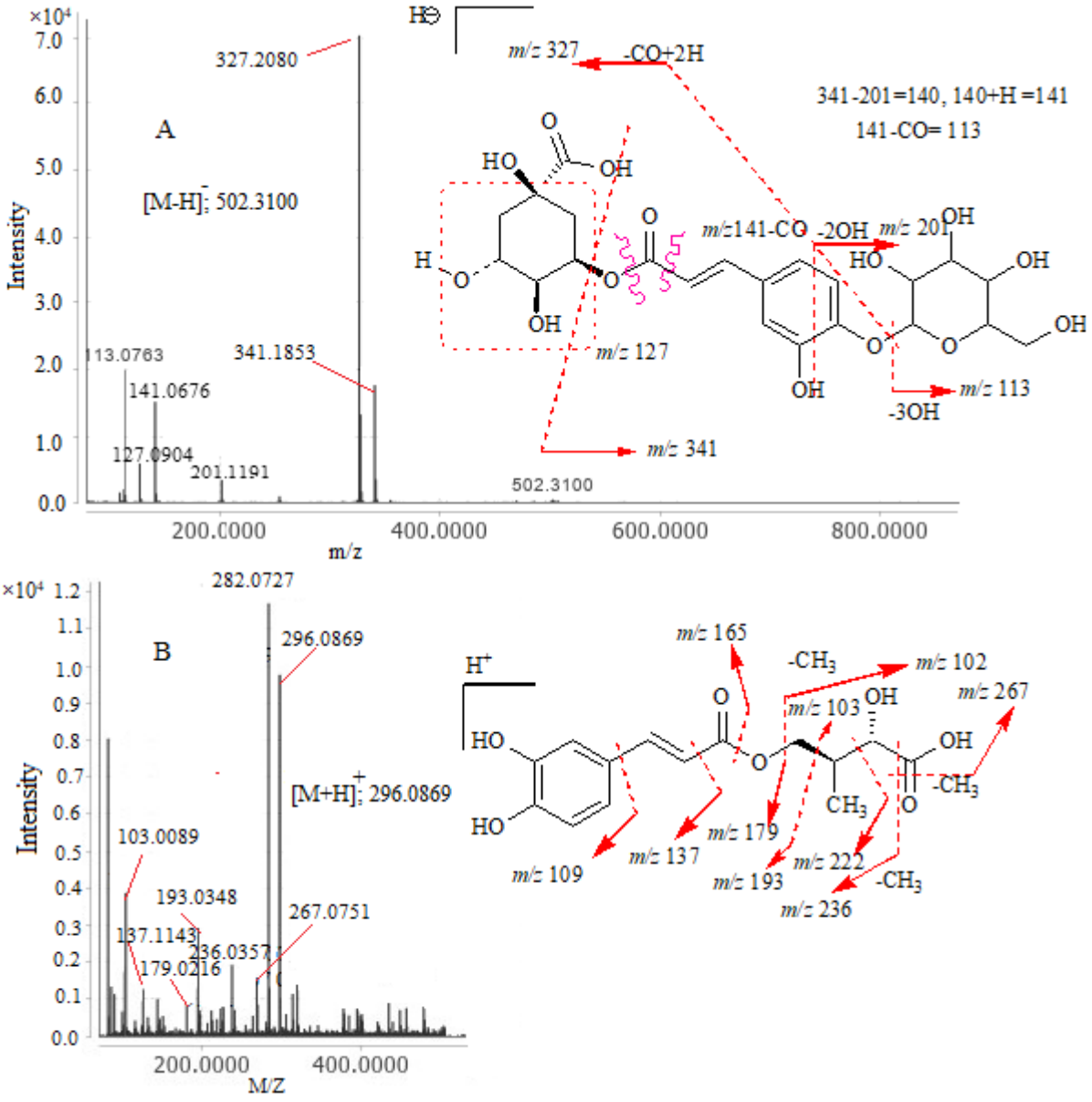
Hydroxybenzoic acid peaks: 24, 47, 54 and 57 were detected. Peak 24 displayed $[M-H]^-$; *m/z* 187.1408 corresponding to that of gallic acid monohydrate (Khallouki et al., 2015), since its MS^2 fragmentation gave high intense peak ions at 169 $[M-H-H_2O]^-$; and *m/z* 125 $[M-H-H_2O-CO_2]^-$. Peak 47 with $[M-H]^-$; 345.2170 was identified to be theogallin (galloylquinic acid, 343Da) (Spínola et al., 2015), with fragments 201 $[M-H-145]^-$, 127 [quinic acid-CO-$2H_2O]^-$, 327 $[M-H-OH]^-$, 145 (Retro Diels Alder (RDA) cleavage through quinic ring forming $C_9H_8O_5^-$), 109 (dihydroxy phenyl radical). Peak 54 was that of protocatechuic acid, displaying both $[M-H]^-$ and $[M+H]^+$ ions. MS^2 fragment ion *m/z* 111 (dihydroxy phenyl

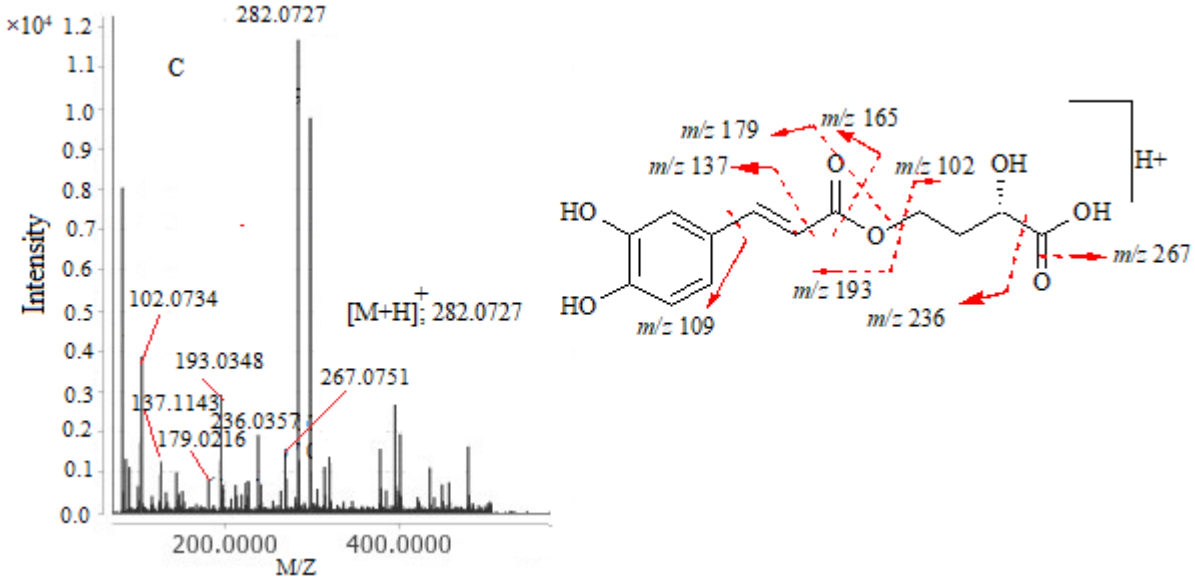
residue, $[M+H-CO_2]^+$) is in line with literature data.[17] Peak 57 with $[M-H]^-$; 535.2920 had MS^2 fragment ions 535, 373 [M-H- hexoside]$^-$, 343 [M-H-191]$^-$, 313 (galloyl hexoside), 211 [M-H- hexoside- 162]$^-$, and thus identified as gallotannin (dimethyl galloyl quinic acid-C-hexoside).

3.4.2.1.2 Characterisation of hydroxycinnamic acid derivatives

Twenty four hydroxycinnamic acids peaks (4, 7, 18, 19, 23, 28, 43, 53, 58, 59, 62, 63, 64, 65 67, 68, 69, and 74) in addition to 1 phenyl propane glycerides (peaks 27), 5 unknown hydroxycinnamic acid derivatives (peaks 25, 60, 61, 64 and 72) were identified. Peak 4 displayed a deprotonated molecular ion, $[M-H]^-$ with *m/z* 223.9371. Its MS/MS fragment ions *m/z* 133 $[M-H-2CH_3O-2OH]^-$, 181 $[M-H-CO_2]^-$, enabled tentative identification as sinapic acid. Peak 43 with $[M-H]^-$; 370.5113 has been tentatively identified as caffeoyl glucaric acid with characteristic ions of the glucaric acid; *m/z* 209 [glucaric acid-H]$^-$; and 191. Fragment, *m/z* 209 formed after loss of a caffeoyl radical (162Da). Peaks 19, 23, 29 and 68 were identified as caffeoylquinic acid (CQA) or its derivatives. Peak 23 was a mono-CQA acid (4-*O*- CQA) identified by a comparison of its mass spectra with that of literature (Gouveia and Castilho, 2011, Clifford et al., 2008). It displayed MS in negative ion mode; ($[M-H]^-$) ion at *m/z* 353.0147. Its MS^2 spectrum gave *m/z* 191 [quinic acid - H]$^-$ ion after losing a caffeoyl radical (179 Da), *m/z* 135 ([caffeoyl-H - CO_2]$^-$) ion and 173 [quinate-H_2O]$^-$, the base peak, is characteristic of 4-OH quinic acid substitution (Clifford et al., 2008). The dimeric ion at [2M-H]$^-$; 707.7141 was detected at peak 28 confirming CQA presence. The precursor ion at *m/z* 516 for peak 68 is 4,5-di-*O*-E-caffeoylquinic acid (4,5-diCQA) due to $[M-H]^-$. Peak 19 was tentatively identified as acetyl-diCQA. After losing acetyl caffeoyl (206 Da) from the precursor ion to give 353 ([M-H-206]$^-$) and *m/z* 397 [M-H-162]$^-$. The quinic moiety came from [M-H-367]$^-$. Peaks 59 and 61 identified from fraction F5 showed ion signals $[M+H]^+$ at *m/z* 530.3372, and 558.4421 respectively, displaying CQA ion moiety; $[CQA+H]^+$ at *m/z* 355 as the base peak ion. Peaks 60 and 72 displayed $[M-H]^-$; 494.2839, 658.4421 respectively with [CQA -H]$^-$, *m/z* 353. In addition, peaks 60 and 72 also had 327 [M-H-28]$^-$, *m/z* 127 (quinic acid residue) and 353 or 355 (CQA) were also formed. In addition to peak 59, were CQA derivatives. Peak 59, 61 and 64 both have a common MS^2 fragment ion *m/z* 141 unit and also *m/z* 341 and *m/z* 113. According to Rivera-Pastrana et al., (2010), the fragment ion *m/z* 341 belongs to that of a deprotonated molecular ion; caffeoyl-hexoside having dehydrated hexose (162Da) and caffeic acid anion at *m/z* 179. Fragment ion at *m/z* 113

belongs to that of the saccharide residue according to Spínola et al., (2015). However, it may arise from the loss of CO from *m/z* 141 (141-CO). This enables peaks 53 and 58 to be identified. Peak 53 $[M-H]^-$; *m/z* 502.3093 is CQA-hexoside orspecifically 4-*O*-(1-*O*-caffeoylglucosyl)quinic acid, with MS^2 fragments; *m/z* 327 $[CQA-CO+2H]^-$, 341 (caffeoylglucosyl), 113 (saccharide residue) and quinic acid residue at *m/z* 127 and other fragments *m/z* 201 and 141. The ion at *m/z* 201 is suggested to be due to retro Dields Alder cleavage through the aromatic ring of the caffeoyl. 113Da may also arise from loss of CO from *m/z* 141 (see the proposed fragmentation pathway in Fig. 3.8A). It is a new hydroxycinnamate.

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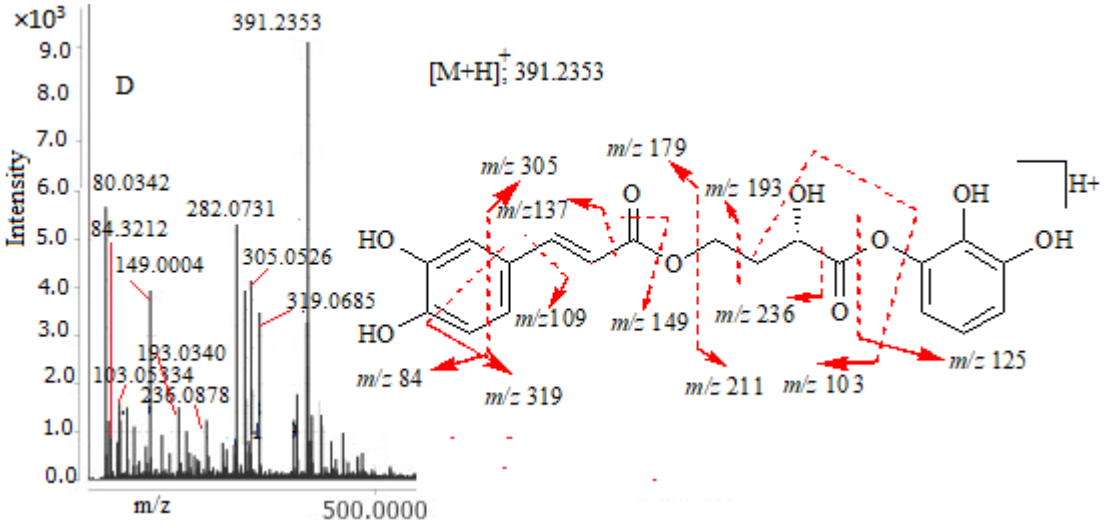
193.0348
267.0751
137.1143
236.0357
179.0216
[M+H]+; 282.0727
Intensity
M/Z
m/z 179
m/z 165
m/z 137
m/z 102
m/z 267
m/z 193
m/z 236
m/z 109
H+


D
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[M+H]+; 391.2353
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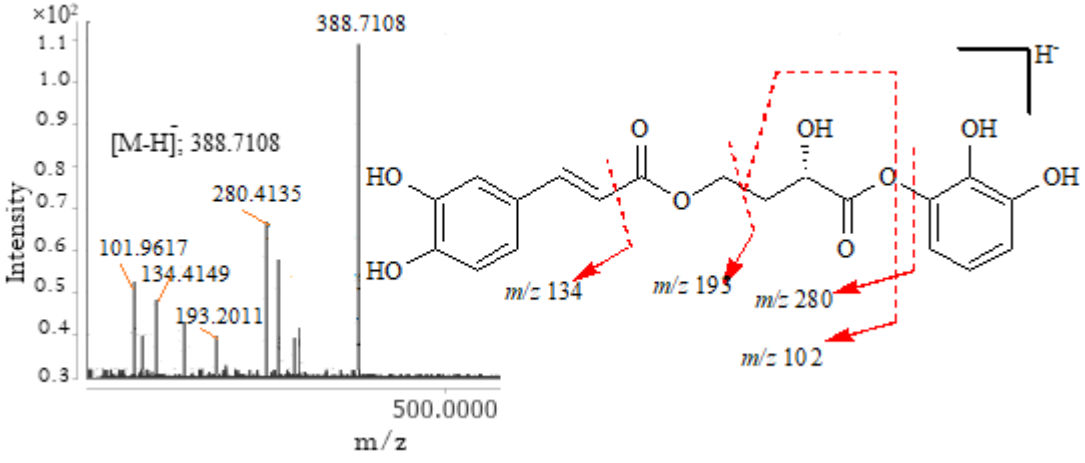
84.3212
149.0004
305.0526
319.0685
193.0340
103.05334
236.0878
Intensity
m/z
m/z 305
m/z 179
m/z 193
m/z 137
m/z 109
m/z 149
m/z 236
m/z 84
m/z 319
m/z 211
m/z 103
m/z 125
H+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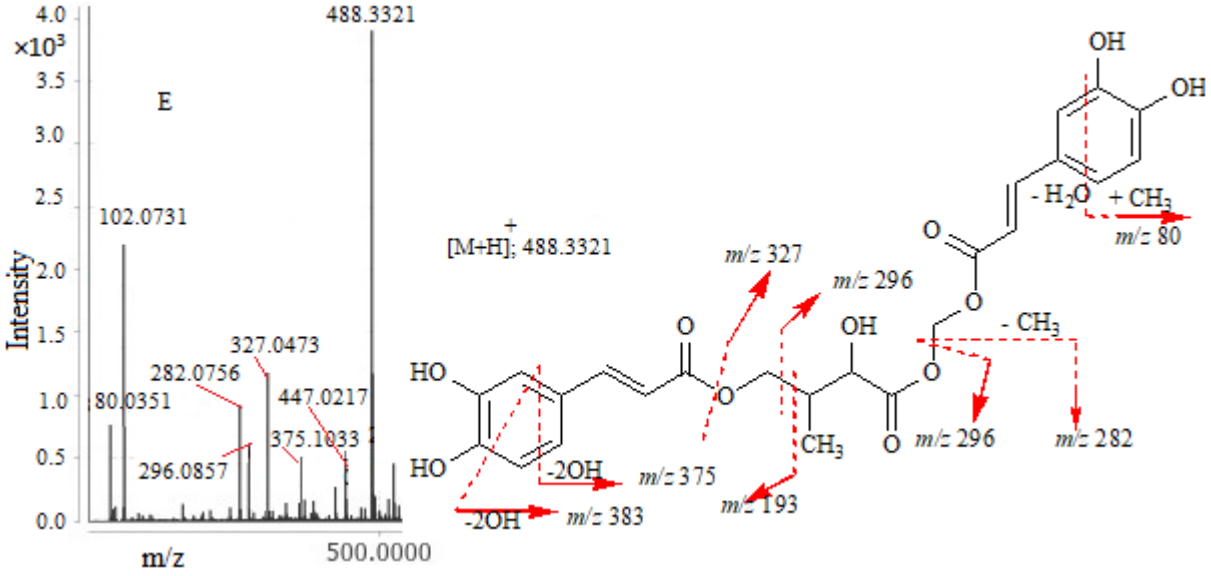
[M-H]-; 388.7108
280.4135
101.9617
134.4149
193.2011
Intensity
m/z
m/z 134
m/z 195
m/z 280
m/z 102
H-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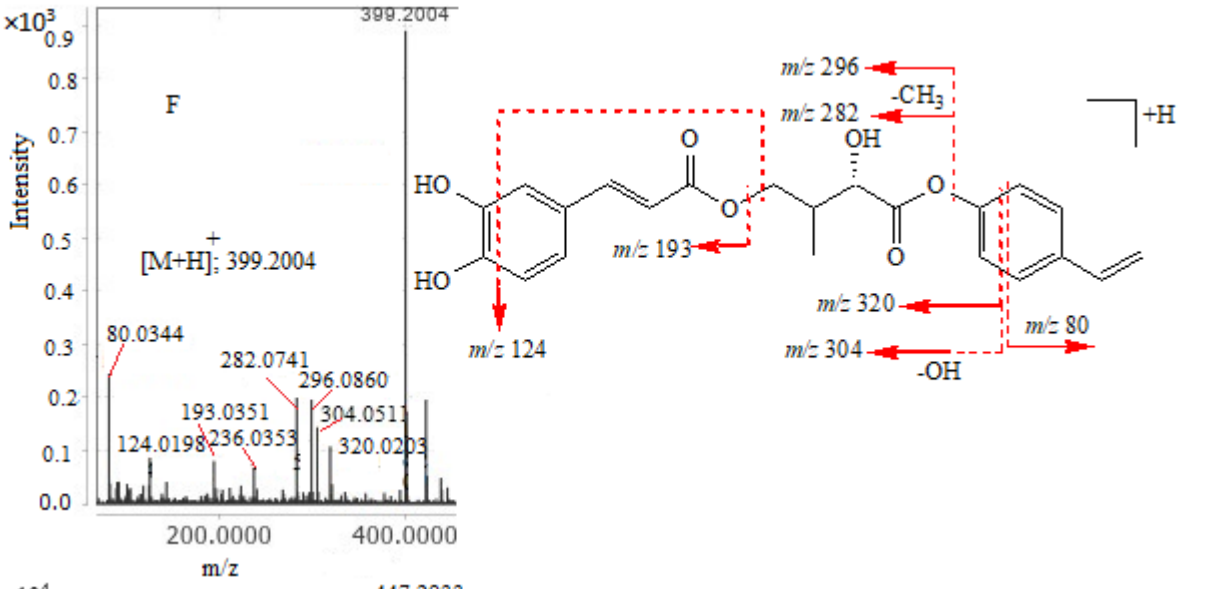
282.0756
447.0217
80.0351
375.1033
296.0857
Intensity
m/z
500.0000
[M+H]+; 488.3321
m/z 327
m/z 296
- H2O
+ CH3
m/z 80
- CH3
-2OH
m/z 375
m/z 193
-2OH
m/z 383
m/z 296
m/z 282


F
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[M+H]+; 399.2004
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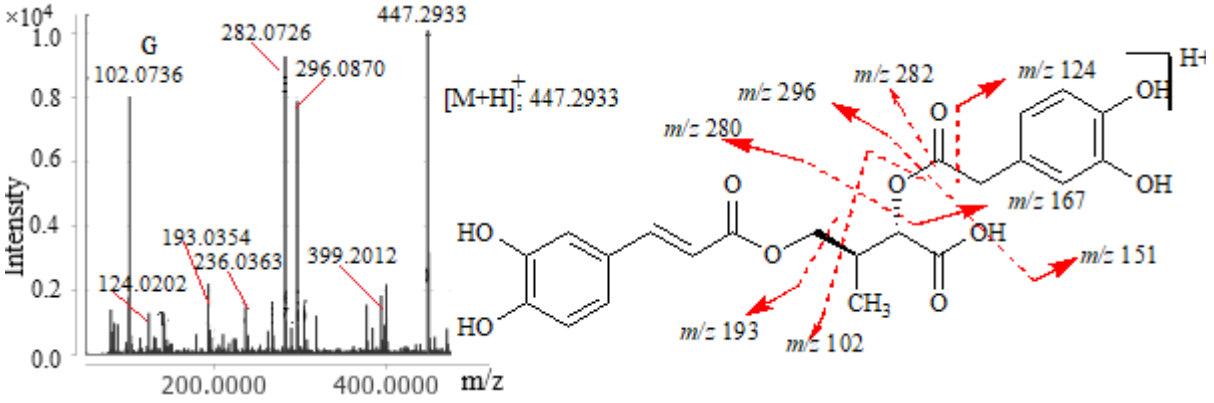
304.0511
124.0198
236.0353
320.0203
Intensity
m/z
200.0000
400.0000
m/z 296
-CH3
m/z 282
+H
m/z 193
m/z 320
m/z 80
m/z 124
m/z 304
-OH


G
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296.0870
102.0736
193.0354
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399.2012
124.0202
Intensity
m/z
200.0000
400.0000
[M+H]+; 447.2933
m/z 296
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m/z 124
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m/z 167
m/z 151
m/z 193
m/z 102

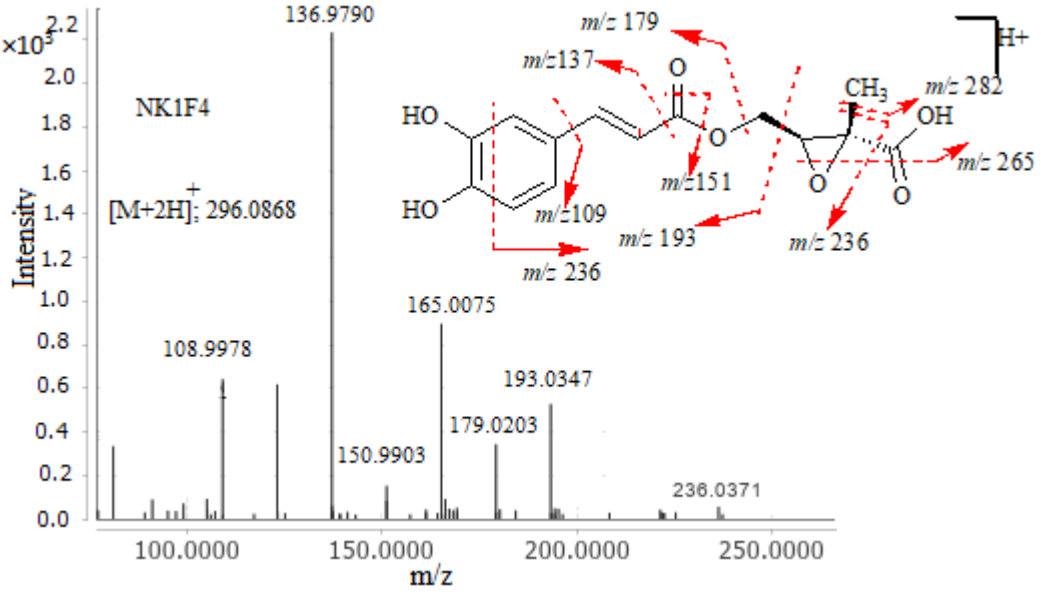


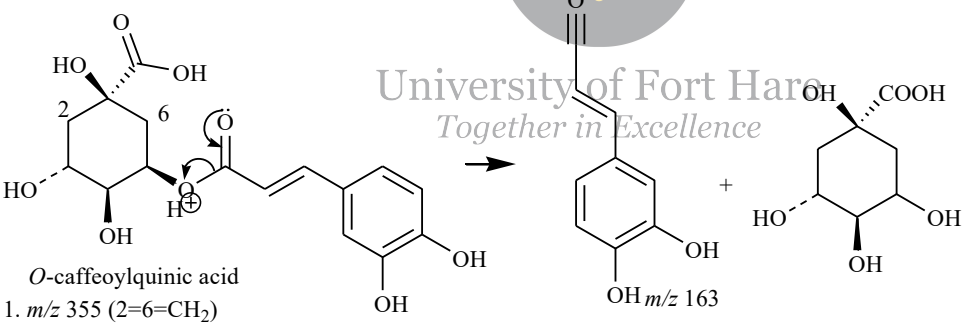
Figure 3.8: MS/MS and fragmentation pathway of 4-*O*-(1-*O*-caffeoylglucosyl)quinic acid (A), 4-*O*-caffeoyl-2-hydroxy-3-methylbutanoic acid (B) 4-*O*-caffeoyl-2-hydroxybutanoic acid (C), 3,4-dihydroxyphenyl-4-*O*-caffeoyl-2-hydroxybutanoate (D), 3-(4-hydroxy-3-methoxyphenyl) acryloyloxy) methyl-4-*O*-caffeoyl-2-hydroxybutanoate (E), 4-vinylphenyl 4-*O*-caffeoyl-2-hydroxy-3-methylbutanoate (F), 4-*O*-caffeoyl-2-(2-(3,4-dihydroxyphenyl)acetoyloxy)-3-methylbutanoic acid (G) 3-(4-*O*-(caffeoyl)methyl)-2-methyloxirane-2-carboxylic acid (NK1F4)

Peaks 18, 62 and 69 identified from fractions 1 and 4 with $[M+H]^+$; 282.0715, 282.0733 and 282.0719 respectively had almost similar fragmentation patterns and ions with that of peak 63 ($[M+H]^+$; *m/z* 296.0872). Chemical inductive dissociation (CID) of compounds in these peaks yielded fragment ions; *m/z* 137, 109, 193, 165, 179, 151, 267 and 236 (Fig. 5B and 5C). The ion at *m/z* 179 represents a caffeoyl moiety obtained through simple inductive cleavage leading to loss of 2-hydroxybutanoic acid (HBA) (104Da) ($[M+H-HBA]^+$ for peaks 18, 62 and 69 or $[M+H-HBA-CH_3]^+$ for peak 63). The fragments *m/z* 137 $[M+H-C_6H_9O_5]^+$, 193 ($[M+H-197]^+$), 165 ($[M+H-118]^+$ or $[M+H-133]^+$ for the methyl derivative), 267 ($[M+H-O]^+$ or $[M+H-O-CH_3]^+$), and 237 ($[M+H-CO_2]^+$ or $[M+H-CO_2 - CH_3]^+$) and 109 (dihydroxy phenyl radical) were CID products of these compounds. Peak 63 had extra characteristic fragment ion *m/z* 222 that is consistent with methyl group presence and thus tentatively described to be 4-*O*-caffeoyl-2-hydroxy-3-methylbutanoic acid (4-CMHBA). Therefore the peaks 18, 62 and 69 with retention times 11.2, 52.0 and 55.9 minutes have been characterised to be 4-*O*-caffeoyl-2-hydroxybutanoic acid (4-CHBA). These compounds are novel hydroxyl

cinnamic acid derivatives described for first time from *T. diversifolia* and nature. The epoxide derivative of this compound (3-((4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid (NK1F4)) was identified from this plant (Fig. 3.8 NK1F4), by Pantoja Pulido et al., (2017) and has been isolated as a pure compound and identified with $UV_{\lambda max}$ of 268 and 300nm (Appendix), characteristic of hydroxycinnamic acids and 1D ^{1}H and ^{13}C NMR data (Appedix). The CID of 3-(4-*O*-(caffeoyloxy)methyl)-2-methyloxirane-2-carboxylic acid gave products shown in Fig. 3.8 NK1F4, which were similar to those identified in the peaks 18, 62, 63 and 69 above. However, careful observation of MS/MS spectra of these novel compounds, showed no fragment series associated with terminal and nonterminal epoxides although the latter was indicated in NK1F4 (*m/z* 101, 115, 143). In nonterminal epoxides, trans annular cleavage with H transfer and elimination of an alkenyl radical to generate $C_nH_{2n+1}O$ fragments (*m/z* 45, 59, 73 …) occurs or γ-cleavage to generate $C_nH_{2n}O$ fragments (*m/z* 44, 58, 72 …) and alkene ($C_nH_{2n;}$ 28, 42, 56...) series for terminal epoxides (Badertscher et al., 2009). The compounds are therefore not epoxide derivatives of these new caffeic acids. The 1D NMR identification of pure compound NK1F4 together with the above fragmentationpatterns, enabled peaks 58, 65, 74 and 76 to be identified. Their spectra are indicated in Fig. 3.8D, E, F. Peak 58 displayed $[M+H]^+$ ion at *m/z* 391.2341 and $[M-H]^-$; 388.7108. Its MS^2 experiment in negative ion mode yielded fragments: *m/z* 134 [caffeoyl-$CO_2]^-$, 191 ($C_{10}H_9O_4^-$) ([M-H-197]$^-$), 280 [M-H-109]$^-$, 102 [HBA]$^-$. The proposed fragmentation mechanism involves both inductive and RDA cleavage in the positive ion mode (Fig. 3.8 D). This enabled identification of this peak as 3,4-dihydroxyphenyl-4-*O*-caffeoyl-2-hydroxybutanoate after addition of 109 Da of 3,4-dihydroxy benzene radical. Peak 65 ($[M+H]^+$; *m/z* 488.3314) displayed other MS^2 fragments; *m/z* 327([M+H-caffeoyl]$^+$, 375 and *m/z* 383 (RDA products through the dihydroxy benzene moiety with subsequent loss of 2OH in each) in addition to HBA moiety (102 Da), 193($C_{10}H_9O_4^-$), 296 [4-CMHBA +H]$^+$ and 282 [4-CHBA +H]$^+$ (See proposed fragmentation scheme in Fig. 3.8E). The fragment at *m/z* 80 involved RDA cleavage, addition of CH_3 (30 Da) and then loss of water (18 Da) through 1,3 McLafferty hydrogen rearrangement. Thus there was an extra 193 Da added to 295 Da of peak 63. This enabled its annotation as caffeoyloxymethyl-4-(caffeoyl)-2-hydroxy-3-methylbutanoate. Peak 74 with precursor ion *m/z* 399.2004 and MS/MS fragment ions; 282 [4-CHBA +H]$^+$, 296 [4-CMHBA +H]$^+$, 193 ($C_{10}H_9O_4^-$), 80 ($C_6H_6^-$) (via RDA through the vinylic ring), 124 ($C_7H_6O_2^-$) (via simple inductive cleavage followed by RDA through the dihydroxybenzene ring), 304 and 318 (formed after RDA cleavage through the vinylic ring and lose of OH for 304Da). This enabled identification as 4-vinylphenyl-4-*O*-caffeoyl-2-

hydroxy-3-methylbutanoate. Its fragmentation pattern is shown in Fig. 3.8F. The alkene ($C_nH_{2n;}$ 28, 42, 56...) series has been detected at *m/z* 84, 99, 113, and according to Badertscher et al., (2009) that supports the existence of the vinyl group in this compound. And lastly peak 64 was tentatively identified to be a 3,4-dihydroxyphenylacetic derivative of the 2-HBA above with the precursor protonated ion $[M+H]^+$; *m/z* 447.2933 and deprotonated molecular ion, $[M-H]^-$; 445.2911 The peak was identified as 4-*O*-caffeoyl-2-(2-(3,4-dihydroxyphenyl)acetoyloxy)-3-methylbutanoic acid from MS^2 ions of *m/z* 282 [4-CHBA $+H]^+$, 296 [4-CMHBA $+H]^+$, 149 and 167 are CID products of 3,4-dihydroxyphenylacetic acid, 399 $[M+H-CH_3-OH]^+$. The proposed fragmentation pathway is shown in Fig. 3.8H.

Peak 58 is proposed to be 3-caffeoyl-5-feruloylquinic acid (MS^2; 369, 355, 193, 179) because of the high intense peak at *m/z* 355 $[CQA +H]^+$ distinguished from 3-caffeoyl-4-feruloylquinic acid where the *m/z* 369 is more intense (Zhang et al., 2016) and base peak detected at *m/z* 369 [feruloyl quinic acid $+H]^+$ and the precursor ion peak at *m/z* 391. ($[M+Na]^+$, *m/z* 341 (caffeoyl glucoside), and *m/z* 201, 141 are other RDA ions. The MS^2 spectrum contained oxidative product ions at *m/z* 355, 369, 383 and 397 of the caffeoylquinic acid (Fig. 3.9).



O-caffeoylquinic acid
1. *m/z* 355 (2=6=CH_2)
2.*m/z* 369 (2=carbonyl,6=CH_2, or 2=CH_2 6=carbonyl)
3.*m/z* 383 (2=6=carbonyl)

Figure 3.9: Fragmentation of caffeoylquinic acid in positive ion mode

The oxidative product at *m/z* 355 is the protonated caffeoylquinic acid; *m/z* 369 represents carbonylated caffeoylquinic acid, *m/z* 383 is for dicarbonylated caffeoylquinicacid while *m/z* 397 appears to be a carbon monoxide derivative of the carbonylated caffeoylquinic acid (369) i.e, (369+ CO). The neutral elimination of the quinic acid moiety from *m/z* 355, carbonyl quinic acid moiety from *m/z* 369 and dicarbonyl quinic acid moiety from *m/z* 383 generated the product ion at *m/z* 163 according to dos Santos et al., (2005) and perhaps with appropriate ion, the adduct with mass ion *m/z* 169 is observed in the MS/MS spectrum. Peaks 25 and 27

at $[M-H]^-$; 255.1030 and $[M-H]^-$; 617.1912 respectively were identified to be phenylpropane glycerides. They had *m/z* 179, *m/z* 161, and *m/z* 135 that indicate the presence of a caffeoyl. MS^2 fragmentation ions of 255: *m/z* 179, m/z 161, and *m/z* 135 including the fragmentation ions *m/z* 179 $[M-H-74]^-$ and *m/z* 161 $[M-H-92]^-$ were characteristic loss of glycerol residue. So this compound must be an isomer of caffeoylglycerol. Based on literature (Ma et al., 2007), peak 26 corresponds to that of 1-*O*-caffeoylglycerol while peak 27 is itsderivative. Peaks 7 and 67 showed some similar characteristic ions of coumaroyl. Peak 7 is identified as para-coumaric acid in comparison with the retention time of the authentic standard and mass spectra (t_R = 4.4 min). It displayed *m/z* 163 ([coumaric acid-H]$^-$) whose MS^2 fragmentation yielded *m/z* 119 ([coumaric acid -CO_2]$^-$) and 75 (C_6H_5•). Peak 67 displayed fragment ions at *m/z* 307 (due to loss of malonic acid), and *m/z* 163, 145, and 119 (due to p-coumaroyl) and 249 (loss of hexose). This compound identified to be p-coumaroyl malonylhexoside using database search and literature (Metlin).

3.4.2.1.3 Characterisation of other hydroxyl compounds

Four other hydroxyl containing compounds such as quinic acid and glucaric acid were detected at peaks 5, 49, 51 and 70. Quinic acid (t_R = 3.6 min) displayed deprotonated ([quinic-H]$^-$) ion at *m/z* 191 in peak 5. Loss of water (18Da) from quinic moiety gave *m/z* 173([quinic-H-18]$^-$). MS^2 fragment ion at *m/z* 127 corresponds to the quinic acid residue; [quinic acid-CO-$2H_2O$]$^-$ (Alakolanga et al., 2014). Peaks 49, 51 and 70 were identified as glucaric acid based on the characteristic ions *m/z* 191, 147, and 85 (Simirgiotis et al., 2009).

3.4.2.2 Identification of flavonoids and other polyphenols

In this study, twelve flavonols (peaks 10, 11, 14, 17, 30, 31, 35, 40, 48, 66, 73 and 77), five flavones (peaks 8, 9, 44, 45 and 71), two flavanones (peaks 28 and 36), four flavanols (peak 12, 33, 34 and 56) and two unknown flavonoids (peaks 6, and 15), one saponin triterpenoid (peak 21) were detected. The flavonoid derivative and other polyphenol (saponin triterpenoids) were identified by loss of 1 or more sugar groups such as rutinoside (308Da), hexoside (glucoside or galactoside, 162Da), caffeoyl (162Da), rhamnoside (146 Da), pentoside (arabinoside or xyloside, 132 Da), coumaroyl (146Da) and glucuronic acid (176 Da) to produce the corresponding aglycone. Due to the successive elimination of small molecules such as one or more water molecules, CO e.t.c. and retro-Diels-Alder (RDA) reaction, aglycone fragment ions are formed. The glucosides under the same category could share identical aglycones such as quercetin, kaempferol and this enabled identification of

flavonoid derivatives. The same could apply to other polyphenolic compounds (saponins, coumarins and furanocoumarins). These could exhibit abundant MS^2 data with appropriate collision energy in positive and negative ionisation modes. One group is for the aglycone fragment ions such as hederagenin and the other is for many sugar moieties linking to aglycone. Two, three, four, five, or six sugars such as glucose (Glc), rhamnose (Rha), arabinose (Ara) or xylose (Xyl) (132Da) could link up to form ions with several fragment ions. For example two or three sugar moieties form ions like *m/z* 279 ($[Rha + Ara + H]^+$), *m/z* 295 ($[Glc + Ara+ H]^+$), *m/z* 309 ($[Glc + Rha + H]^+$), *m/z* 325 ($[Glc + Glc+ H]^+$), *m/z* 441 ($[Glc + Rha + Ara + H]^+$), *m/z* 471 ($[Glc+ Glc + Rha + H]^+$), and *m/z* 487 ($[Glc + Glc + Glc + H]^+$), four *m/z* 603 $[Glc + Glc + Rha + Ara + H]^+$, *m/z* 633 [Glc + Glc + Glc + Rha + H]+, *m/z* 649 [Glc + Glc + Glc + Glc + H]+), could be formed. Five sugar molecules; *m/z* 765[Glc + Glc + Glc + Rha + Ara + H]+, *m/z* 795 [Glc + Glc + Glc + Glc + Glc + Rha + H]+), and six; *m/z* 956 [Glc + Glc + Rha + Glc + Glc + Glc + H]+) sugar moieties. Using this information together with mass defect theory (where an approximate 0.05 Da increase after addition of sugar moiety occurs), it was possible to some extent identify the nature of the aglycone from some ions consisting of four, five and six sugar moieties. Thus those with six sugars are expected to have ions like *m/z* 1055.8701, 1103.7688, 907.6294, 914.2336, five sugars; 711.4149, 740.6864, 739.2314, 756.2996, 866.9083, 893.5775, 893.5775 and four or three sugars; *m/z* 658.4421 in positive ion mode ($[M + H]^+$ ion).

3.4.2.2.1 Characterisation of flavonols

Twelve flavonol peaks; 10, 11, 14, 17, 30, 31, 35, 40, 48, 66, 73 and 77) could be detected. Peak 10 eluted at 5.6 min was identified to be kaempferol-dihexoside; $[M-H]^-$; 609.1452. MS^2 experiment gave high-intensity peak ions at *m/z* 447 [M-H-hexoside]$^-$, 285 [M-H-hexoside-hexoside]$^-$ and 255 [kaempferol-H- CH_2OH(30Da)]$^-$ as supported by literature (Gouveia and Castilho, 2010). Thus peak 17 was identified as kaempferol rutinosyl dirhamnoside in agreement with annotation by Bresciani et al., (2015). Peaks 30 and 31 identified with similar retention times, 20.4 minutes shows that the compound is the same detected in both positive and negative ionization modes. Peak 31 with fragment ion at $[M-H]^-$; *m/z* 739.2314 displayed MS^2 fragment ions *m/z* 593 [M-H- rhamnoside]$^-$, 575 [M-H- 2 glucose-2H]$^-$, 285 [M-H-2rhamnoside-glucoside]$^-$. According to this study and literature, (Hasler et al., 1992) this loss sequence is similar to that of kaempferol dirhamnosyl glucoside. Peak 40 displayed $[M+H]^+$; 1080.7521. Its MS^2 produced fragment aglycone at *m/z* 285 of

the kaempferol after the loss of *m/z* 795 and also loss of 4glucosyl residue (648Da) and a rhamnosyl (146Da) in positive ion mode. The molecule can tentatively be described as kaempferol tetra glucosylrhamnoside. Peak 71 was identified as a kaempferol derivative whose MS^2 fragments; 285 $[Y_0]^-$, 257 $[Y_0\text{-}2CO]^-$, 202 $[Y_0\text{-}CO]^-$, 155 $[^{1,3}A]^-$, (formed through retrocyclization cleavage of the C-ring of the aglycone involving 1 and 3 bond) (bonds 1 and 3 refer to the O—C-2 and C-3—C-4 bonds of the C-ring) (ma et al, 1997). This fragmentation pattern shows that of a kaempferol aglycone (Tsimogiannis et al., 2007, Ma et al., 1997). Peaks 11, 14, 34, 48, and 64 were identified to be quercetin derivatives. Peak 11 with $[M\text{-}H]^-$; 1055.8701 gave MS^2 fragmentation ions *m/z* 755 due to the loss of fragment ion at *m/z* 301 (quercetin aglycone). MS^2 formed fragments *m/z* 609, 343, 301 by loss of 146Da and 308 Da. When compared with literature (Simirgiotis et al., 2009), this compound is tentatively identified as quercetin dirhamnosyl arabinosylsophoroside. Peak 14, $[M\text{-}H]^-$; 893.5775 gave MS^2 fragments *m/z* 548, 293, 301, 255, 271, 179, 44. The fragment ion *m/z* 293 is due to [M-H-hexoside-pentoside]$^-$, carbon dioxide (44Da), *m/z* 301 [quercetin-H]$^-$, *m/z* 271 [quercetin-CH_2O-H]$^-$, 255 [quercetin - CHO - OH]$^-$ and 179 ([M-H-OH-B ring]$^-$) product ions of the quercetin (Ma et al., 1997). The ion *m/z* 548 is formed after loss of 345 Da [M-H-quercetin - CO_2]$^-$. This compound is tentatively identified as adduct of quercetin pentosylhexoside and quercetin. Peak 34, 48 and 64 were identified to be quercetin-3-*O*-(2,6-di-*O*-rhamnosylglucoside), quercetin-7-*O*-hexosyl-3-*O*-(malonyl) hexoside and quercetin-3-*O*-rhamnoside having $[M\text{-}H]^-$; 756.2998, $[M+H]^+$; 711.4149 and $[M\text{-}H]^-$; 447.4021 respectively. The parent ion for peak 48 (*m/z* 711.4149) had fragments at *m/z* 667 and 301 corresponding to a neutral loss of CO_2 (44), *m/z* 667 [M-H-CO_2] and a quercetin aglycone or *m/z* 301 i.e. [M-H-hexose-malonyl- hexose]$^-$. MS^2 fragment of *m/z* 367 (667-367=300) is due to the loss of quercetin aglycone radical. The MS^2 fragmentation ions at *m/z* 151 $[^{1,3}A^-]$ (due to C-ring degradation by fission of the 1,3 bond to produce the RDA fragment $[^{1,3}A^-]$ of the deprotonated molecule), 179 ([M-H-OH-B ring]$^-$), 255 ([M-H-H_2O-CO]$^-$) and 271 ([M-H-CH_2O]$^-$), are a result of RDA cleavage consistent with *m/z* ion 301 of quercetin (Tsimogiannis et al., 2007; Ma et al., 1997). Therefore this compound is tentatively identified as quercetin-7-*O*-hexoside-3-*O*-(malonyl) hexoside consistent with literature data of Gouveia and Castilho et al., (2010). The peak at $[M\text{-}H]^-$; 447.4021 corresponded to that of quercetin-3-*O*-rhamnoside because of the formation of characteristic *m/z* 301 from MS^2 fragmentation of *m/z* 447 in which a rhamnosyl moiety was lost. Further MS^2 fragmentation of *m/z* 301 yielded *m/z* 271, 255 product ions of the quercetin in agreement with the literature (Ma et al., 1997, Ye et al., 2005).

3.4.2.2.2 Characterisation of flavones

Peaks 8 and 9 with retention time 4.6 minutes in both positive and negative ion modes point to that of apigenin. Chemical inductive dissociation of apigenin yielded *m/z* 225 [apigenin -CO_2]$^-$, and 201 [apigenin -C_3O_2]$^-$. This fragmentation pattern is consistent with the literature about the standard solution of the flavone apigenin (Ye et al., 2005). Peaks 44 ([M + H]$^+$; 975.8624), displayed ions *m/z* 358, 271. The ion at *m/z* 358 was due to the consecutive loss of two glucuronyl (176×2 Da) and two pyranosyl (132×2 Da) in positive ion mode. The ion at *m/z* 271 was due to apigenin aglycone. MS^2 [271]: 227 [apigenin -CO_2]$^+$, 203 [apigenin -C_3O_2]$^+$ and 153 [$^{1,3}A^+$] consistent with apigenin fragmentation ion in positive ionisation mode. MS^2 fragmentation of *m/z* 358 could have formed *m/z* 193 in which its ions *m/z* 178, 149, 134 consistent with CID of ferulic acid thus a feruloyl moiety. The compound is tentatively identified as apigenin-*O*-feruloyl-*O*[glucuronopyranosyl]-*O*-glucuronopyranoside. Peak 45 was identified as 3^1,4^1-dimethylluteolin with CID product ions 135 [$^{1,3}B^+$], 153[$^{1,3}A^+$], 243 ([M+H-H_2O-CO]$^+$). Peak 71, [M+H]$^+$; 668.7289 had MS^2 fragment at *m/z* 493 (after the loss of glucuronic acid moiety (176Da)) and 331 after the loss of glucose residue (162Da). The ion at *m/z* 331 belongs to tricin aglycone [tricin+H]$^+$. This compound is tentatively identified as tricin-*O*-glucuronopyranosyl-(1-2)-*O*-glucopyranoside when compared with database search (Metlin).

3.4.2.2.3 Characterisation of flavanones

Peaks 26 and 36 were identified as flavanones. Peak 28 displayed [M+H]$^+$; 907.6294 with MS^2 fragments *m/z* 618 [M+H- eriodictoyl]$^+$, 289 [eriodictoyl+H]$^+$ due to [M+H- 618]$^+$. The fragments *m/z* 618 is an indication of the loss of three hexose sugars and one pentose sugar i.e. trihexosyl pentoside. The peak is thus identified as eriodictoyl trihexosyl pentoside. Peak 36 with parent ion [M-H]$^-$; 271.0616 was identified as naringenin from its MS^2 characteristic fragment ions *m/z* 227 [M-H-CO_2]$^-$, 177 [M-H- C_3O_2- CO]$^-$, 165 ([M-H]$^-$ retrocyclisation to form $C_9H_{10}O_3$), 151 (RDA cleavage through C ring), 125, 107(151-CO_2) (Xu et al., 2009)

3.4.2.2.4 Characterisation of flavanols

Peak 34 is identified as (epi)catechin gallocatechin-C- hexoside from its parent ion, [M-H]$^-$; 756.2996. Its MS/MS experiment yielded *m/z* 505 [M-H-hexoside-90]$^-$, 450 [M-H-epigallocatechin (305Da)]$^-$, to give epicatechin hexoside (451Da), 287 [epicatechin-H]$^-$. The ion at *m/z* 287 represents that of isotopic epicatechin. The peak at 56 having a parent ion

$[M+H]^+$; 466.2556 was identified as gallocatechin hexoside with the gallocatechin at *m/z* 305 after losing a hexoside (161Da). Peak 12 was identified as a B-type proanthocyanidin trimer, due to pseudomolecular $[M+H]^+$; ion at *m/z* 866.9083, which yielded MS^2 ions at *m/z* 739 from cleavages of heterocyclic ring fission of ring C of one pyran ring losing 127 Da; 695 $[M+H-170]^+$, 408 $[M+H-695-170]^+$ and 287((epi)catechin); compared with standards, NIST data base and previous work (Sun et al., 2007). The fragments 695 and 408 are due to RDA of the heterocyclic ring and loss of H_2O. The sequence in this trimer was identified as (epi)catechin-(epi)catechin-(epi)catechin due to loss of 288 amu. Peak 33, $[M+H]^+$; 739.3146 was identified as sugar moiety of proanthocyanidin dimer i.e. (epi)catechin-4,8′-(epi)catechin-C-hexoside) (Metlin and MassBank)

3.4.2.2.5 Characterisation of coumarin and furanocoumarins

Eight coumarins and furocoumarins (peaks, 2, 3, 16, 20, 22, 37, 38, 39, 41, and 42) were identified. Peak 2 with $[M+H]^+$; 335.1832; was identified as byakangelicin. This furanocoumarin displayed MS/MS fragment ions *m/z* 179, 225, 173, 149, 147, 103, 299, 265, 221, 215, 205 and 133. Its fragmentation involves simple inductive cleavage and RDA cleavage through the furano and lactone rings as illustrated in Fig.3.10, which is in agreement with Dugrand et al., (2013) report. Peak 3 was identified as 5,7-dihydroxy-4-methylcoumarin. It displayed main fragments *m/z* 177 $[M+H-CH_3]^+$, 148 $[M+H-CO_2]^+$, 131, 162 $[M+H-OH-CH_3]^+$, 157 $[M+H-2OH]^+$. Peak 16 was proposed to be as 5-methoxy-8-hydroxypsoralen with protonated ion $[M+H]^+$; 232.8862 (Yang et al., 2010). The fragment at *m/z* 190 was formed from RDA cleavage of the furano ring losing $C_2H_2O^-$ fragment ion. Further loss of the acetylene ($C_2H_2\bullet^+$) in the lactone ring generated *m/z* 166 fragment. The isotopic ion at *m/z* 151 was formed after successive loss of acetylenyl in the furano ring followed by the loss of $C_3H_2O\bullet^+$ in the lactone ring in a retro DA cleavage manner. Peak 20 was proposed to be that of the coumarin derivative, dimethyl coumarin due to the addition of 30Da to *m/z* 146 for coumarin. Peaks 38 and 42 were identified to be two isomeric furanocoumarins; xanthotoxol (8-hydroxypsorale) and bergaptol respectively (Yang et al., 2010).

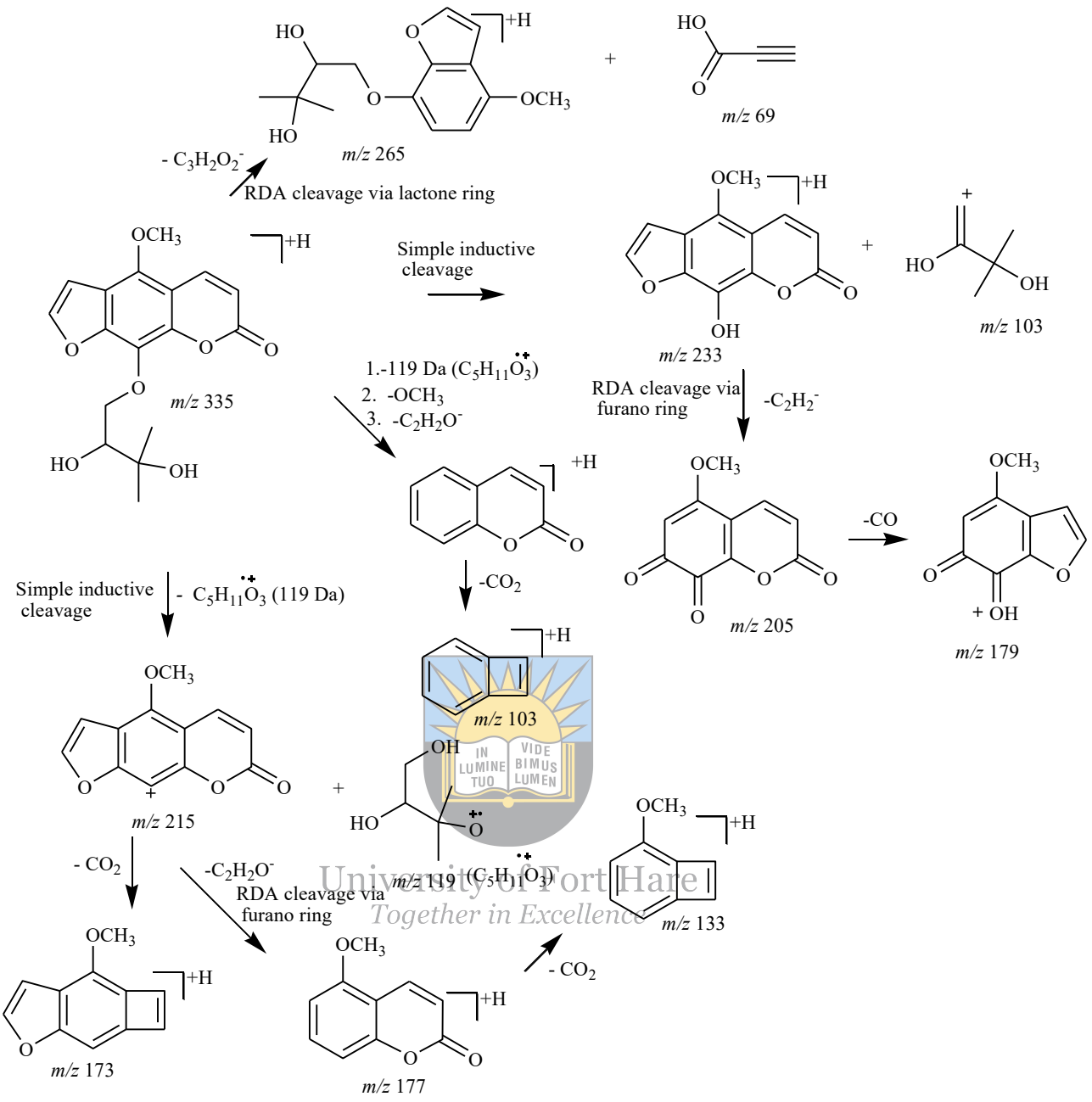


Figure 3.10: Proposed fragmention scheme for byakangelicin in positive ion mode

CID of xanthotoxol yielded products; *m/z* 141 $[M+H- CO_2 – H_2O]^+$, 133 $[M+H- C_3H_2O•^+]^+$ (via retro DA cleavage in the lactone ring), 118($C_8H_5O•^+$) $[M+H- C_3H_2O•^+- OH]^+$ while bergaptol yielded MS/MS *m/z* 133 $[M+H- C_3H_2O•^+]^+$, 124 $[M+H- CO_2 -H_2O-O]^+$, 118 $[M+H- C_3H_2O•^+- OH]^+$, 179 $[M+H-^{13}CO]^+$ and *m/z* 149 $[M+H-2^{13}CO]^+$ (via retro DA cleavage in the lactone ring leading to the loss of $C_3H_2O•^+$, distinguishing it from 8-hydroxypsorale). Peak 41 was identified as leptophyllin (Yang et al., 2010). with $[M+H]^+$; 263.8508 and MS^2 fragments *m/z* 141 $[M+H- CO_2 – H_2O]^+$, 237 $[M+H- C_2H_2^-]^+$ (via retro DA in the lactone ring), 194 $[M+H- C_2H_2^- - CHO_2]^+$, 137 $[M+H- C_2H_2^- - CHO_2 - C_3H_7O]^+$ (or through RDA cleavage in the aromatic ring), 217 $[M+H- CO_2]^+$, 182 $[M+H- C_{10}H_{10}O_4$ -2

$CH_3]^+$ (successive loss via retro DA occurred in the lactone ring followed by loss of 30Da). Peak 37 was identified as xanthotoxol but the precursor ion was its ammonium adduct $[M+NH_4]^+$ (Yang et al., 2010). Peak 22 was considered as 5,7-dihydroxy-4-methylcoumarin derivative due to the precursor ion fragment at *m/z* 191 for 5,7-Dihydroxy-4-methylcoumarin and *m/z* 144 for coumarin and lastly peak 39 formed MS/MS fragments *m/z* 137, 121, 153, 109, 116 and considered to be a coumarin residue with *m/z* 121 fragment resulting from the retro DA reaction through the lactone ring in which the $C_2H_2O^-$ is lost.

3.4.2.2.6 Characterisation of other polyphenols

One saponin triterpenoid (peak 21), $[M+H]^+$; 914.2336. Peak 19 displayed ions *m/z* 565, 473, 453, 437, 409, 309, 279, 147. The ion *m/z* 279 is a sugar sequence, showing how glucose and an arabinose/xylose are linked to the aglycone. The ions *m/z* 473 455, 437, 409 are characteristic fragment ions of hederagenin aglycone (Shi et al., 2014). The ion *m/z* 147 was generated from the rhamnoside in positive ion mode. The fragmentation sequence of this compound is comparable to that of 3-*O*-α-L-arabinopyranosyl(2→1)-*O*-α-L-rhamnopyranosyl-hederagenin-28-*O*-*β*-D-glucopyranosyl ester (Shi et al., 2014).

3.4.2.2.7 Other compounds and unknowns

One fatty acid compound (peak 50) with deprotonated precursor ion $[M-H]^-$; 327.9418 was identified to be oxo-dihydroxy-octadecenoic acid consistent with Spínola et al, (2014). In addition, unknown flavonoid peaks were detected in peaks 6 and 15. Peak 6 with $[M+H]^+$; *m/z* 1103.7688 could display fragment *m/z* 815 due to loss of *m/z* 289. The MS^2 of *m/z* 1103 was *m/z* 653 and MS^2 of *m/z* 653 formed *m/z* 345 after the sequential loss of 2 glucosyls (162×2 Da) and one rhamnosyl units. The MS^2 of *m/z* 289 and 345 could not display aglycone fragment ions. This, therefore, is an unknown diglucosylrhamnosyl flavonoid. Similarly, peak 15, $[M-H]^-$; 750.5167 was considered to be an unknown rhamnosyl flavonoid. Several unknown peaks (1, 13, 25, 32, 46, 52, 55 and 75) were also detected.

To sum up, a total of 76 peaks (Table 3.6) could be detected with 55 well characterised phenolic compounds. These include; 4 hydroxybenzoic acids, 19 hydroxycinnamic acids and their derivatives, 12 flavonols, 5 flavones, 2 flavanones, 4 flavanols, 1 saponin triterpeneoid and 9 coumarins and furanocoumarins. The other peaks were 4 unknown hydroxycinnamic acid derivative, 2 unknown flavonoids, unknown coumarin metabolite, 4 other hydroxyl

compounds that included quinic acids and glucaric acids, one fatty acid compound and nine unknown peaks.

Table 3.6: Identification of phenolic compounds using UHPLC-ESI- QTOF- MS/MS

Peak	t_R (m)	UV(nm)	Proposed ion; Experimental *m/z*	MF	*m/z* of main fragment(s) (intensity, % of precursor ion) of MS/MS	Tentative identification	Fraction
1	0.9	271	$[M+H]^+$; 379.7831	$C_{16}H_{12}O_{11}$	327(11), 295(25), 274(16)	Unknown	F1
2[b]	2.7	278	$[M+H]^+$; 335.1832	$C_{17}H_{18}O_7$	335(96)179(96), 173(104), 149(64), 147(72), 103(48), 299(49), 265(52), 221(81), 215(75), 205(72), 133(130)	byakangelicin	F1
3[b]	3.0	277	$[M+H]^+$; 192.3243	$C_{10}H_8O_4$	157(41), 177(58), 148(57), 131(31), 162(25)	5,7-Dihydroxy-4-methylcoumarin	F2
4[b]	3.3	300	$[M+H]^-$; 223.9371	$C_{11}H_{12}O_5$	133(53), 123(12), 181(12), 183(4)	Sinapic acid	F1
5[b]	3.6	265	$[M-H]^-$; 190.6290	$C_7H_{12}O_6$	127(70), 85(34), 173(56), 93(2)	Quinic acid	F1, F2
6	4.2	257, 368	$[M-H]^-$; 1103.7688	$C_{45}H_{61}O_{33}$	653(11), 345(24)	Unknown diglucosylrhamnosyl flavonoid	F2
7[a]	4.4	275, 308	$[M-H]^-$; 163.0398	$C_9H_8O_3$	119(50), 75(26)	p-coumaric acid	F1, F2
8[b]	4.6	290, 310	$[M-H]^-$; 269.0928	$C_{15}H_{10}O_5$	225(56), 227(25), 201(23), 151(5), 149(3)	Apigenin	F1, F5
9[b]	4.6		$[M+H]^+$; 271.0616	$C_{15}H_{10}O_5$	171(32), 221(22), 181(21), 199(5)	Apigenin	F2
10[b]	5.6	254, 414	$[M-H]^-$; 609.1452	$C_{27}H_{30}O_{16}$	447(100), 285(16), 284(85), 327(10), 255(8)	Kaempferol dihexoside	F2
11[b]	8.0	287, 352	$[M-H]^-$; 1055.8701	$C_{44}H_{61}O_{31}$	755(78), 301(100)	Quercetin dirhamnosyl arabinosylsophoroside	F2
12[b]	8.2	282, 329	$[M-H]^-$; 866.9083	$C_{45}H_{35}O_{18}$	739 (100), 695 (40), 677(12), 408(8), 547 (70), 455 (56)	Proanthocyanidin trimer ((epi)catechin- (epi)catechin-(epi)catechin)	F2
13[b]	8.7	275	$[M+H]^+$; 374.3900	$C_{22}H_{29}O_5$	374(100), 181(12), 193(10)	CQA derivative	F2, F5
			$[M-H]^-$; 372.4109		179(48), 191(17)	CQA derivative	
14[b]	9.8	255, 352	$[M-H]^-$; 893.5775	$C_{41}H_{38}O_{23}$	548(107), 293(24), 44(10), 151(13), 255(43), 179(10)	Adduct of quercetin pentosylhexoside and quercetin	F2
15	10.3	354	$[M-H]^-$; 750.5167	$C_{46}H_{70}O_8$	605(100)	Unknown rhamnosyl flavonoid	F1
16[b]	10.6	277	$[M+H]^+$; 232.8862	$C_{12}H_8O_5$	151(32), 182(32),166(32), 190(32), 83(41)	5-Methoxy-8-hydroxypsoralen	F2
17[b]	11.0	328, 416	$[M+H]^+$; 886.4406	$C_{51}H_{65}O_{13}$	869(23),737(71), 591(14), 429(8), 411(2), 287(2), 269(3), 357(3), 627(2), 133(1), 463(1), 413(1), 160(1), 763(1), 574(1), 186(1)	Kaempferol-rutinosyl-dirhamnoside	F1
18[c]	11.2	268, 300	$[M+H]^+$; 282.0715	$C_{13}H_{13}O_7$	133(43), 175(23), 179(20), 193(11), 265(10)	4-*O*-Caffeoyl-2-hydroxybutanoic acid (4-CHBA)	F1
19[b]	13.8	288, 325	$[M-H]^-$; 395.5144	$C_{18}H_{21}O_{10}$	375(17), 351(22), 335(30), 327(11), 295(25), 274(16)	3-Acetyl-5- CQA	F5
20[b]	14.1	274	$[M+H]^+$; 175.8471	$C_{11}H_{10}O_2$	125(32), 144(32), 147(33), 151(32)	Dimethyl coumarin	F2
21[b]	14.1	288, 351	$[M+H]^+$; 914.2336	$C_{47}H_{78}O_{17}$	565(76), 473(43), 453(23), 437(13), 409(10), 309(9),	3-*O*-Arabinopyranosyl-*O*-rhamnopyranosyl-	F1

					279(23), 147(2)	hederagenin-28-*O*-β-D glucopyranosyl ester	
22[b]	14.3	277, 311	$[M+H]^+$; 314.6463	$C_{12}H_8O_7$	242(72), 130(48), 285(40), 144(40), 191(40), 91(48)	5,7-Dihydroxy-4-methylcoumarin derivative	F1
23[b]	14.5	268, 329	$[M+H]^+$; 355.0873	$C_{16}H_{18}O_9$	137(28), 109(10), 193(8), 165(13), 123(5), 81(4),179(3), 151(2), 236(2)	4-*O*-CQA	F2
		268, 329	$[M-H]^-$; 353.0147	$C_{16}H_{18}O_9$	173(77), 179(35), 191(10) ,135(10)	4-*O*-CQA	F2
24[b]	16.3	266	$[M-H]^-$; 187.1408	$C_7H_8O_6$	125 (75), 169(12)	Gallic acid monohydrate	F5
25[b]	17.3	290, 309	$[M-H]^-$; 617.1912	$C_{30}H_{34}O_{14}$	306 (59), 288 (14), 272 (10), 160 (18), 210 (35), 179 (98), 161 (34), 135(30)	Caffeoyl glycerol derivative	F1
26[b]	18.5	254,348	$[M+H]^+$; 907.6294	$C_{39}H_{55}O_{25}$	618 (59), 288 (14), 289 (10)	Eriodictoyl trihexosyl pentoside	F2
27[b]	19.3	266, 326	$[M-H]^-$; 255.1030	$C_{11}H_{12}O_7$	253(75), 179(32), 181(29), 180(3), 163(15), 162(13), 161(20), 137(49), 136(32), 135(78)	1-*O*-Dihydrocaffeoylglycerol	F1
28[b]	20.1	300, 326	$[M-H]^-$; 707.6523	$C_{39}H_{32}O_{13}$	179(87), 119(32), 149(12), 434(11), 496(5)	CQA dimer	F2
29[b]	20.4	351,414	$[M-H]^-$; 737.2314	$C_{33}H_{40}O_{19}$	593(23), 285(43)	Kaempferol dirhamnosyl glucoside	F1
30[b]	20.4		$[M+H]^+$; 739.2314	$C_{33}H_{40}O_{19}$	595 (34), 287(54)	Kaempferol dirhamnosyl glucoside	F2
31	20.6	277	$[M+H]^+$; 990.2627	$C_{56}H_{45}O_{17}$	314(32), 397(32), 461(33), 693(32), 177(32), 815(32)	Unknown	F2
32	22.5		$M-H]^-$; 241.1070	$C_{12}H_{17}O_5$	241(3), 204(5), 141(2), 123(1), 121(1)	Unknown	F5
33[b]	24.0	282, 329	$[M+H]^+$; 739.3146	$C_{43}H_{47}O_{11}$	222(49), 600(49), 324(48), 616(48), 590(48), 181(48), 318(42)	Proanthocyanidin dimers ((epi)catechin-4,8′-(epi)catechin C-hexoside)	F1
			$M-H]^-$; 737.1145		221(25), 324(23), 598(12), 289(4)		F1
34[b]	25.7	283, 329	$[M-H]^-$; 756.2996	$C_{36}H_{36}O_{18}$	505 (40), 450 (27), 449 (101), 287 (67)	(Epi)catechin gallocatechin hexoside	F2
35[b]	25.9	287, 352	$[M-H]^-$; 756.2998	$C_{33}H_{40}O_{20}$	253(34), 301(24), 365(14), 310(14), 388(11), 187), 181(4), 184(3), 156(1), 139(1)	Quercetin-3-*O*-(2,6-di-*O*-rhamnosylglucoside)	F2, F3, F4
36[b]	27.5	254, 412	$[M-H]^-$; 271.0616	$C_{15}H_{12}O_5$	227(2), 177(20), 165(8), 151(100), 125(5), 107(3)	Naringenin	F1
37[b]	28.1	277	$[M+NH_4]^+$; 220.4131	$C_{11}H_8O_5$	131(120), 173(34), 175(91), 194(108), 195(50)	Xanthotoxol	F2, F3, F4
38[b]	29.2	275	$[M+H]^+$; 202.8380	$C_{11}H_6O_4$	141(32), 192(32), 197(33), 133(24), 118(25), 125(25)	Xanthotoxol (8-hydroxypsorale)	F1
39	29.7	274	$[M+H]^+$; 173.8972	$C_{10}H_5O_3$	137(65), 121(58), 153(54), 109(56), 116(32)	Unknown	F1
40[b]	31.5	351,414	$[M+H]^+$; 1080.7521	$C_{45}H_{60}O_{30}$	285(57), 795(21), 810(10), 146(23)	Kaempferol tetraglucosylrhamnoside	F1
41[b]	31.7	274	$[M+H]^+$; 263.8508	$C_{14}H_{14}O_5$	141(40), 238(40), 194(32), 237(34), 182(32), 147(32), 217(34), 137(33)	Leptophyllin	F2
42[b]	31.8	272	$[M+H]^+$; 202.8820	$C_{11}H_6O_4$	133(55), 149(40),179(32), 192(56), 124(32), 118(56)	Bergaptol	F2
43[b]	36.1	303	$[M-H]^-$; 370.5113	$C_{15}H_{16}O_{11}$	210 (47), 209 (106), 191 (33)	Caffeoylglucaric acid	F1, F3, F4
44[b]	36.9	288, 351	$[M+H]^-$; 975.8624	$C_{43}H_{42}O_{26}$	358(111), 271 (59), 227(23), 203(12), 153(12) and 151(34), 193(13), 178(67), 149(78), 134(13)	Apigenin-*O*-feruloyl-*O*[glucuronopyranosyl] -*O*glucuronopyranoside	F1
45[b]	37.5	254, 348	$[M+H]^+$;	$C_{17}H_{14}O_6$	259(8), 135(6), 93(5),	3^1,4^1-Dimethyluteolin	F1, F5

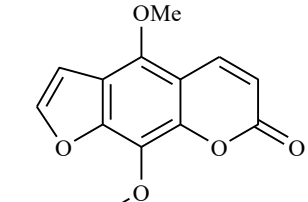
			315.1890		235(4), 153(4), 221(1), 181(1), 231(1)		
46	40.3		[M-H]⁻; 327.2086	$C_{21}H_{28}O_3$	327(56), 127(22), 109(12)	unknown	F1, F5
47[b]	40.6	265	[M-H]⁻; 345.2170	$C_{14}H_{16}O_{10}$	201(38), 127(14), 327(8), 145(3), 109(2)	Theogallin (galloylquinic acid)	F5
48[b]	40.8	272, 345	[M+H]⁺; 711.4149	$C_{30}H_{32}O_{20}$	667(37), 367(236), 151(34), 179(22), 255(23), 271(12)	Quercetin-7-*O*-hexosyl-3-*O*-(malonyl)hexoside	F5
49[b]	41.3		[M-H]⁻; 209.2988	$C_{10}H_9O_5$	191 (98), 147(37) 85 (34)	Glucaric acid	F1
50[b]	43.2		[M-H]⁻; 327.9418	$C_{18}H_{32}O_5$	259(25), 113(15), 315(12), 141(10), 200(4), 220(4), 265(4), 293(4), 277(3), 307(2)	Oxo-dihydroxy-octadecenoic acid	F1
51[b]	44.4		[M-H]⁻; 209.3110	$C_{10}H_9O_5$	127(37), 192(28), 134(16), 177(15), 184(3)	Glucaric acid	F2
52	47.3		[M+H]⁺; 805.8614	$C_{55}H_{112}O_2$	639(53), 806(111)	Unknown	F1, F3, F4
53[c]	47.6	300	[M-H]⁻; 502.3093	$C_{21}H_{27}O_{14}$	327(86), 113(23), 341(21), 201(17), 141(17), 127(10)	4-*O*-(1-*O*-caffeoylglucosyl)quinic acid	F5
54[b]	47.7	257, 294	[M+H]⁺; 155.970	$C_7H_6O_4$	111(49)	Protocatechuic acid	F1, F2, F5
		257, 294	[M-H]⁻; 153.1970	$C_7H_6O_4$	111(37)	Protocatechuic acid	F1
55	47.9		[M+H]⁺; 423.2522	$C_{20}H_{38}O_9$	423(17), 386(1)	unknown	F5
56[b]	48.1	262, 294	[M+H]⁺; 466.2556	$C_{14}H_{27}O_7$	305(50), 327(30),105(25), 127(6)	Gallocatechin hexoside	F5
57[b]	48.8	263	[M-H]⁻; 535.2920	$C_{33}H_{42}O_6$	535(48), 373(13), 343(42), 313(12), 211(10)	Gallotannins (dimethyl galloyl quinic acid-C-hexoside?)	F5
58[c]	49.4	282, 311	[M+H]⁺; 391.2341	$C_{19}H_{18}O_9$	282(12), 296(11), 149(9), 193(14), 279(8), 304(5), 305(3), 318(8), 319(3) 102(43), 124(3), 125(2), 84(3), 376(2)	3,4-dihydroxyphenyl-4-*O*-caffeoyl-2-hydroxybutanoate	F4, F3
			[M-H]⁻; 388.7108		280(56), 294(44), 193(34), 316(11), 121(2), 102(34)		F2
59[b]	49.7	328	[M+H]⁺; 530.3372	$C_{26}H_{26}O_{12}$	355(58), 113(20), 141(14), 201(6), 369(9), 341(9), 327(5), 193(4), 179(3), 517(9)	Caffeoyl-5-feruloylquinic acid	F5
60[b]	50.5	300	[M-H]⁻; 494.2839	$C_{23}H_{25}O_{12}$	305(34), 353(33), 333(29), 105(25), 127(3), 155(2)	CQA derivative	F5
61[b]	51.4	300	[M+H]⁺; 558.4421	$C_{27}H_{26}O_{13}$	355(78), 141(22), 169(20), 369(17), 397(9), 383(9)	Acetyl diCQA	F5
			[M-H]⁻; 557.0421	$C_{27}H_{26}O_{13}$	353(67), 395(7), 367(15)	Acetyl diCQA	F2
62[c]	52.0	268, 300	[M+H]⁺; 282.0733	$C_{13}H_{13}O_7$	137(28), 108(10), 193(8), 165(13), 123(5), 81(4),179(3), 151(2), 236(2), 267(6)	4-CHBA	F1, F3, F4, F5
			[M-H]⁻; 281.0011		135 (33), 179(10), 265(5)		F3
63[c]	52.0	268, 300	[M+H]⁺; 296.0872	$C_{14}H_{16}O_7$	137 (20), 109(6), 193(5), 165(8), 123(6), 81(3), 179(3), 267(5), 236(1), 222(1), 103(3)	4-*O*-Caffeoyl-2-hydroxy-3-methylbutanoic acid (4-CMHBA)	F3, F4, F5
			[M-H]⁻; 293.5544		135 (33), 109(7), 191(7), 163(8), 179(3), 265(5), 102(2)		F4
64[c]	52.9	300	[M+H]⁺; 447.2933	$C_{30}H_{39}O_3$	149(100), 167(16), 309(15), 141 (23.8), 282(115), 296(91), 103(17)	4-*O*-Caffeoyl-2-(2-(3,4-dihydroxyphenyl)acetoyloxy)-3-methylbutanoic acid	F3, F4

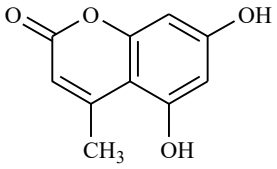
			[M-H]⁻; 445.2916		149(100), 167(16), 307(15), 141 (23.8)		F3
65[c]	53.0	301	[M+H]⁺; 488.3314	$C_{24}H_{24}O_{11}$	102(44), 193(27), 282(11), 296(10), 327(12), 375(5), 383(3)	4-*O*-Caffeoyl-caffeoyloxymethyl-2-hydroxy-3-methylbutanoate	F5
			[M-H]⁻; 486.5367		191(37), 294(14), 327(12), 373(2), 380(3)		F2
66[b]	53.1		[M-H]⁻; 447.4021	$C_{21}H_{20}O_{11}$	301(79), 271(13), 255(11)	Quercetin-3-*O*-rhamnoside	F2
67[b]	53.3	276, 325	[M-H]⁻; 411.7831		307(30), 163(26), 145(12),119(7), 117(1)	p-Coumaroyl malonylhexoside	F2
68[b]	54.9	301	[M+H]⁺; 516.3594	$C_{25}H_{24}O_{12}$	355(48), 327(27), 127(7), 137 (47), 109(20), 193(10), 165(123), 123(9), 81(7),179(10), 151(5), 236(4)	4,5-di-*O*-*E*-CQA	F5
			[M-H]⁻; 515.3174		353(51), 179(45), 191(12), 127(13)		
69[c]	55.9	267, 300	[M+H]⁺; 282.0719	$C_{13}H_{13}O_7$	137 (26), 109(10), 193(8), 165(9), 123(5), 81(4),179(3), 267(2), 236(2), 81(5), 91(2), 102(4)	4-CHBA	F1, F3, F4, F5
70[b]	56.1		[M-H]⁻; 209.7556	$C_{10}H_9O_5$	191 (76), 147(51) 85 (31)	Glucaric acid	F1
71[b]	58.8	295, 350	[M+H]⁺; 668.7289	$C_{29}H_{32}O_{18}$	493(67), 331(76)	Tricin-7-*O*-glucuronopyranosyl-(1-2)-*O*-glucopyranoside	F1
72[b]	58.9		[M-H]⁻; 658.4421	$C_{32}H_{33}O_{15}$	327(220), 127(4), 353(30), 173(78), 179(41), 191(14), 135(11)	CQA derivative	F5
73[b]	60.4	254, 351 ,414	[M-H]⁻; 308.2784	$C_{14}H_{27}O_7$	270(13), 155(10), 202(9), 296(4), 221(3), 285(5), 257(4)	Kaempferol derivative	F1
74[c]	60.8	302	[M+H]⁺; 399.2004	$C_{22}H_{22}O_7$	282(15), 296(14), 193(11), 124(6), 304(5), 318(2)	4-Vinylphenyl-4-*O*-caffeoyl-2-hydroxy-3-methylbutanoate	F1, F3, F4
75	61.4	300, 324	[M+H]⁺; 405.3187	$C_{22}H_{44}O_6$	405(57), 85(11)	unknown	F5
76[b]	63.2	254, 348	[M+H]⁺; 303.1121 [M-H]⁻; 301.0874	$C_{15}H_{10}O_7$	301(120), 205(121), 149(43), 279(10), 80(45) 299(67), 277(15)	Quercetin	F1, F3, F4

t_R (min) = retention time in minutes. MF = molecular formula

[a] Identified with reference standard, [b]determined with the aid of literature, accurate mass match, reference compound and number of carbon atoms, [c]novel compound deduced from fragmentation pattern and 1D NMR of the reference compound.

The available information gives us a picture of the distribution of phenolic compounds in the fractions of *T. diversifolia* (Fig. 3.11). Previous reports also show that some flavonoids detected here have also been previously been characterised from *T. diversifolia*. These include dimethoxyluteolin and quercetin 3-*O*-rutinoside (Pereira et al., 1997, Kuroda et al., 2007, Ambro´sio, et al., 2008). In addition, luteolin, nepetin and hispidulin have also been earlier reported from *T. diversifolia* (Pereira et al., 1997). Despite this previous report, the present study gives the first comprehensive analysis of phenolic compounds from *T. diversifolia*. The extent of distribution of flavonols in the plants determines the extent of antioxidant potential since quercetin and kaempferol (flavonols) are the best anti-oxidant compounds (Agati et al., 2011; Tattini et al., 2004).


byakangelicin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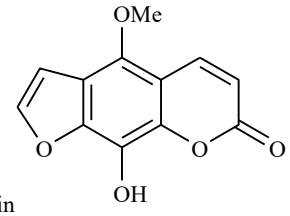
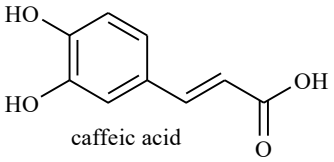
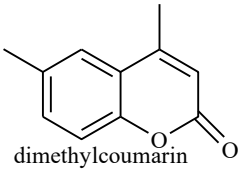

5-methoxy-8-hydroxypsoralen


caffeic acid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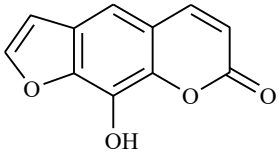
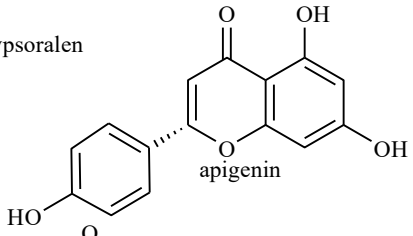
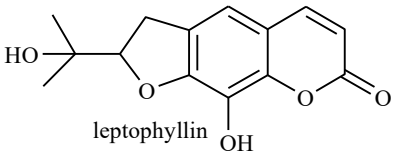
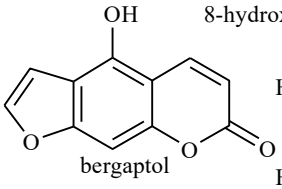

8-hydroxypsorale


apigenin


leptophyllin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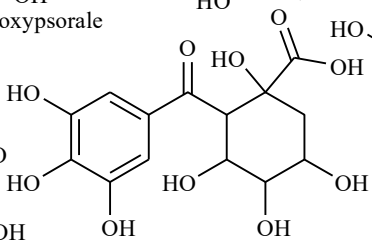
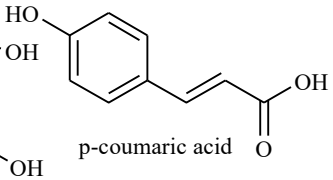
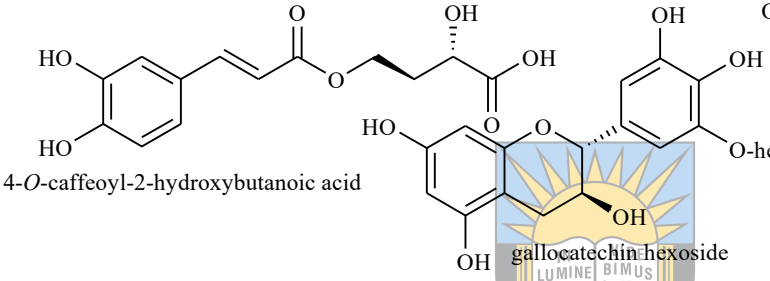
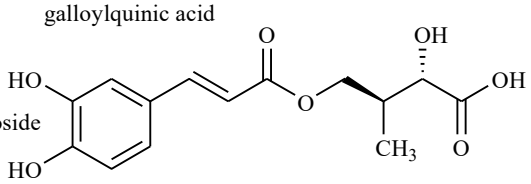
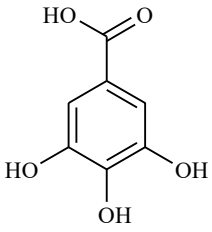

galloylquinic acid

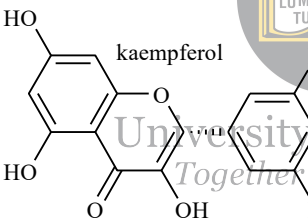

p-coumaric acid

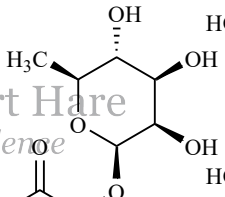

4-*O*-caffeoyl-2-hydroxybutanoic acid

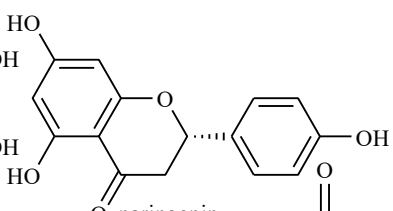
gallocatechin hexoside

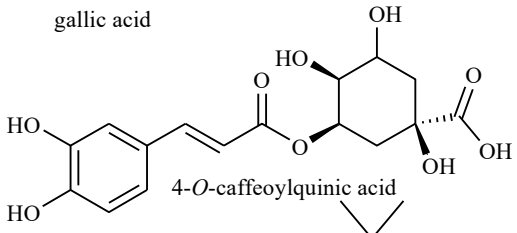

4-*O*-caffeoyl-2-hydroxy-3-methylbutanoic acid

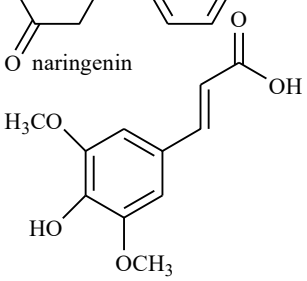

gallic acid

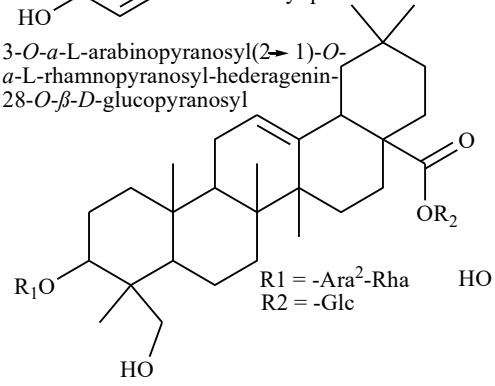

kaempferol


quercetin-3-O-rhamnoside

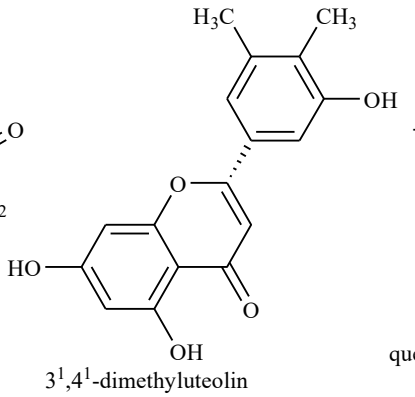

naringenin

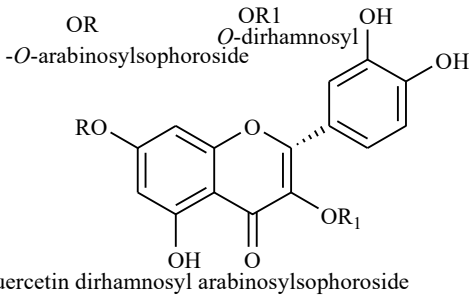

4-*O*-caffeoylquinic acid


Sinapinic acid


3-*O*-*a*-L-arabinopyranosyl(2→ 1)-*O*-*a*-L-rhamnopyranosyl-hederagenin-28-*O*-*ß*-*D*-glucopyranosyl

R1 = -Ara^2-Rha
R2 = -Glc


3^1,4^1-dimethyluteolin


-*O*-arabinosylsophoroside

quercetin dirhamnosyl arabinosylsophoroside

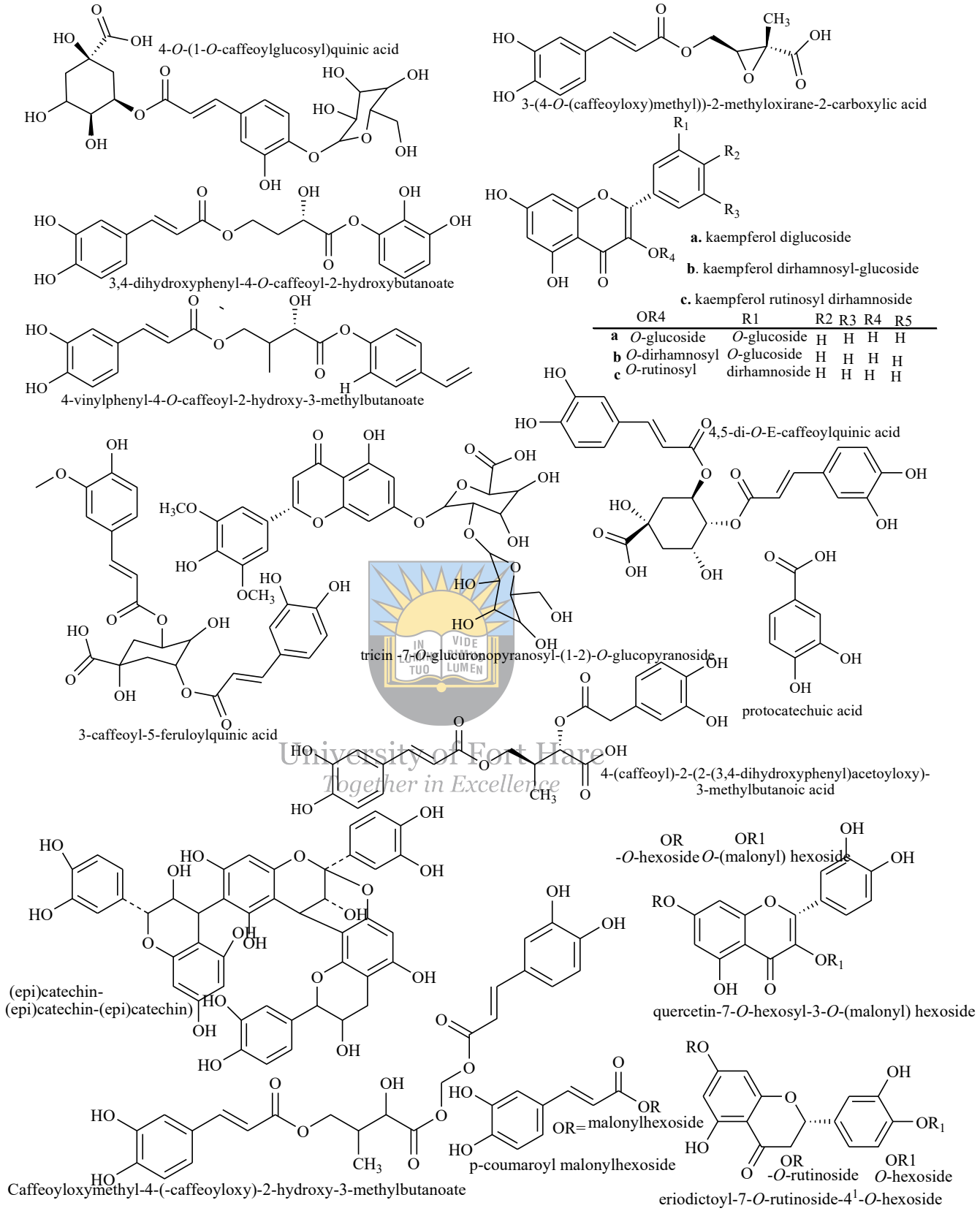


Figure 3.11: Phenolic compounds identified from aqueous methanol active fractions

3.5 Characterisation of isolated bioactive compounds from *T. diversifolia*

The isolated compounds were characterised using spectral data; 1D and 2D ^{1}H and ^{13}C-NMR, FTIR, UV and LC-MS or LC-MS-MS (see appendix 3 to 8). Assignments were confirmed by 2D NMR. Tables 3.7, 3.8 and 3.9 show the spectral data used to elucidate isolated compounds. Figure 3.12 shows structures of the bioactive compounds isolated from *T. diversifolia*.

Table 3.7: ^{1}H NMR, ^{13}CNMR, COSY, HSQC and HMBC spectral data for compound NK1F5

#	**δH (J in Hz) (400 MHz & $CDCl_3$)**	δC **(151MHz, $CDCl_3$)**	**HMBC**	**HSQC**	**COSY**
1	4.06 (1H, d, 10.9)	76.7	C-10	C-1/ H-1	
2	2.88 (H-2a, dd, 13.3, 9.2) 1.70 (H-2b, d, 10.9)	27.3	C-10, C-4, C-1		H-2a, H-15
3	4.45 (1H, s)	110.1	C-1[1]		H-2b, H-9a
4		137.5			
5	5.88 (1H, s)	133.1		C-5/ H-5	
6	4.29 (1H, d, 7.8)	78.3		C-6/ H-6	H-2b, H-2a
7	2.45 (1H, dddd, 11.1, 2.3, 9.2, 1.9)	55.3	C-6, C-3, C-9	C-7/ H-7	H-6
8	4.17 (1H, d, 11.0)	71.1			H-9ab
9	1.86 (2H, ddd, 4.8, 6.8, 7.1)	39.3	C-7, C-14	C-9/ H-9a,b	
10		77.5			
11		137.0			
12		166.1			
13	6.23 (1H, d, 13.7) & 5.60 (1H, d, 11.8)	127.7	C-7		H-13b,H-2b, H-7, H-2a, H-2[1]
14	1.15 (3H, m)	22.8	C-10		
15	1.30 (3H, s)	17.6	C-4		H-2b, H-15
1[1]		174.4			
2[1]	1.28-1.22 (1H, m)	35.5	C-4[1]		H-3[1]
3[1]	1.03 (3H, s)	14.7			
4[1]	1.08 (3H, s)	25.0		C-4[1]/ H-4[1]	

Table 3.8: ^{1}H NMR, ^{13}CNMR, COSY and HMBC spectral data for compound NK3F4

#	**δH (J in Hz) (400 MHz & CDCl3)**	δC **(151MHz , $CDCl_3$)**	**HMBC**	**HSQC**	**COSY**
1	3.53 (1H, ddddd, 66.2, 9.2,3.9, 4.9, 3.7)	71.8		3.55/71.2	H-2, H-3
2	2.30 (2H, ddddd, 3.9,	43.2		2.30/43.2	H-3, H-1, H-11

	6.6, 2.2, 8.7, 10.2)				
3	1.68-1.44 (1H, m)& 1.19-1.08 (1H, m)	28.2		1.68/28.7	H-2, H-1
4		36.5			
5	1.19-1.08 (1H, m)	45.9		0.95/45.7	H-6, H-3, H-18, H-19
6	1.35-1.25 (2H, dddd, 7.2, 14.7, 6.2, 11.0)	23.1,	C-7	1.27/22.8	H-7, H-5, H-3, H-18
7	2.01 (1H, ddd,6.7, 3.4, 17.4) & 1.89 (1H, d, 7.5)	29.8		2.00/31.0	H-5, H-6
8		140.8			
9		74.4			
10		50.6			
11	1.89 (1H, d, 7.5)& 1.68-1.44 (1H, m)	31.9		1.87/31.5 2.00/31.7 1.53/31.5	H-12, H-2
12	2.01 (1H, ddd,6.7, 3.4, 17.4)&1.19-1.08 (1H, m)	39.8	C-15	2.03/39.8	H-11, H-2
13		56.1	C-17, C-15		
14	5.36 (1H, s)	121.7		5.36/121.6	H-7, H-12
15	5.16 (1H, dd, 5.6, 7.0)	127.8			H-16
16	5.03 (1H, d, 5.6) & 4.99 (1H, s)	119.2			H-15
17	0.92 (3H, d, 5.1)	26.1	C-13, C-11, C-15		H-12, H-11
18	0.88- 0.79 (3H, m)	28.2	C-19, C-3,C-5		
19	0.7 (3H, d, 7.3)	19.4	C-2		
20	1.03 (3H, d, 7.5)	18.9	C-4, C-10	1.03/19.0	

Table 3.9: ^{1}H NMR, ^{13}CNMR spectral data for compound NK1F4

#	**δH (J in Hz) (400 MHz & CDCl3)**	δC **(100MHz, D2O, CD3OD)**	**δH* (J in Hz) (400 MHz & CD3OD -d4)**	δC * **(100MHz, CD3OD -d4**
1		128.8		127.8
2	7.39 (1H, d, 4.4)	104.5	7.05 (d, 1.8)	115.2
3		143.4		146.9
4		151.0		149.6
5	7.38(1H, s)	118.3	6.78 (d, 8.3)	116.5
6	7.36 (1H, s)	125.0	6.95 (dd, 8.3, 1.8)	122.9
7	5.38 (1H, s)	143.1	7.59 (d, 16.1)	147.2
8	4.97-4.64 (1H, m)	110.2	6.29 (d, 16.1)	114.9
9		171.4		169.3
10	4.25 (1H, br s), 3.6-3.1(1H, m)	65.3	4.20 (dd, 11.7, 7.9) 4.46(dd, 11.7, 3.3)	65.9
11	3.82(1H, s)	77.3	4.05 (dd, 7.9, 3.3)	74.9
12		75.3		77.3
13		171.4		178.2
14	1.43 (3H, m)	20.2	1.42 s	22.8

*comparative data from Pantoja Pulido et al., 2017

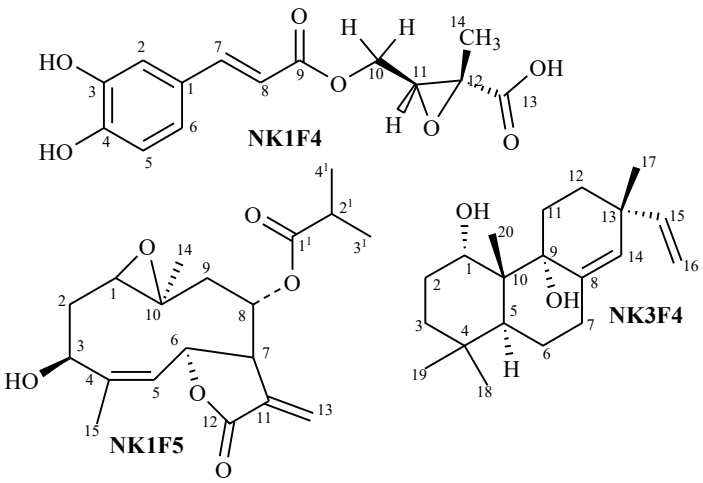


Figure 3.12: Structures of isolated and active compounds of *T. diversifolia*

Compound **NK1F5** was a colourless crystalline solid. The spectral data (**(**^{1}H and ^{13}C NMR: 1D and 2D, IR, and MS; Table 3.7, Appendix 3, 4, 7) obtained and literature information from Baruah et al., (1979) enabled identification of this compound as deacetylviguiestenin. Deacetylviguiestenin is an epimer of tagitinin E and was earlier reported from *T. diversifolia* (Baruah et al., 1979). The high-resolution electronspray ionisation mass spectrometry (HRESIMS) signal showed at *m/z*, 352 $[M+2H]^{+}$. The molecule is thus formulated as $C_{19}H_{26}O_6$. The IR spectrum of NK1F5 suggested the presence of olefinic sp^2 C-H (824 cm^{-1}) bond, an alkoxy C-O (1173 cm^{-1}), C-O stretch of an ester (1368 cm^{-1}) and an α -methylene γ-lactone (1766, 1781 cm^{-1}) group in the molecule. The IR absorption broadband also indicated the presence of a hydroxyl group (3419 cm^{-1}). The NMR spectra are shown in appendix 4. The ^{1}H NMR (600 MHz, $CDCl_3$, δ_H) spectrum gave a multiplet at δ_H 2.45 (m, 1H, H-1), doublets at δ_H 6.23(J = 13.7, 1H, H-13a) and δ_H 5.60 (J = 11.9, 1H, H-13b) with coupling constants J = 13.7 Hz and J = 11.9 Hz which are indicative of an α-methylene γ-lactone moiety. Tertiary methyl groups at δ_H 1.30 (H-15, s) and 1.15 (H-14, m) each 3H were revealed. Multiplets of δ_H 2.44-2.40 (H-2^1) and and two methyl singlets at δ_H 1.08 (H-4^1) and δ_H 1.03 (H-3^1) confirmed the presence of the isobutyrate ester side chain. 3H singlet protons each at δ_H 1.30 and multiplet at δ_H 1.15 (3H, m) confirm positions H-15 and H-14 respectively. The ^{13}C NMR (151 MHz, $CDCl_3$, δ) showed 19 carbons. The classification based on ^{13}C NMR could show 4 tertiary, 3 secondary, 7 primary and 5 quarternary carbons. The ^{13}C NMR also showed olefinic methylene carbons at δ_C 127.7 (C-13), carbonyl of ester at δ_C 166.1 (C-12) and 174.4 (C-1^1), oxomethine carbon at 76.7 (C-1) and quarternary oxygenated carbon at

77.5 (C-10). The HSQC data confirmed carbons at positions 1, 5, 6, 7 and 9 i.e. δ_C (ppm) 76.6 (C-1), 133.1 (C-5), 78.3 (C-6), 55.3 (C-7), and 39.3 (C-9) respectively with correlation spectroscopy experiment of protons at positions C-1 to C-9. The olefinic quarternary at δ_C 137.5 (C-4), 137.0 (C-11) and 133.1 (C-5), methyl and methylene carbons at δ_C 27.3(C-2), 22.8 (C-14), 25.0(C-3^1), 17.6 (C-15), 14.7(C-4^1); methines at δ_C 34.0(C-2^1), 36.8(C-1) were also noted. The correlations from protons at positions 2 and 15 to the chemical shift at C-4 were confirmed by HMBC. Besides, the chemical shifts at C-10 (δ_C 77.5) correlated well with those of the protons at positions 14, 2 and 1.

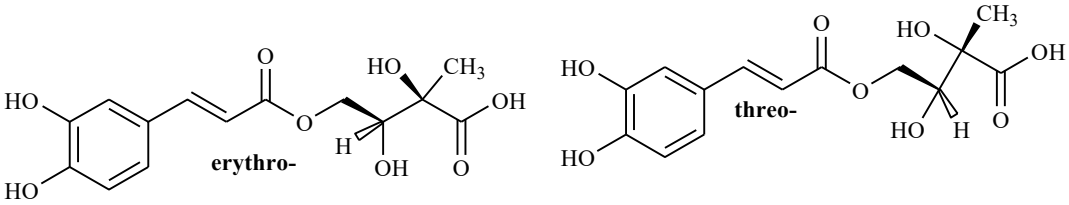
Compound **NK3F4** was isolated as a powder. The molecular mass of NK3F4 was determined to be 304 giving a molecular formula of $C_{20}H_{32}O_2$. The HRESIMS signal showed $[M-H]^-$ ion at *m/z* 303 with fragments; 289 $[M-H-CH_3]^-$ and 274 $[M-H-2CH_3]^-$(Appendix 8). The FT-IR spectrum of NK3F4 (Appendix 3) suggested the presence of sp^3 C-H stretch (2936 cm^{-1}), an alkoxy C-O (1056 cm^{-1}), a trisubstituted sp^2C-H bond (3014, 1645 and 801 cm^{-1}), a weak sp^2 C-H stretch (1466cm^{-1}), alcohol O-H, stretch bonds (3327 cm^{-1}) and a C=C (1645cm^{-1}) stretch in the molecule. The ^{1}H NMR (400 MHz, $CDCl_3$: δ_H) together with the ^{13}C NMR (151 MHz, $CDCl_3$, δ_C) showed signals similar to those of sandaracopimaradiene-1α,9α-diol (Prawat et al., 1993) (Table 3.8, Appendix 5). ^{13}C NMR spectrum revealed four sp^3 methyls (δ_C: 26.1, 28.2, 19.4 and 18.9), six sp^3 methylenes (δ_C: 28.2, 29.7, 31.9, 24.6, 39.8 and 42.3), two sp^3 methines including one oxygenated (δ_C: 71.8, and 45.9), one sp^2 methylene (δ_C 119.2), one sp^2 methine (δ_C 127.8), four sp^3 quaternary carbons including one oxygenated (δ_C 36.5, 50.6, 56.1 and 74.4), and one sp^2 quaternary carbon (δ_C 140.8). The ^{1}H NMR spectrum displayed signals for a pimarane diterpene type skeleton including terminal vinylic protons [δ_H 5.16 (dd, J = 5.6, 7.0 Hz, H-15), 5.01 (d, J = 5.6 Hz, H-16a), 4.99 (s,H-16b)], an olefinic methine [δ_H 5.36 (s, H-14)], and four tertiary methyl signals [δ_H 0.92 (ddd, J = 4.6, 7.2, 5.1) (H-17), 0.88- 0.79 (m, (H-18), 0.70 (d, J = 7.3) (H-19)] and 1.03 (dd, J = 10.8, 7.5) (H-20). The HMBC data showed correlations between H-18 and C-3, C-19, C-5, H-20 and C-4, C-2 and between H-6 and C-7. The HMBC between H-17 and C-13 and H-17 and C-15 comfirm the attachment of the vinyl group at position 13. There were COSY between H-1 and H-3 at position 2 and between H-2 and H-3 at position 1. In addition, COSY data corfirmed correlations between H-5 through H-7 and H-11 with H-12 also existed. The HSQC data confirmed carbons at positions 1, 2, 3, 5, 6, 7, 11, 12, 14 and 20. The aforementioned spectroscopic data support those of a pimarane diterpene type compound, sandaracopimaradiene-1α,9α-diol to a very large extent. It is the first time this compound is

identified from *T. diversifolia,* however sandaracopimaradiene, was present in its flower oil (Moronkola et al., 2007).

Compound **NK1F4** was identified to be 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid from its spectral data (^{1}H and ^{13}C NMR, FTIR, MS and MS/MS) and also from literature (Pantoja Pulido et al., 2017). The HRESIMS showed signals at $[M+2H]^{+}$; *m/z* 296Da giving molecular mass 294Da which is formulated as $C_{14}H_{14}O_7$. Other LC-MS fragments were: *m/z* 282 $(M + 2H\text{-}CH_3]^{+}$, *m/z* 102, 80. The MS^2 fragmentation of high intense peaks; *m/z* 296 and 282 gave similar fragment ions; *m/z* 137, 109, 193, 165, 179, 151, 265 and 236 (Appendix 9). The ion at *m/z* 179 represents a caffeoyl moiety. The ions *m/z* 193 and 102 represent fragments due to breakage of the bridge through methylene carbon and the epoxide. Other ions in the mass spectrum are *m/z* 447 and *m/z* 393. Loss of 296 from *m/z* 447 gives *m/z* 151 while the loss of 282 from *m/z* 447 yields 165, indicating dihydrop-coumaroyl adduct of NK1F4 (see appendix 7).

The IR spectrum of NK1F4 (Appendix 3) suggested the presence of sp^2 C-H (824 cm^{-1}), an alkoxy C-O (1173 cm^{-1}). IR band at 1373 cm^{-1} shows the presence of sp^3 C-H bend and the broad band between 1436 and 1117 cm^{-1}. A sharp IR band at 1232 cm^{-1} was representative of an epoxy group (oxirane ring). Carbonyl groups in the molecule were represented at (1765cm^{-1}) while carboxylic acid O-H stretch occurred at 2363 cm^{-1}. Careful examination of FTIR data reveals the presence of aromatic (para, meta) sp^2 C-H (876 cm^{-1}) bend and another 824 cm^{-1}, para aromatic sp^2 C-H bend. There were also absorption bands between 3745-3628 and 3023 cm^{-1} indicating hydroxyl groups. Comparing the LC-MS and FTIR data, with the information by Pantoja Pulido et al., (2017), NK1F4 should be (*E*)-3-(((3-(3,4-dihydroxyphenyl)acryloyl)oxy)methyl)-2-methyloxyrane-2-carboxylic acid or simply 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid. The ^{1}H NMR (400 MHz, D_2O, δ_H) (Table 3.9) showed aromatic system of δ_H 7.39 (1H, d, 4.4, H-2), 7.38 (1H, s, H-5), 7.36 (1H, s, H-6) including resonance of two coupled protons, 5.38 (1H, s) and 4.97-4.64 (1H, m) that indicate a caffeoyl moiety. It also displayed two signals 4.25 (1H, br s, H-10a), 3.64-3.18(1H, m, H-10b) and 3.82(1H, s) corresponding to the epoxy residue methylene bridge and oxymethine protons. The ^{13}C NMR (100 MHz, CD_3OD, δ_C) gave carbon resonances at δ_C: 77.3, 75.3, confirming an epoxide or oxirane ring. Additionally, the olefinic carbon at δ_C 151.0, 128.8, 104.5, 143.4, 118.3, 125.0 143.4 and 110.2 and two carbonyl carbons at δ_C 171.4 was noticed. As noticed from more two methyl resonances in the ^{1}H NMR spectrum

i.e. δ_H 1.12, (3H, s) and 1.04 (3H, s), compound NK1F4 is a caffeic acid derivative in which the epoxide ring opens to produce two diastereoisomers i.e. erythro- and threo- 4-*O*-caffeoyl-2,3-dihydroxy-2-methylbutanoic acid



This compound was previously reported in *T. diversifolia* (Pantoja Pulido et al., 2017).

In general, the relative stereochemistry of the above isolated compounds was assigned by comparing ^{13}C NMR chemical shift with the data of known compounds i.e deacetylviguiestenin (Baruah et al., 1979), sandaracopimaradiene-1α,9α-diol (Prawat et al., 1993) and 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid (Pantoja Pulido et al., 2017) and analysis of the coupling constants.

Using the bioassay guided isolation, these three compounds were tested for feeding deterrence characteristics and/or antioxidant potential (Chapter 4). 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid was previously found to have antioxidant properties (Pantoja Pulido et al., 2017) but the extent of its pesticidal potential had not been reported before. The sesquiterpene lactones are known for several biological activities but in particular deacetylviguiestenin had not been tested for these activities. Likewise the diterpenes' insecticidal activities are quite well reported but specific pesticidal and anti-oxidant potential and extent of such properties has been non existent. In the same capacity, the essential oils and some pure compounds of its component identified using the GC/MS/MS were tested for repellency, insecticidal or feeding deterrence potentials.

Chapter Four

Biological activity studies on *T. vogelii* and *T. diversifolia*

In this chapter, the results of the biological investigations carried out on the extracts and isolates of the plant materials of *T. vogelii* and *T. diversifolia* collected from different places in Butaleja district, eastern Uganda will be presented and discussed.

4.1 Pesticidal evaluation of *T. vogelii* and *T. diversifolia* against *S. zeamais*

4.1.1 Evaluation of the pesticidal potential of the chemotypes of the volatile constituents of *T. vogelii*

Different concentrations: 10, 20 and 40 µl/ml of the *T. vogelii* oil corresponding to 21.5, 43.0 and 86.1 µg/L of air for TV4Kyc (density, 1.076g/mL) and 20.6, 41.2 and 82.4 µg/L of air for TV4Muya (density, 1030g/mL) respectively were tested for fumigant toxicity against *S. zeamais* to establish the effect of chemical variation in the components of the chemotypes. Results indicated that there was a significant difference in the fumigant toxicity between the farnesol chemotype and the springene chemotype ($P < 0.05$, Fisher LSD test) (Table 4.1). At all treatment concentrations, springene chemotype exhibited a higher fumigant effect of the two chemical varieties against *S. zeamais*. The LC_{50} of the farnesol chemotype and springene chemotype was respectively $8.8\times10^{1}\pm3.7\times10^{1}$ µg/L of air and $13.0\times10^{1}\pm14.0\times10^{1}$ µg/L of air after 24h exposure. This shows that chemotype 1 is about five times more toxic to *S. zeamais* than methyl bromide, the positive control ($LC_{50} = 4.3\times10^{2}\pm1.1\times10^{2}$ µg/L of air after 24h). Chemotype 2 is three times more toxic to *S. zeamais* compare to methyl bromide after 24 h. This therefore means that chemotype 2 is about three times more toxic to *S. zeamais* than chemotype 1. After a long time of exposure, however, the gap between the fumigant toxicities of the two chemotypes seems to narrow according to the LC_{50} values. This means that with an increase in exposure time, the toxicities of the two chemotypes are almost the same. The higher toxicity of chemotype one compared to that of two, maybe attributed to the fact that oxygenated substances have higher toxicity values.

Table 4.1: Percentage fumigant mortality (mean±SE) due to chemotype1 and chemotype2 and their LC_{50} values

Time (h)	control	Sample	10µl/ml 20(µL/L)	20µl/ml 40(µL/L)	40µl/ml 80(µL/L)	Regression Equation	R^2	LC_{50} (µg/Lair)	SE of LC_{50}	95% upper FL
6	0.0±0.0b	**TV4 Kyc**	2.7±2.2hi	7.6±3.4i	0.7±3.8h	y = 4.3 -0.7x	0.238	1.0×10^{-1}	2.1×10^{1}	2.1×10^{1}
		TV4 muya	0.0±0.0i	3.0±0.7i	5.1±2.4h	y = 1.2 +1.1x	0.970	2.1×10^{3}	2.0×10^{3}	4.1×10^{3}
12	0.6±0.6b	**TV4 Kyc**	6.1±2.9ghi	21.7±2.8h	17.2±2.6gh	y = 2.3 +1.0x	0.541	5.1×10^{2}	6.5×10^{2}	1.2×10^{3}
		TV4 muya	11.9±3.5fghi	25.1±4.4h	26.5±4.6fg	y = 2.7 +0.9x	0.828	3.0×10^{2}	4.4×10^{2}	7.4×10^{2}
24	1.2±0.7b	**TV4 Kyc**	8.9±3.1fghi	40.6±3.9g	39.5±6.1def	y = 1.5 +1.8x	0.736	8.8×10^{1}	3.7×10^{1}	1.3×10^{2}
		TV4 muya	17.0±3.5defghi	38.6±3.2g	37.8±2.2ef	y = 2.8 +1.1x	0.715	1.3×10^{2}	1.4×10^{2}	1.7×10^{2}
36	1.2±0.7b	**TV4 Kyc**	13.9±4.0efghi	51.7±4.1ef	47.8±7.6cde	y = 1.9 +1.7x	0.679	4.1×10^{0}	3.1×10^{1}	3.5×10^{2}
		TV4 muya	20.4±4.6defg	47.3±3.3fg	40.6±3.0def	y = 2.7+1.2x	0.697	8.1×10^{1}	7.3×10^{1}	1.5×10^{2}
48	1.7±0.8b	**TV4 Kyc**	16.1±4.7efghi	53.1±5.1def	53.3±6.7abc	y = 1.9+1.8x	0.751	5.8×10^{1}	2.7×10^{1}	8.5×10^{1}
		TV4 muya	26.6±6.4cdef	54.7±4.5def	40.6±3.0def	y = 0.6+3.7x	0.264	9.8×10^{1}	4.8×10^{2}	5.8×10^{2}
60	1.7±0.8b	**TV4 Kyc**	17.8±4.5defgh	61.7±3.2bcd	62.8±6.6abc	y = 1.6+2.1x	0.763	4.5×10^{1}	1.7×10^{1}	6.2×10^{1}
		TV4 muya	34.0±8.1bcd	56.7±3.7cdef	56.9±4.6bcd	y = 3.4+1.0x	0.750	4.2×10^{1}	5.7×10^{1}	9.9×10^{1}
72	1.7±0.8b	**TV4 Kyc**	19.4±5.1defgh	65.6±2.8bc	62.8±6.6abc	y = 1.7+2.0x	0.702	1.6×10^{0}	1.6×10^{1}	1.8×10^{1}
		TV4 muya	40.7±8.1abc	59.5±2.3cde	47.4±10.2cde	y = 0.7+3.9x	0.663	3.3×10^{1}	1.1×10^{2}	1.4×10^{2}
84	2.2±0.7ab	**TV4 Kyc**	25.1±9.4cdef	69.4±4.2ab	73.3±7.6a	y = 1.7+2.1x	0.815	3.5×10^{1}	1.2×10^{1}	4.7×10^{1}
		TV4 muya	48.1±8.9ab	64.3±4.5bcd	67.7±6.9ab	y = 3.8+0.9x	0.926	2.1×10^{1}	3.3×10^{1}	5.4×10^{1}
96	5.1±2.5a	**TV4 Kyc**	30.1±10.6cde	78.2±3.0a	76.7±7.4a	y = 2.0+2.1x	0.733	2.8×10^{1}	1.0×10^{1}	3.8×10^{1}
		TV4 muya	57.7±10.6a	69.7±4.0ab	74.5±8.0a	y = 4.2+0.8x	0.958	1.1×10^{1}	2.6×10^{1}	3.7×10^{1}
P > F	0.077		<0.0001	<0.0001	<0.0001					
F	1.94		6.05	35.83	13.66					
Sig.	No		Yes	Yes	Yes					
LSD	2.95		17.74	9.82	16.57					
R^2 (model)	0.26		0.53	0.88	0.74					
		MeBr						4.3×10^{2} (0.43 mg/Lair)*	1.1×10^{2}	4.9×10^{2}(UFL)

UFL= Upper Fiducial Limit, * Data from Wang et al, 2015. The density of TV4 Kyc (chemotype1) is 1.076±0.049. The density of TV4 muya (chemotype2) =1.030±0.030. Data expressed as means±SE of 2 replicates. *Values sharing the same letters in the column means they are not significantly different from each other (P < 0.05) (Fisher LSD test).*

The repellency potential of TV4 Kyc (Farnesol chemotype) and TV4 Muya (*β*-Springene) was also evaluated and results indicated that there is no much difference in their repellency effect against *S. zeamais* (Table 4.2). The preference index of TV4kyc (farnesol type) oil against *S. zeamais* ranged between 0.0 to -0.7, 0.0 to -0.5 and -0.3 to -1 for 0.03, 0.16 and 0.31 µL of oil per cm^2 of air respectively. That of TV4muya (springene type) could vary between 0.1 to -0.4, -0.2 to -0.5 and -0.4 to -1 for 0.03, 0.16 and 0.31 µL of oil per cm^2 of air treatment. These results indicate that the oils had a repellency effect against the maize weevil based on the preference index scale of *S. zeamais*. Chemotype3 showed a lower effect. The composition of farnesol and springene was very low; partly explaining the less repellency activity for this chemotype. These observations underline the vital role that both farnesol and springene play in the repellency potential.

Table 4.2: Percentage repellency (mean± SE) of *T. vogelii* essential oils against *S. zeamais*

		Dose (µL/cm^2)							
Time (h)		**0.03**	**0.16**	**0.31**	**Regression Equation**	**R^2**	**RD_{50} µL/ cm^2**	**SE of RD_{50}**	**95% UFL**
1	Chemotype 1	12.5±10.0[b]	35.2±6.3[b]	39.2±12.3	y = 0.9x +5.2	0.969	0.6	1.0	1.6
	Chemotype 2	3.8±6.8[b]	50.5±9.3[b]	62.8±4.9[b]	y = 2.2x +6.6	0.973	0.2	0.3	0.4
	Chemotype 3	-15.6±26.2	2.2±15.5	40.0±10.2	y = 0.4x +6.3	0.979	25.4	7.8	33.2
	Farnesol	-4.9±9.2	-4.9±9.2	48.1±5.3	y = 0.6x +6.5	0.711	250.5	11.4	261.5
12	Chemotype 1	12.7±2.5[b]	9.1±1.8	42.0±8.4[b]	y = 0.7x +4.8	0.364	1.9	6.7	8.6
	Chemotype 2	22.8±4.6[b]	23.1±4.6[b]	51.6±10.3[b]	y = 0.7x +5.1	0.536	0.7	2.5	3.2
	Checotype 3	-2.2±8.0	4.4±2.2	28.9±2.2[b]	y = 0.8x +5.3	0.910	29.7	10.6	40.3
	Farnesol	-0.5±-0.1	4.4±0.9	18.4±3.7	y = 2.6x +6.5	0.945	0.3	0.5	0.8
24	Chemotype 1	51.1±13.1[b]	24.9±7.9[b]	33.3±14.7[b]	y = -0.6x +4.1	0.657	0.1	0.2	0.2
	Chemotype 2	30.4±4.3[b]	15.1±12.8[b]	47.4±19.0[b]	y = 0.2x +4.7	0.061	31.6	4.1×10^5	-
	Checotype 3	10.6±9.4[b]	0.9±16.2	20.0±3.8	y = 0.4x +4.4	0.006	31.6	1.3×10^3	-
	Farnesol	3.9±19.7	-8.2±27.3	18.3±3.9	y = 1.5x +5.4	0.996	0.5	0.3	0.8
	IR3535*					0.962	0.3	0.2	0.4

* Data from Nerio et al, 2009, UFL = upper fiducial limits. [b] *Mean repellency of farnesol differs significantly from that of chemotypes (P< 0.05), at the same exposure period, according to paired t-test.*

Chemotype 2 (RD_{50} = 2 0.2±0.4µL/cm^2) showed almost the same repellency potential as that of IR3535 while chemotype 1 (RD_{50} =0.6±1.6) µL/cm^2 is two times less potent than the positive control. Chemotype 3 with RD_{50} = 25.4±33.2µL/cm^2 is however much less potent compared to IR3535 i.e about 85 times less repellent. The RD_{50} values here represent the maximum possible amount of the oil in the air and they are therefore the median repellency doses for the oils. The repellency potential of farnesol was also evaluated against the weevil and results show that farnesol is effective at a higher concentration as a repellent against *S. zeamais*. Compared to the positive control, farnesol is less effective against *S. zeamais*. The preference index evaluated for 0.31µL of farnesol per cm^2 of air varied between 0.0 to -0.4 while that of the lower concentration ranged between 0.1 to 0.6 and -0.2 to 0.3 for 0.16 µL and 0.03 µL of farnesol per cm^2 of farnesol respectively. This implied that lower concentrations of farnesol had a very limited repellent effect against *S. zeamais*. Repellency activity roughly increased with an increase in the amount of farnesol. The repellency effect of farnesol isomers implies that the farnesol chemotype could have its repellency activity further enhanced with a larger amount of farnesol in the oil and probably would have an advantage over the springene chemotype. In both cases, however, the synergistic or complementary part of all other constituents found in the oils play crucial role in the overall repellent and insecticidal effect of *T. vogelii* oil.

4.2 Comparison of toxicity and repellency potential of *T. vogelii* and *T. diversifolia* essential oils against *S. zeamais*

4.2.1 Repellence potential of the essential oils against *S. zeamais*

The results of comparative repellency of the oils of the two plants against *S. zeamais* are shown in Table 4.3. The two plants had some repellent activities at all treatments. Compared to the positive control, IR3535 (ethyl-3-(N-acetyl-N-butylamino)proportionate) (median repellence dose, RD_{50} =0.3±0.2 µL/cm^2), *T. vogelii* essential oil was 3 times less active against *S. zeamais*. *T. diversifolia* was atmost 3 times more active than IR3535. The percentage repellence (PR) value of *T. vogelii* essential oil significantly increased with the increase in dose level. Unlike *T. vogelii* essential oil, *T. diversifolia* essential oil showed a dose-independent profile.

Table 4.3: Mean repellence (PR), preference index (PI) (mean ± SE) and median repellence dose (RD_{50}) data for *T. vogelii* (TV) and *T. diversifolia* (TD) essential oils against *S. zeamais*

		Treatment (Dose)						R^2	RD_{50}	SE of RD_{50}
		0.03 µL/cm²		0.16 µL/cm²		0.31 µL/cm²			µL/cm²	
Time (h)		PR ±SE	PI±SE	PR±SE	PI±SE	PR±SE	PI±SE			
1	TD	44.4±12.4	-0,4±0.1	17.8±35.8	-0.2±0.4	31.1±11.1	-0.3±0.1	0.412	8.0×10^{-3}	0.1
	TV	-15.6±26.2[b]	0.2±0.3[b]	2.2±15.5[b]	0.0±0.2[b]	40.0±10.2	-0.4±0.1	0.658	0.9	0.4
12	TD	22.2±19.0	-0.2±0.2	4.4±11.8	0.0±0.1	0.8±3.8[b]	0.0±0.1[b]	0.995	8.0×10^{-3}	5.6×10^{-3}
	TV	-2.2±8,0[b]	0.0±0.1[b]	4.4±2.2	0.0±0.0	28.9±2.2	-0.3±0.0	0.831	1.0	0.5
24	TD	10.6±9.4	-0.1±0.1	10.9±16.2	0.0±0.2[b]	20.0±3.8[b]	-0.2±0.1	0.536	316.2	0.0
	TV	2.2±9.7[b]	0.0±0.1[b]	26.7±10.2[b]	-0.3±0.1	39.3±7.7	-0.4±0.1	0.006	31.6	1.3×10^{3}
48	TD	13.3±6.7	-0.1±0.1	11.1±11.8	-0.1±0.1	11.1±8.0[b]	-0.1±0.1	0.914	1.0×10^{-11}	0.0
	TV	6.7±7.7	-0.2±0.1	33.3±3.9[b]	-0.3±0.1	28.9±2.2	-0.3±0.0	0.853	0.8	1.0
	IR3535*							0.962	0.3	0.2

* Data from Nerio et al, 2009

Results are presented as mean values ± SE. Negative PR indicates attraction while the positive PR shows repellence. [b]Mean repellency /preference index of T. vogelii differs significantly from that of T. diversifolia (P< 0.05), at the same exposure period, according to paired t-test.

Previous researchers have profiled the extent of repellency of essential oils as indicated in Table 4.4 according to this scale, the dosage of 0.03 µL/cm² of *T. vogelii* exhibited class 1 repellency in the first hour of exposure, while the same treatment level for *T. diversifolia* was of class 2. Compared with the positive standard which showed class 1 for doses (0.06 and 0.126 µL/cm²), it means at this level the oils of *T. vogelii* and *T. diversifolia* were more potent against the weevils (Nerio et al, 2009).

Table 4.4: The scale used to assign repellency of the essential oils of *T. vogelii* and *T. diversifolia*

Class	Percent repellency	Class	Percent repellency	Class	Percent repellency
0	>0.01 to <0.1	I	0.1–20	II	20.1–40
III	40.1–60	IV	60.1–80	V	80.1–100

Source: Juliana and Su, 1983; Ogendo et al., 2003; Viglianco et al., 2008; Lü et al., 2011).

A 0.16 µL/cm² *T. vogelii* treatment to weevils showed class 1 just like that of *T. diversifolia*. When weevils were treated with 0.31 µL/cm² of *T. vogelii*, a repellent class 2 was observed whereas the same concentration of the oil for *T. diversifolia* was class 1. This implies that at a

higher dosage, *T. vogelii* exhibited higher repellency efficacy especially over an exposure period of 48hr from 12hrs. The preference index (PI) rating varies between -1.0 and -0.1 indicate repellent, -0.1 to +0.1 neutral and finally within +0.1 to +1.0 qualifies it an attractant oil to insects (Chaubey, 2017b). Therefore, the essential oils of *T. vogelii* had a repellence effect against *S. zeamais* for 0.31 µL/cm^2 treatment for nearly all the exposure hours. For 0.16 µL/cm^2 dose, repellency occurred from the 12th hour up to the end. However, in the beginning, it had no repellent effect on the maize weevil. Likewise, 0.03 µL/cm^2 treatments showed no repellent effect on the weevil at the beginning however it reduced towards the end of the exposure period. The reduction was probably due to the reduction in the amount the active substances. Owing to the high volatility of essential oils, the effect tends to quickly dissipate. The repellency is therefore active only for a short while. The PI values of *T. diversifolia* essential oils were between -1.0 and -0.1 for 0.03 µL/cm^2 treatment indicating repellent property but for 0.16 µL/cm^2 and 0.31 µL/cm^2, -0.1 to +0.1 or +0.1 to +1.0 showed that the oil had a neutral or attractive effect. The repellency effect for *T. vogelii* increased with an increase in the treatment concentration. The overall repellency was greatest for 0.31 µL/cm^2 followed by 0.16 µL/cm^2 and finally 0.03 µL/cm^2 over an exposure period of 48 hours. Thus repellency was dose-dependent. At lower concentrations, *T. diversifolia* essential oils appeared to show more repellent efficacy than that of *T. vogelii*.

At 0.20% v/w, *S. zeamais* showed negative (-5.3%) repellency (attraction) 24 h after exposure to *Eucalyptus saligna* leaf essential oil. The PR values for *E. saligna* leaf essential oil, at 0.20% v/w was -10%, respectively 24 h after exposure *Cupressus lusitanica* (Bett et al., 2016). Othira et al. (2009) reported a low dose higher repellent effect of *Hyptis spicigera* steam distilled essential oil against *S. zeamais* adults. The overall percentage repellency was however relatively small thus giving a short protection time. A repellence of nearly 100% at a relatively long time would be preferred. The oils can be formulated to retain the active ingredients on the insect for a long. This can be made possible by formulating cream and polymer mixtures of oils. An example of this technology was employed on lemongrass oil mixed in petroleum jelly that resulted in longer protection against insects, without side effects (Oyedele et al., 2002).

4.2.2 Contact toxicity of essential oils of *T. vogelii* and *T. diversifolia* against *S. zeamais*

Table 4.5 shows the results of the toxicity of maize weevils when maize grains were treated with *T. diversifolia* and *T. vogelii* essential oils. Results show that contact mortalities of *S. zeamais* due to *T. vogelii* was greater than that due to *T. diversifolia*. The LC_{50} value (maximum possible amount of the *T. diversifolia* oil on the grains) was 2200 ± 3300 µL/g and thus the LC_{50} value. The LC_{50} value of *T. vogelii* is 0.001±0.013 µL/g after 48 hours. Compared with Delthametrin, the positive control (LC_{50} = 0.41 ± 0.15 µL/g), *T. diversifolia* oil was at least 5000 times less toxic to weevils while that of *T. vogelii* was atmost 4 times more toxic to *S. zeamais*. *T. vogelii* was more toxic to *S. zeamais* compared with *T. diversifolia* oil**.** The reason for this observation could be attributed to the nature and varying concentrations of the active compounds present in these plants. The percentage mortalities of weevils were however very low probably due to less sensitivity of these oils due to contact exposure. It may also be due to the nature of the positive standard versus the nature of essential oils. Delthametrin is more environmentally stable and thus can be associated with the ability to withstand degradation generating a better response to weevils.

The contact toxicity of these plants' essential oils as compared to other plants show that *T. vogelii* is more toxic to weevils than crude neem seed oil (LC_{50} = 1.46 µL/gram of seeds) (Nukenine et al. 2011), *Schinus terebinthifolia* Raddi (Anacardiaceae) ((LC_{50} = 57.7 µL 40 g^{-1}) and *Melaleuca leucadendron* L. (Myrtaceae) (LC_{50} = 75.8 µL 40 g^{-1} grain) (Coitinho et al., 2011), *Piper marginatum* Jacq. (Piperaceae) (LC_{50} = 21.1 µL 40 g^{-1} grain), *Cupressus sempervirens* L. (Cupressaceae) (LC_{50} = 38.05 µL 40 g^{-1} grain) (Tapondjou et al., 2005). These oils are however more toxic to *S. zeamais* compared with *T. diversifolia* oil.

Table 4.5: Cumulative mortality of *S. zeamais* due to contact toxicity from *T. vogelii* (TV) and *T. diversifolia* (TD) essential oils

Time (h)		0.25µL/g	0.5 µL/g	1.0 µL/g	0.0 µL/g	Regression equation	R^2	LC_{50} µL/g	SE of LC_{50}
48	TD	1.1±1.9	3.3±1.9	4.4±1.1	0.0±0.0[a]	y= 1.8+1.0x	0.908	2.3×10^{3}	3.3×10^{3}
	TV	4.4±1.1[b]	4.4±2.9	3.3±1.9		y= 3.5-0.5x	0.750	1.0×10^{-3}	1.3×10^{-2}
60	TD	1.1±1.1	3.3±1.9	4.4±1.1	1.1±1.1[a]	y= 1.8+1.0x	0.908	2.3×10^{3}	3.3×10^{3}
	TV	5.6±1.1[b]	4.4±2.9	3.3±1.9		y= 4.0-0.6x	0.985	1.4×10^{-2}	9.6×10^{-2}
72	TD	2.2±1.9	6.7±1.9	5.6±2.2	1.1±1.1[a]	y= 2.2+0.8x	0.647	2.2×10^{3}	4.5×10^{3}
	TV	6.7±1.9[b]	4.4±2.9[b]	3.3±1.9[b]		y= 4.2-0.7x	0.961	5.3×10^{-2}	1.2×10^{0}
84	TD	2.2±1.9	6.7±1.9	5.6±2.2	1.1±1.1[a]	y= 2.2+0.8x	0.647	2.2×10^{3}	4.5×10^{3}
	TV	6.7±1.9[b]	5.6±4.0	3.3±1.9[b]		y= 4.2-0.7x	0.877	6.8×10^{-2}	5.8×10^{-2}

96	TD	5.6±2.9	8.9±1.1	7.8±2.2	1.1±1.1[a]	y= 3.3+0.2x	0.429	3.7×10^{7}	6.8×10^{11}
	TV	7.8±2.9[b]	6.7±5.1[b]	4.4±1.1[b]		y= 4.2-0.6x	0.897	2.9×10^{1}	2.8×10^{-2}
	Delthametrin*							4.1×10^{-2}	1.5×10^{-2}

* Data from Ribeiro et al, 2017, obtained when weevils were exposed for 48 hours.

Results are presented as mean values ± SE. [a] Mean mortality due to the negative control is not significantly different ((Fisher's LSD test, P < 0.05). [b] Mean mortality of T. vogelii differs significantly from that of T. diversifolia (P< 0.05) at the same exposure period, according to paired t-test.

4.2.3 Fumigant toxicity of essential oils of *T. vogelii* and *T. diversifolia* against *S. zeamais*

The essential oils of *T. diversifolia* and *T. vogelii* exhibited fumigant toxicity against *S. zeamais*. Compared to the negative control treatment, all the dosages were significantly active against the maize weevils for the oils of the two plants ($P < 0.05$, Fisher LSD test, $R^2 = 70.9$, $F= 22.8$) (Table 4.6). The LC_{50} for the *T. diversifolia* oil after 24-hour exposure was $1.5\times10^{1} \pm 7.0\times10^{0}$ µL/L (33.5 ± 6.0 mg/L of air) while that of *T. vogelii* was $3.9\times10^{1} \pm 1.5\times10^{1}$µL/L (39.0 ± 15.0 mg/L of air). Compared with the positive control, methyl bromide (LC_{50} = 0.43 ± 0.11 mg/L of air), *T. diversifolia* oil after 24 hours was about 78 times less toxic than methyl bromide fumigant against *S. zeamais* while *T. vogelii* was about 91 times less toxic than this synthetic fumigant. This means that *T. diversifolia* oil has a slightly higher fumigant efficacy against *S. zeamais* compared to that of *T. vogelii*. The higher fumigant mortality was further observed when *T. diversifolia* oil could achieve 100% mortality in the 60th hour at a concentration of 80 µL/L. For which at this exposure time, only 57.8 ± 21.2% mean mortality was attained for *T. vogelii* oil. The cumulative mortalities at either the 84th or 96th hour when 40 µL/L and 80 µL/L was treated to weevils for the two oils were however not very different ($P < 0.05$, t-test). This would probably mean that with the increase in concentration at a certain point, the efficacy of the two plants against *S. zeamais* does not differ.

Table 4.6: Mean percentage mortality of weevils due to fumigant toxicity of *T. vogelii* (TV) and *T. diversifolia* (TD) essential oils

Time (h)		20 µL/L	40 µL/L	80 µL/L	0µL/L	Regression Equation	R^2	LC_{50} (mg/Lair)	**SE of LC_{50}**	**95%FL** $\times10^{1}$
6	TD	17.8±2.9	30.0±1.9	46.7±7.7	0.0±0.0a	y = 2.7 +1.4x	0.999	3.9×10^{1}	2.4×10^{1}	1.5-6.3
	TV	4.4±4.4	10.0±6.9	1.1±1.1b		y = 0.5 –1.2x	0.295	2.2×10^{-4}	1.5×10^{-4}	(0.75-3.7)$\times10^{-5}$
12	TD	30.0±2.9	57.8±10.6	70.0±15.8	1.1±1.1a	y = 2.8 +1.7x	0.953	1.5×10^{1}	7.9×10^{0}	0.7-2.3
	TV	5.6±4.0	24.4±4.8b	16.7±1.9b		y = -2.2 +1.7x	0.334	1.5×10^{-4}	2.8×10^{2}	$<2.8\times10^{2}$
24	TD	37.8±9.5	63.3±9.6	75.6±12.2	1.1±1.1a	y = 3.0 +1.7x	0.979	1.1×10^{1}	5.2×10^{0}	0.6-1.6
	TV	10±5.1b	48.9±4.0	42.2±13.1b		y = 2.2 +1.8x	0.634	3.9×10^{1}	1.5×10^{1}	2.4-5.4

36	TD	44.9±10.4	84.2±8.1	91.0±4.9	1.1±1.1a	y = 2.6 +2.4x	0.907	8.6×10^{0}	2.5×10^{0}	0.6-1.1
	TV	14.4±6.8b	60.0±3.3b	51.1±15.7b		y = 2.3 +1.8x	1.000	2.8×10^{1}	1.4×10^{1}	1.4-4.2
48	TD	50.6±14.4	87.6±6.8	95.5±2.9	1.1±1.1a	y =2.3 +2.9x	0.963	7.7×10^{0}	1.6×10^{0}	0.6-0.9
	TV	15.6±5.9b	64.4±4.0b	55.6±14.4b		y = 2.4 +1.9x	0.616	2.5×10^{1}	9.2×10^{0}	1.5-3.4
60	TD	58.4±14.8	93.3±3.8	98.9±1.1	1.1±1.1a	y = 1.7 +3.5x	0.987	7.3×10^{0}	1.4×10^{0}	0.6-0.9
	TV	17.8±6.2b	66.7±3.3b	57.8±12.2b		y = 2.5 +1.9x	0.613	2.3×10^{1}	8.2×10^{0}	1.4-3.1
72	TD	65.3±14.7	95.5±2.9	98.9±1.1	1.1±1.1a	y = 2.3 +3.2x	0.949	5.9×10^{0}	1.2×10^{0}	0.5-0.7
	TV	20.0±8.4b	70.0±3.8b	57.8±12.2b		y = 2.7 +1.7x	0.535	2.1×10^{1}	1.1×10^{1}	1.0-3.2
84	TD	68.7±14.5	95.5±2.9	98.9±1.1	2.3±1.1a	y = 2.6 +3.0x	0.957	5.4×10^{0}	1.2×10^{0}	0.4-0.7
	TV	29.2±19.1b	77.8±4.0	73.3±15.0		y = 2.8 +1.9x	0.648	1.4×10^{1}	5.7×10^{0}	0.9-2.0
96	TD	72.1±16.5	95.5±2.9	98.9±1.1	6.9±5.3b	y = 2.8 +2.9x	0.963	5.0×10^{0}	1.1×10^{0}	0.4-0.6
	TV	36.8±21.6b	81.0±3.9	78.9±14.5		y = 3.0 +1.9x	0.704	1.2×10^{1}	4.5×10^{0}	0.7-1.6
	MeBr							4.3×10^{-1}*	1.1×10^{-1}	38.0-49.0

Results are presented as mean values ± SEM. Values in the same column for the negative control followed by the same letters are not significantly different (P<0.05) (Fisher LSD test). LC_{50}= lethal concentration 50% (µL/L). * Data from Wang et al, 2015. Density of *T. vogelii*; TV =1.001 ±0.024, density of *T. diversifolia*; TD= 0.860±0.019. FL = fiducial limits. [b] *Mean mortality of T. vogelii differs significantly from that of T. diversifolia (P< 0.05) at the same exposure period, according to paired t-test.*

Both *T. diversifolia* (LC_{50} = 14.5 µL/L) and *T. vogelii* LC_{50} = 38.6 µL/L) essential oils showed higher efficacy against *S. zeamais* than the essential oils of *Minthostachys verticillata* (LC_{50} = 28.2 µL/L air) and *Eucalyptus globulus* (LC_{50} = 335.7 µL/L air) against the same pest at 24 hours (Arena et al., 2017). The oils of these plants were also stronger fumigant than those of *Hyptis spiciger*; LC_{50} = 48.11 µL/Lair at 48 h (Othira et al., 2009).

4.2.4 Repellency and toxicity potential of the major components found in the oils of the two plants against maize weevils

Monoterpenoids, sesquiterpenes, and alcohols have been proven to contribute to the repellent properties of essential oils (Sathantriphop et al. 2015). The major oil constituents of the two plants xylene, ethylbenzene and farnesol (for *T. vogelii*) and α-pinene (for *T. diversifolia* oil) were evaluated for repellent effect after 2h exposure to obtain their corresponding median repellent dose (RD_{50}) (Table 4.7) and then compared with that of the positive control. Results show that α-pinene exhibited higher repellency than o-xylene, farnesol and ethylbenzene. A similar effect was noticed with that of the positive control. Ethylbenzene and farnesol however were more potent than o-xylene in repelling *S. zeamais* after 2h exposure.

Table 4.7: RD_{50} ± SE and LC_{50} ± SE data of some major compounds found in the oils of the two plants

Repellency potential			
Compound	**RD_{50}±SE ($\mu L/cm^2$)**	**R**	**95%FL**
Farnesol	1.15±0.56[b]	0.732	1.71-0.59
α-Pinene	0.41±0.58	0.899	≤ 0.99
o-Xylene	21.5±103.9[b]	0.806	≤ 125.4
Ethylbenzene	1.47±0.17[b]	0.999	1.67-1.30
IR3535[*]	0.31±0.08	0.962	0.39-0.26
Toxicity potential			
Compound	**RD_{50}±SE**	**R**	**95% FL**
	µL/L air (mg/L)		
α-Pinene	18.12±3.08	0.777	15.04-21.39
	(15.55±2.64)[b]		(12.91-18.19)
o-Xylene	7.31±0.49	0.950	6.82-7.80
	(6.43±0.43)[b]		(6.00-6.86)
MeBr[**]	(0.43±0.15)		(0.58-0.69)

[b] *Value differs significantly from that of positive control (P< 0.05) according to paired t-test.* *Data from Nerio et al, 2009, ** Data from Wang et al, 2015. Density: *α*-Pinene =0.858g/mL, o-Xylene =0.879. FL = fiducial limits

This would indicate that *T. diversifolia* oil would show a higher repellency effect than *T. vogelii* given the higher percentage these compounds contributed to the overall composition of the oil. The results however indicated otherwise. The higher repellency effect of *T. vogelii* essential oil is therefore attributed to the joint effect of the components (synergistic effect) against the weevils whose over all effect brought about the higher repellent effect of *T.*

vogelii essential oil. In addition, this report and the previous reports also indicate that α-pinene and limonene as components of many essential oils exhibit some repellent potential (Bendera, 2007). *Tephrosia vogelii* leaves essential oils were mostly dominated by alkylbenzenes especially the xylenes (47.9±3.8% of total oil composition) whose repellent effect is found to be more at a higher dose (Class II, at 80µL/L). Not to mention their alcohols (> 13.6% of total oil) and aldehydes (>4.6%) present an enhanced repellent effect. Some of these compounds including aromatic hydrocarbons (alkylbenzenes), linalool, caryophyllene oxide and α-terpineol that were more represented in *T. vogelii* essential oils than in *T. diversifolia*. This implied that their joint effect was instrumental in bringing about higher repellency. Wang et al, (2015) showed that 4-terpineol; repellency (62%) and D-limonene (78%), evaluated against *S. zeamais* after 4 h exposure, showed high repellency (Class IV). Compounds that are oxygenated and have the hydroxyl group connected to a primary, secondary or aromatic carbon such as linalool, perillaldehyde, geraniol, citronellal, caryophyllene oxide, and myrcene have also been found to have repellent activities in arthropods (Nerio et al. 2010). Some of these compounds have been evaluated for repellent or fumigant activities or both against *Sitophilus* species (Lee et al., 2001). The fumigant toxicities of α- pinene (the major constituent in *T. diversifolia* (31.1±4.7%)) and *o*-xylene (major constituent in *T. vogelii* essential oil, 25.6±2.3); were evaluated against the adult *S. zeamais* after 24 hours of exposure. Their LC_{50} values were compared with that of the positive control, methyl bromide. Results indicate that o-xylene was more toxic than *α*-pinene. *o*-Xylene was however 15 times less toxic than that of methyl bromide. *α*-Pinene was about 36 times less toxic than methyl bromide. Fumigation toxicity data also indicated that *o*-xylene was dose dependent against the weevil while *α*-pinene was effective at all the concentrations. This perhaps explains the higher fumigant effect of *T. diversifolia* oils since o-xylene was miserably low in this oil. Additionally, toxicity would also be due to the synergistic effect of all the fumigant potent compounds against *S. zeamais*. Wang et al, (2015) established that D-Limonene (LC_{50} = 4.55 mg/L air) exhibited stronger fumigant toxicity against *S. zeamais* than *α*-terpineol (LC_{50} = 20.15 mg/L air) and 4-terpineol (LC_{50} =14.94 mg/L air). Additionally, Herrera et al., (2014) showed that compounds like R-carvone (LC_{50} =17.56 ±2.56 µL/L air), *S*-carvone (LC_{50} =28.10 ±5.16 µL/L air), *α*-thujene (LC_{50} = 65.53±7.87 µL/L air) and ocimenone (LC_{50} = 43.30 ±4.25µL/L air) were fumigants. Some report also indicates that pinene and myrcene and many monoterpene vapors have fumigant effects on many insects (Cook and Hain 1988). The huge amount of pinene in the *T. diversifolia* oil (close to 40% of total oil) and synergistic effect in the oils could, therefore, be

responsible for the higher fumigant toxicity effects of this oil compared to that of *T. vogelii*. Furthermore, Yildirim et al. (2013) established that linalool, terpinen-4-ol, *α*-terpineol (alcohols group), epoxides group such as limonene oxide, esters group (neryl acetate) and ketonic and aldehydic group oils like carvone, citronellal, could attain 100 % mortality of *S. zeamais* after the 96th hour of exposure when compounds were individually applied. The report further noted that monoterpene hydrocarbons were found to have a lower toxicity effect than those of oxygenated monoterpene especially of the ketone and aldehyde and epoxide (Yildirim et al., 2013). The lower contact toxicity of both oils is attributed to the lower sensitivity of weevils towards these oils. Additionally, studies have linked xylene isomers to organismal toxicity, particularly to giant cockroach *Blaberus giganteus* L. (Maliszewska and Tęgowska, 2018). This was also reflected in the toxicity against *S. zeamais*.

It should be noted that the results obtained here for investigating insecticidal and repellence potential of the oils of two plants and the standard compound may suffer from the shortcomings of experiment designs used here although these designs have been used for decades in insect biology. Figure 4.1 shows the experimental setup for repellency and toxicity effects of essential oils against weevils. In the repellency design, a choice design was used while for toxicity studies, a no choice experimental set up was used. For petri dish assay design, the petri dish is very small and weevil behaviour will be affected when exposed for a very long time. Within minutes the air in the petri dish will saturate and the choice will essentially be between a slightly lower and slightly higher concentration of the test samples. Within hours the two sides may be rather indistinguishable. Additionally, bioassays used with multiple individuals whose aggregation behaviour is well-known is nearly impossible to establish whether individuals have settled in one part of the dish because of an environmental preference across all individuals in the group, or because of one or two individuals who can release aggregation pheromone and/or other queues which caused the others to aggregate due to conspecific attraction. This confounds results. It is therefore recommended that repellency assay designs different from the petri dish assay design such as olfactory design be used in future investigative work on the pesticidal activities for a better comparison of results. The no-choice design for insecticidal activity means that weevils are left with no choice so, in case of suffocation, they may die. This therefore is not due to the toxicity effect of the substance.

a b

e

Figure 4.1: Set up of repellence and insecticidal experiments: (a) repellence bioassay, (b) fumigant toxicity bioassay and contact toxicity bioassay (c)

4.3 Effects of *T. diversifolia* treated samples on feeding deterrence and nutritional indices against weevils

4.3.1 Effect of the volatile substances on feeding deterrence and nutritional indices

The feeding deterrence index (FDI) and the nutritional indices: relative growth rate (RGR), relative consumption rate (RCR), the efficiency of conversion of ingested food (ECI) for essential oils are shown in Table 4.8. The possibility of feeding deterrence effect in the oil whose concentration is increasing may be noticed by the reduction in the growth rate of weevils and disk consumption without affecting the efficiency of conversion of the ingested food i.e. a behavioural action) (Dethier et al., 1960; Beck, 1965). If however there is a drop in the efficiency of conversion of ingested food then it is a post-ingestive toxicity effect that

occurs (Xie et al., 1996). The essential oil of *T. diversifolia* did not significantly affect the nutritional and feeding deterrence indices and mortality of maize weevils ($P > 0.05$) suggesting that the oil did not inhibit feeding and the growth of weevils for the tested concentration range of 0 to 0.29 µL/mg of flour disks.

Table 4.8: Effects of essential oils of *T. diversifolia* on RGR, RCR, ECI and its FDI against *S. zeamais*

Concn (uL/g)	D(mg)	X(mg)	Y(mg)	(RGR) mg/mg day-1	RCR mg/mg day-1	ECI%	FDI*100	percentage dead
0.00	21.77±6.91a	2.92±0.04a	3.08±0.10a	-0.02±0.01a	2.32±0.70a	-0.70±0.34a		4.00±2.31a
0.03	20.97±1.45a	3.01±0.05a	3.23±0.12a	-0.02±0.01a	2.18±0.21a	-1.14±0.49a	-41.38±71.90a	2.67±1.33a
0.06	18.70±1.47a	2.98±0.10a	3.08±0.10a	-0.01±0.00a	2.02±0.15a	-0.58±3.60a	-20.64±55.80a	2.67±1.33a
0.11	12.03±3.02a	2.83±0.09a	3.11±0.24a	-0.03±0.03a	1.35±0.40a	-4.91±5.26a	13.49±49.29a	4.23±2.5a
P > F (model)	0.4	0.407	0.874	0.939	0.431	0.628	0.813	0.903
F	1.30	1.09	0.23	0.13	1.03	0.61	0.22	0.19
Sig.	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**

Data expressed as means±SE of 2 replicates. RGR = Relative Growth Rate; RCR = Relative Consumption Rate; EC1 = Efficiency of Conversion of Ingested Food; FDI = Feeding Deterrence Index. *Values sharing the same letters in the column means they are not significantly different from each other (P < 0.05) (Fisher LSD test).*

To establish the intensity of feeding deterrence of diets, Liu et al. (2007) adopted criteria to classify diets to insects i.e. no feeding deterrence (FDI < 20%), weak (50% > FDI ≥ 20%), moderate (70% > FDI ≥ 50%) and strong (FDI ≥70%). Based on this classification, the essential oil of *T. diversifolia* did not have a feeding deterrence effect. This could be due to the absence or low composition of well-known feeding deterrents of genus *Sitophilus* which act especially through the synergism effect such as 1,8-cineole, linalool, α-terpineol, eugenol, carvacrol and thymol (Koul et al., 2008). *α*-Pinene, *β*-pinene, *α*-phellandrene, *α*-thujene and D-limonene which dominated the essential oil of *T. diversifolia* have not yet been known for feeding deterrent activity against *Sitophilus* species. In addition this, the essential oil was not toxic to weevils, $LC_{50} = 8.3\times10^{3} \pm 3.1\times10^{6}$ µL/g (Regression equation; $Y = 0.225x + 3.441$, $R^2 = 0.715$). Compared with the positive control; delthametrin ($LC_{50} = 0.41 \pm 0.15$ µL/g) this oil was over 20,000 times less toxic when impregnated on the flour disks. The feeding deterrence effect of essential oil of *T. diversifolia*, when compared to that of other plants, reveals different scenarios. For example, *Evodia rutaecarpa* Hook f. et Thomas oil showed feeding deterrent activity and had an effect on nutritional indices against *S. zeamais* (Liu and.

Ho, 1999). The essential oil reduced the RGR and RCR of *S. zeamais* at a concentration of 1.5 mg/disk and 3 mg/disk respectively (Liu and. Ho, 1999). In the same study (Liu and. Ho, 1999), the essential oil of *E. rutaecarpa* inhibited the RGR and RCR of *S. zeamais* at a concentration of 1.5 mg/disk and 2.2 mg/disk respectively. In a related study, the essential oil of *Croton rudolphianus* Müll. Arg. leaves was toxic to *S. zeamais* (LC_{50} = 102.66±9.55 µL/g) upon ingestion and had effects on RCR, relative biomass gain, and ECR (de Araújo Ribeiro, et al., 2020). The effect of feeding deterrence of volatile substances like that of *T. diversifolia* may sometimes seem difficult to distinguish from that of repellence if an appropriate experimental design is not set up. In this study, the effort was taken to segregate the two by using a no-choice experimental design for feeding deterrence.

4.3.1 Effects of the non-volatile substances of *T. diversifolia* on feeding deterrence and nutritional indices

The feeding deterrence of the crude extracts, fractions and the pure isolated compounds was determined to establish whether specificity against *S. zeamais* by specific compounds would improve the efficacy over the activity of the crude extract. Table 4.9 shows the results of their feeding deterrence and effect on nutritional indices. Following the criteria by Liu et al. (2007), the crude extract exhibited weak feeding deterrence only at 5% (w/w). Fraction F1 could show a moderate feeding deterrence effect at 10% w/w and a weak one at 5%w/w. The rest of the treatment concentrations did not show significant feeding deterrence against the maize weevils. Similarly, Fraction 3 (F3) was not significantly active at all concentrations given that the FDI was less than 20%. Generally, the crude and fractions could only show feeding deterrence effect at a treatment level of 10%w/w and not often at 5% w/w. Neemazal extract that contains 10% azadirachtin could exhibit a feeding deterrence effect at all concentrations except at 0.5%w/w. Sandaracopimaradiene-1α,9α-diol, deacetylviguiestenin and 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid showed weak to moderate and even strong feeding deterrence against *S. zeamais* at certain concentrations. Sandaracopimaradiene-1α,9α-diol showed FDI of 81.19±5.94% at 0.1%w/w compared to that of neemazal extract at 10%w/w (containing 10% of the bioactive compound) (97.45±0.43). The EC_{50} of 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid; 18.27±13.58 mg/g of food (3.65±2.72 mg/mL or 3654.28± 2715.09 ppm) (Table 4.10) was lower than that of other diets except for fraction 5 (F5) (0.41±0.22 mg/g of food) and azadirachtin (0.11±0.05 mg/g of food).

Treatment of different non-volatile diets has also shown effects on RGR, RCR and ECI at different concentrations. The crude extract of *T. diversifolia* inhibited the relative growth rate of weevils and there was a reduction in consumption rate from 0.5%w/w to 1%w/w. At higher concentrations, the reverse was true for RCR. However, the efficiency of ingested food was not affected. These changes signify a behavioural action (feeding deterrence), therefore, the crude extract exhibited feeding deterrence activity. Fraction F_1 showed a reduction in the growth rate of weevils and the utilisation of food. The ECR was feasibly not affected, indicating manifestation of feeding deterrence potential. The weevils would die of starvation rather than post ingestive toxicity effect. Fraction F_2 resulted in a slight increase in RGR but consumption of food was not affected by an increase in its concentration in the diet given to weevils. The ECR increased from 1%w/w concentration to 10%w/w. This implies that the feeding deterrence effect was not manifested significantly. Fraction 3(F3) did not reduce the relative food consumption, but there was an increase in relative growth rate with reduced utilisation of food. This was not a manifestation of antifeedant activity (behavioural change) but rather a growth-regulating change (physiological change). The negative value of FDI at a lower concentration of 0.5%w/w for fraction F_3 means that the diet was stimulating feeding. Similarly, fraction F4 did not reduce diet consumption at all tested concentrations and did not affect the growth rate of weevils. The available data do not indicate a drop or increase in ECI. However, its EC_{50} value (98.5 ± 158.00 mg/g food) (Table 4.10) indicates that fraction F4 was many times weaker than the commercial antifeedant, azadirachtin. F5 did not affect disk consumption and inhibited the relative growth rate of the weevils. The EC_{50} value of 0.41±0.22 mg/g of food compared to that of the positive control indicates that F5 was weaker than azadirachtin but exhibited a very good deterrence effect. F6 similarly showed weaker feeding deterrence activity. There was no clear trend of a decrease in RGR with any effect on RCR in F6. This was more of a physiological change.

Table 4.9: Effects of non-volatile substances of *T. diversifolia* on nutritional indices and their feeding deterrence index against *S. zeamais*

Test sample	% w/w	D(mg)	X(mg)	Y(mg)	RGR mg/mg mean weight day-1	RCR mg/mg mean weight day-1	ECI%	FDI*100
Control	0	0.38±0.11defg	1.92±0.11b	2.31±0.12bcdefg	-0.06±0.01cd	0.06±0.02cde	-35.29±4.62	
Crude	10	0.63±0.45cde	2.21±0.46ab	2.55±0.28ab	-0.05±0.03bcd	0.07±0.05cde	-4.16±4.77ab	8.28±32.32bcde
(C)	5	0.25±0.12defg	2.07±0.13ab	2.44±0.06abcde	-0.05±0.02bcd	0.03±0.02de	-6.43±1.22b	48.82±9.50abcde
	1	0.05±0.01fg	1.78±0.12b	2.28±0.15bcdefg	-0.07±0.00d	0.01±0.00e	-9.73±0.29b	*
	0.5	0.14±0.11efg	1.84±0.13b	2.32±0.26bcdefg	-0.07±0.01d	0.02±0.01de	*	23.12±62.04abcde
F_1	10	0.13±0.17defg	2.23±0.44ab	2.50±0.27abc	-0.04±0.03abcd	0.02±0.02de	-2.13±2.17ab	65.79±15.28abcde
	5	0.19±0.11defg	2.01±0.09b	2.40±0.05abcdef	-0.05±0.01bcd	0.03±0.01de	-16.62±16.54	41.35±9.65abcde
	1	1.87±0.02ab	2.02±0.17b	2.00±0.14bcdefg	0.00±0.01abc	0.31±0.02a	1.51±1.83ab	-15.07±43.72e
	0.5	0.33±0.17defg	1.87±0.15b	2.45±0.41bcdefg	*	*	-69.05±17.22	*
F_2	10	0.27±0.34defg	2.19±0.35ab	2.35±0.17bcdefg	-0.03±0.03abcd	0.04±0.04de	27.98±1.57a	55.65±22.17abcde
	5	0.19±0.32defg	1.93±0.10b	2.34±0.06bcdefg	-0.06±0.01cd	0.03±0.04de	*	20.24±29.86abcde
	1	0.92±0.40bc	1.71±0.07b	1.78±0.29g	0.00±0.06abc		19.81±13.25ab	17.76±46.73abcde
	0.5	0.11±0.04efg	1.98±0.17b	2.47±0.25abcd	-0.07±0.01d	0.02±0.01de	*	39.35±17.73abcde
F_3	10	0.38±0.22defg	2.80±0.90a	2.41±0.28abcdef	0.03±0.08a	0.05±0.02de	*	15.11±39.78bcde
	5	0.06±0.03fg	1.93±0.10b	2.25±0.10bcdefg	-0.05±0.00bcd	0.01±0.01e	-26.44±15.92	*
	1	0.26±0.05defg	1.76±0.10b	2.09±0.06bcdefg	-0.05±0.01bcd	0.01±0.03e	*	17.30±13.30abcde
	0.5	0.28±0.18defg	1.95±0.09b	2.39±0.18abcdef	-0.06±0.01cd	0.05±0.03de	-47.85±5.62	-28.44±55.63e
F_4	10	0.09±0.12defg	1.90±0.33b	2.53±0.28ab	-0.09±0.02d	0.01±0.01e	-9.04±0.00b	76.32±20.46abcde
	5	0.18±0.13defg	1.84±0.04b	2.46±0.16abcde	-0.08±0.01d	0.02±d0.02e	-26.52±40.62	12.06±23.61bcde
	1	0.19±0.02defg	1.86±0.08b	2.33±0.10bcdefg	-0.07±0.02d	0.03±0.00de	*	*
	0.5	0.25±0.13defg	1.72± 0.10b	2.37±0.12bcdef	-0.09±0.01d	0.04±0.02de	*	16.67±16.67abcde
F_5	10	0.56±0.21cdef	2.07±0.24b	2.50±0.31abc	-0.06±0.01cd	0.07±0.02cde	-66.87±4.12	33.45±9.70abcde
	5	0.25±0.08defg	1.94±0.07b	2.44±0.10abcde	-0.07±0.00d	0.03±0.01de	-23.75±0.21	31.93±6.65abcde
	1	0.29±0.08defg	1.82±0.15b	2.33±0.03bcdefg	-0.07±0.02d	0.04±0.01de	*	*

	0.5	0.30±0.15defg	1.96±0.13b	2.48±0.24abc	-0.07±0.01d	0.05±0.03de	-38.42±0.00	0.89±33.33cde
F_6	10	0.15±0.01defg	2.30±0.16ab	2.97±0.05a	-0.08±0.02d	0.02±0.00de	*	25.76±62.94abcde
	5	0.15±0.06defg	1.91±0.10b	2.34±0.05bcdefg	-0.06±0.01cd	0.02±0.01de	-16.50±22.17	37.88±30.81abcde
	1	1.18±0.34b	1.87±0.15b	1.89±0.15defg	*	*	-1.70±28.37ab	-7.86±34.35de
	0.5	0.09±0.06fg	1.89±0.20b	2.32±0.28bcdefg	-0.06±0.01cd	0.02±0.01de	*	*
NK1F4	10	0.03±0.09defg	2.10±0.28ab	2.39±0.24abcdef	-0.04±0.02abcd	0.00±0.01e	*	92.11±15.23ab
	5	0.01±0.01g	1.77±0.19b	2.08±0.63bcdefg	-0.03±0.06abcd	0.00±0.00e	*	48.52±0.00 abcde
	1	0.20±0.02defg	1.89±0.06b	2.36±0.08bcdefg	-0.07±0.01d	0.03±0.00de	-2.32±0.40ab	46.38±5.02abcde
	0.5	0.30±0.10defg	1.89±0.00b	2.12±0.00bcdefg	-0.04±0.00abcd	0.05±0.02cde	-0.87±0.29ab	21.01±21.01abcde
NK3F4	0.1	0.10±0.04efg	1.67±0.01b	1.94±0.09cdefg	-0.03±0.01abcd	0.01±0.01e	*	81.19±5.94ab
NK1F5	10	0.67±0.17bcd	1.87±0.09b	1.87±0.20efg	0.01±0.02ab	0.14±0.04bc	1.97±9.51ab	43.03±30.60abcde
	5	0.25±0.08defg	1.68±0.09b	2.22±0.14bcdefg	-0.08±0.00d	0.04±0.01de	-2.53±1.03ab	38.41±10.87abcde
	1	0.29±0.08efg	1.82±0.15b	2.33±0.03bcdefg	-0.07±0.02d	0.04±0.01de	-1.79±0.21ab	16.43±20.92bcde
	0.5	0.48±0.42cdefg	1.62±0.14b	1.85±0.15fg	-0.04±0.05abcd	0.09±0.08cd	-4.04±4.11ab	*
Neemazal (azadirachtin, 10%)	10	0.00±0.01g	1.80±0.23b	1.81±0.17fg	-0.01±0.01bcde	0.00±0.01e	-1.51±0.53ab	97.45±0.43a
	5	0.05±0.02fg	1.74± 0.14b	1.79±0.32g	-0.01±0.02bcde	0.01±0.00e	0.01±0.00bc	75.62±13.32ab
	1	0.17±0.06defg	2.13±0.22ab	1.91±0.31cdefg	0.04±0.03a	0.03±0.02de	0.02±0.01bc	40.12±12.71 abcde
	0.5	0.18±0.08defg	2.20±0.10ab	2.02±0.04bcdefg	0.03±0.02a	0.03±0.21de	0.05±0.08bc	19.3±15.10 abcde
P > F		<0.000	0.81	0.14	0.11	<0.0001	0.000	0.008
F		2.34	0.78	1.31	1.35	3.23	3.04	0.80
Sig.		Yes	No	No	No	Yes	Yes	yes
LSD		0.28	0.39	0.31	0.04	0.04	18.10	59.33
R^2 (model)		0.46	0.22	0.32	0.33	0.54	0.68	0.27

NK1F4= 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid, **NK3F4**= sandaracopimaradiene-1*α*,9*α*-diol, **NK1F5=** deacetylviguiestenin. *Data not available. (RGR = Relative Growth Rate; RCR = Relative Consumption Rate; EC1 = Efficiency of Conversion of Ingested food; FDI = Feeding Deterrence Index). *Data expressed as means ± SE of 2 replicates. Values sharing the same letters in the column means they are not significantly different from each other (P < 0.05) (Fisher LSD test). Positive values expressed a feeding deterrent effect and negative values expressed a feeding stimulant effect.*

Table 4.10: Effective concentrations for 50% deterrence (EC_{50}) for non-volatile substance of *T. diversifolia* against *S. zeamais*

	Ec_{50} ppm (mg/g)	SE	R^2	Regression equation	χ^2	p-value
Crude extract	1.01×10^{5} (5.04×10^{2})	1.40×10^{7} (7.00×10^{4})	0.403	y = 0.33x +3.33	8.63	0.003
F_1	1.02×10^{4} (5.10×10^{1})	1.96×10^{4} (9.80×10^{1})	0.836	y = 0.87x +1.51	0.62	0.432
F_2	3.58×10^{5} (1.79×10^{3})	5.03×10^{4} (2.52×10^{2})	0.601	y = 0.91x +0.95	10.67	0.005
F_3	4.16×10^{4} (2.08×10^{2})	6.52×10^{4} (3.26×10^{2})	0.287	y = 0.92x +0.75	10.52	0.001
F_4	1.97×10^{4} (9.85×10^{1})	3.51×10^{4} (1.58×10^{2})	0.351	y = 0.90x +1.15	6.71	0.010
F_5	8.26×10^{1b} (4.13×10^{-1})	4.38×10^{1} (2.19×10^{-1})	0.957	y = 1.56x +1.94	2.21	0.137
F_6	2.06×10^{4} (1.03×10^{2})	6.88×10^{3} (3.44×10^{1})	0.829	y = 2.14x -4.23	5.86	0.015
$NK1F_4$	3.65×10^{3} (1.83×10^{1})	2.72×10^{3} (1.36×10^{1})	0.760	y = 1.35x +0.20	3.52	0.172
$NK1F_5$	2.21×10^{4} (1.11×10^{2})	9.10×10^{3} (4.55×10^{1})	0.819	y = 1.97x +2.35	1.26	0.263
Azadirachtin	1.46×10^{1} (1.00×10^{-1})	5.56×10^{0} (5.00×10^{-2})	0.959	y = 1.95x +2.73	1.35	0.508

EC_{50} = the concentration needed to inhibit insect feeding by 50% relative to the negative control. Data is presented as mean of EC_{50}± standard error (SE) in ppm or mg/g of food (in parentheses)

[b] Value does not differ significantly from that of the positive control (P< 0.05) according to paired t-test.

The consumption of the flour disks allowed the comparison of the different compounds' efficacy that was extracted from *T diversifolia*. The amount of the disk consumed was expressed as a percentage of the negative control (Fig. 4.2). Generally, F3, F4, F5, 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid, deacetylviguiestenin and neemazal extract (positive control) experienced a reduction in the amount of the disk with a concentration increase.

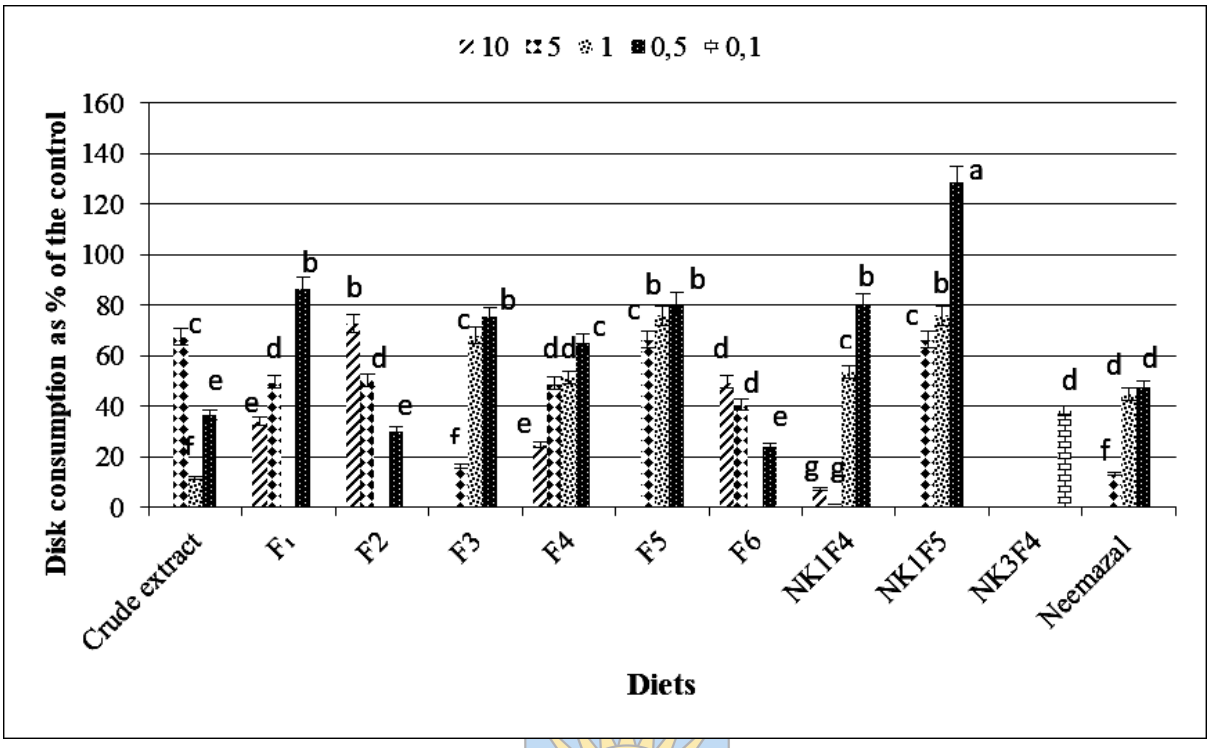


Figure 4.2: Disc consumption by *S. zeamais* expressed as a percentage (%) of the control sample for different treatments (10, 5, 0.5, or 0.1%w/w) of the sample

Percentages in the same column followed by the same letters do not differ significantly (P > 0.05) in ANOVA and LSD.

Besides, they showed a mean diet relative consumption below that of the negative control (with 100% consumption). The crude extract of *T. diversifolia* and fraction F1 only showed this at 0.5% and 1% concentrations. Fractions F2 and F6 did not show the dependence of concentration on an increase in the amount of disk consumed. This concentration independence would mean that their feeding deterrence was a result of the synergistic effects from their constituents. The fractions would also be possessing feeding stimulating characteristics since the amount of disk consumption increased with an increase in their concentration. It was also noted that for *T. diversifolia*, some isolated compounds such as 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid and sandaracopimaradiene-1α,9α-diol exhibited higher efficacy specificity against *S. zeamais* as compared to the fraction from which they were isolated. Deacetylviguiestin and 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid inhibited the growth of weevils and reduced the consumption of food without a drop in the efficiency of conversion of ingested food. This was a clear manifestation of the feeding deterrence effect. Deacetylviguiestin (EC_{50} = 22140.23± 9103.29 ppm) was a less active feeding deterrent compared to 3-(4-*O*-

(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid (EC_{50} = 3654.28± 2715.09 ppm). Deacetylviguiestin and 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid were both less effective against *S. zeamais* as compared to the commercial anti-feedant, azadirachtin (EC_{50} = 14.59±5.59 ppm). Skimmianine isolated from *Orixa japonica* Thunb showed an EC_{50} of 1000 ppm against *S. zeamais* adults (Yajima et al., 1977). This shows that skimmianine is about 3.6 times more effective than 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid and 22 times more effective than deacetylviguiestin. Liu et al., (2002) showed that fraxinellone (EC_{50} = 71.2 ppm) and dictamnine (EC_{50} = 91.7 ppm) also significantly reduced the RGR and RCR of *S. zeamais* at concentrations of at least 30 ppm. Their FDI at 30-300 ppm varied between 30.8% to 77.8% and 30.9% to 68.1% respectively for the *S. zeamais* adults (Liu et al., 2002). In comparison with this study, fraxinellone and dictamnine are more active as feeding deterrents as compared to deacetylviguiestin and 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid but appears to be far less active than the isolated diterpene; sandaracopimaradiene-1α,9α-diol (FDI at 0.1%w/w is 81.19±5.94%).

In this study, the no-choice experimental design used in evaluating feeding deterrents could have far-reaching consequences on the death of weevils. Here weevils were left with no choice implying that insects were forced to either eat the food or starve to death (absolute deterrence). In such circumstances, the extent of feeding deterrence of the diet against insects and mortality was compromised. Figure 4.3 shows the results of the percentage mortality of weevils. The mortality for these samples may be attributed to starvation as a result of weevils avoiding food, external factors such as temperature difference leading to high heat or internally due to post-ingestion toxicity. Generally, there was no statistical difference in mortality due to the treatment from that of the negative control ($P > 0.05$) except in a few exceptions such as due to fractions F3, F4 and deacetylviguiestin.

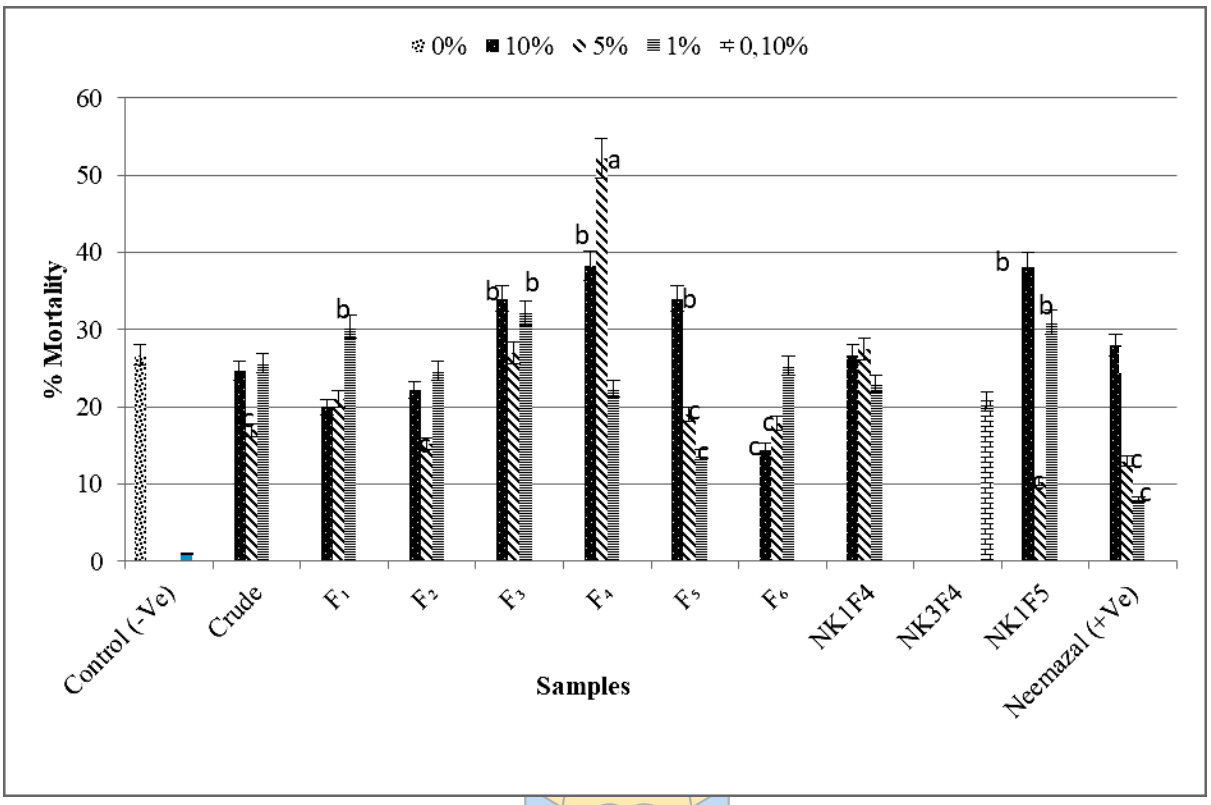


Figure 4.3: Mean percentage (%) mortality of weevils in the treated samples and control.

Means with letters are different from that of the control. Mean mortalities with different letters are statistically different (P < 0.05) in ANOVA and LSD.

There were also some differences in the toxicities on the weevils upon treatment. Lower concentrations for F5 and neemazal extract were less toxic compared to the negative control. For F6, it was its higher concentrations that were less toxic. From all these observations, the mortality due to post-ingestion effects remains unclear. It is therefore paramount to investigate the post-ingestion toxicity of the treated samples against the weevils. That is, whether, upon ingestion, weevils could die from the toxic effects or recover.

In this study, the weighing method was used in a no-choice set up (Figure 4.4). Although the gravimetric technique is believed to be highly accurate and could lead to reproducibility, weevils were left with no-choice implying that insects were forced to either eat the food or starve to death. In such circumstances, the extent of feeding deterrence of the diet against insects was compromised. Therefore the use of other assays such as leaf disc assay, glass fibre assay, wafer disc e.t.c would generate comparative data and ascertain the level of accuracy.

Figure 4.4: Feeding deterrence setup for: essential oils (A) non-volatile sample (B) of *T. diversifolia*

4.4 Evaluation of the antioxidant potential of *T. diversifolia* non-volatile substances

The phytochemical study of phenolic compounds in the crude, fractions and isolated compounds of *T. diversifolia* (section 3.4) revealed the presence of anti-oxidant compounds. The quantity of individual phenolic compounds is still not yet known. For purposes of estimating the phenolic compounds, this section aims at investigating the total phenolic content and flavonoid activity and their relationship with total anti-oxidant potential. It should be noted that an enormous amount of work exists about the anti-oxidant assay studies on the anti-oxidant potential of *T. diversifolia* (Ojo et al., 2018, Pantoja Pulido et al., 2017; Orsomando et al., 2016 and Giacomo et al., 2015).

4.4.1 Total anti-oxidant capacity estimation

Phenolics play a vital role in protecting humans from free radicals when they scavenge them through the donation of electrons or hydrogen to stabilize the radical (Gyamfi et al., 1999; Oboh et al., 2007). The DPPH radical scavenging and antioxidant reducing power were used to examine the antioxidant capacity of non-volatile substances of *T. diversifolia.* Figure 4.5 shows a graph of the proportion of percentage DPPH scavenging activity of tested samples. Statistically, scavenging potential of the tested solutions differed ($p < 0.05$, Fisher LSD Test) in the decreasing order: F1 ~F2 ~BHT > ascorbic acid > MeOH crude extract > NK1F4 ~ F3 > F4 ~ F6~F5 > NK1F5. The IC_{50} of BHT, F1 and F2 were lowest followed by that of ascorbic acid and MeOH crude extract (Table 4.11). The lower the IC_{50} the stronger the sample to act as DPPH free radicals scavenger. This means that these fractions had more phenolic free radical scavenging compounds. This is in agreement with the high amount of caffeic acids, quercetin and kaempferol and their derivatives found in F1 and F2 in the chemical analysis study (section 3.4). The differences in the distribution of phenolic compounds affected the anti-oxidant potential of the *T. diversifolia* samples. Many phenolic compounds have been characterised from *T. diversifolia* in this study. F1 and F2 both had kaempferol and quercetin derivatives such as kaempferol-dihexoside, quercetin-glucosylpentoside, quercetin-rhamnosylglucoside, quercetin-3-O-rhamnoside and kaempferol-rhamnosyl glucoside. Caffeic acids and their derivatives were also hugely abundant in these fractions. In addition, protocatechuic acid was also detected. Quercetin and kaempferol (flavonols) are the best anti-oxidant compounds (Agati et al., 2011;

Tattini et al., 2004). Their abundance in F1 and F2 explains the higher anti-oxidant potential because of the more phenolic free radical scavenging. It was also evident that IC_{50} values generally decreased with fractionation. This observation is similar to the one made by Pantoja Pulido et al., (2017). Comparatively, fraction 5, F5 mostly had caffeic acid derivatives and so was fraction 4, F4. Most of the phenolic compounds characterised in these samples are not excellent anti-oxidants and these are no surprise showed small joint free radical scavenging compared to that of the ascorbic acid.

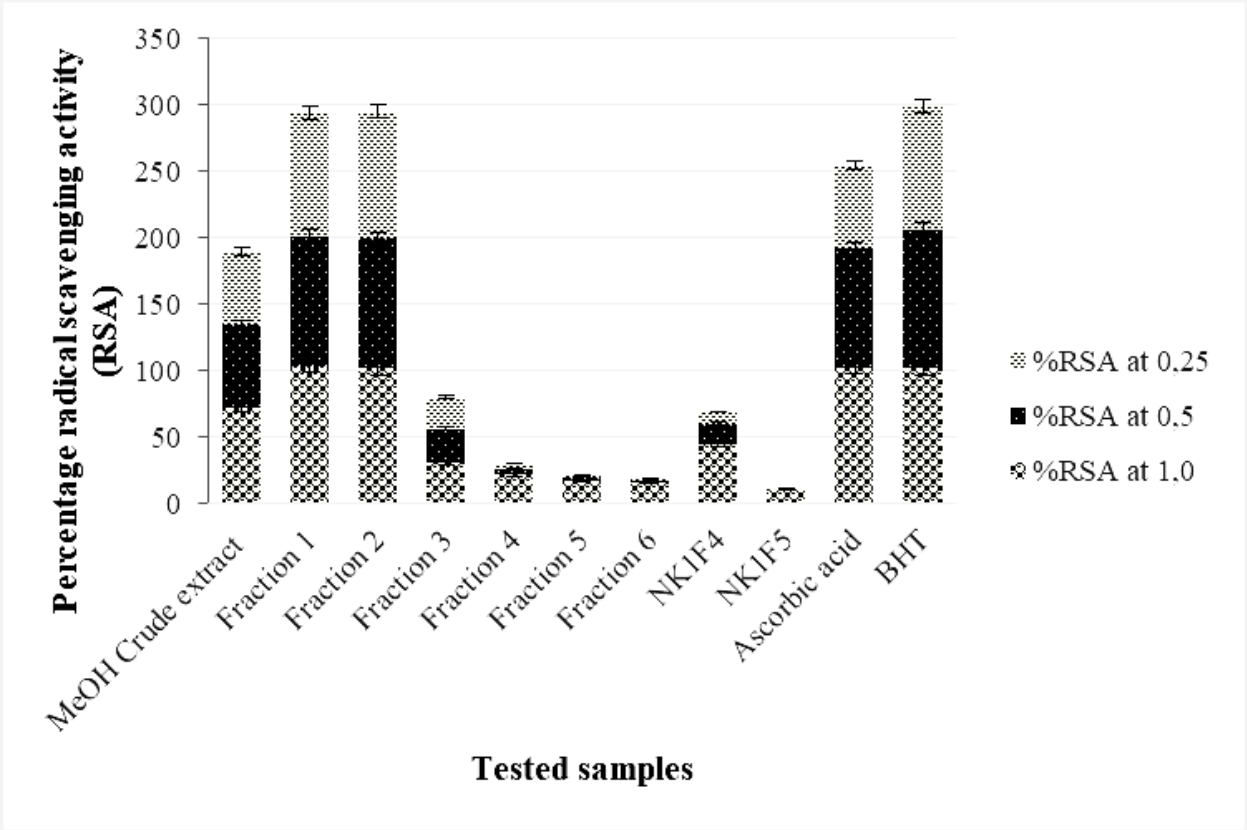


Figure 4.5: Mean DPPH percentage scavenging activity for non volatile samples.

It is evident that IC_{50}, values generally decreased with fractionation. This observation is similar to the one made by Pantoja Pulido et al., (2017). The scavenging activity of various leaf extracts of *T. diversifolia*; aqueous, methanolic and dichloromethane have been previously studied (Giacomo et al., 2015). Giacomo et al., (2015) reported that aqueous extract gave the strongest scavenging effect because of the polar phenolic compounds present in the aqueous extract. Other studies have noted higher radical scavenging activity of the ethanolic extract of *T. diversifolia* (IC_{50} = 0.93 ± 0.20 µg/mL) compared to that of standard ascorbic acid (IC_{50} = 0.48 ± 0.10

µg/mL) (Juang et al., 2014). A study by Giacomo et al., (2015) indicated that aqueous extract scavenged free radicals most (equivalent 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging = 94.89±2.69 mmol Trolox), followed by methanolic and then dichloromethane. The authors further noted that the scavenging activity can be compared to that of 80 mU of superoxide dismutase at 0.04 µg/mL. Similarly, Juang et al., (2014) reported that ethanolic leaf extract scavenged DPPH free radicals with IC_{50} = 0.93±0.20 µg/mL compared to that of the positive control, ascorbic acid IC_{50} = 0.48±0.10 µg/mL. The equivalent ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-radical scavenging activity of ethanolic extract was determined to be 93.09±37.91 µM TEAC (trolox equivalent antioxidant capacity) (Thongsom et al., 2013). In a related report, free radical scavenging potential reached 241.04±11.93 mmol Trolox of dry extraction weight (Hiransai et al., 2016). Although these reports attribute the differences to be due to variation in the phenolic content from *T. diversifolia* extract (Hiransai et al., 2016), it is more than the phenolic content. The essential oils too of *T. diversifolia* scavenged DPPH free radicals (IC_{50} = 108.8 µg/mL) and ABTS (IC_{50} = 41.7 µg/mL) (Orsomando et al., 2016).

Table 4.11: Total antioxidant activity, total phenol content and total flavonoid content of the tested samples of *T. diversifolia*.

Sample	IC_{50}	R^2	AAE*	TPC mg GAE/100g dry sample	TFC RFE/100g of dry sample
MeOH crude extract	0.17±0.00c	0.9839	0.41±0.02cd	220.00±80.00ab	200.00±180.00a
Fraction 1	0.03±0.02d	0.9944	0.59±0.00abc	240.00± 40.00ab	80.00± 0.00b
Fraction 2	0.03±0.02d	0.9418	0.23±0.00d	320.67± 80.00a	80.00± 200.00b
Fraction 3	78.51±17.12abc	0.9347	0.42±0.04cd	200.81± 25.80ab	60.60±96.49b
Fraction 4	12.20 ±1.71bcd	0.8256	0.78±0.05a	280.11± 60.00bc	40.20± 25.27b
Fraction 5	204.74±150.62ab	0.7530	0.63±0.03a	125.00±40.44c	39.50±10.02b
Fraction 6	5.25±0.00bc	0.5036	0.34±0.50c	200.10± 100.00ab	21.90± 41.81bc
NK1F4	1.65±0.51cd	0.7288	0.57±0.05bc	120.00± 20.64c	20.30± 10.23bc
NK1F5	483.69±278.95a	0.8506	0.69±0.07ab	120.00±59.92c	39.50±10.02b
AA	0.16±0.01c	0.9567			
BHT	0.02±0.01d	0.9999			
NC			0.58±0.12bc		
P > F				0.008	0.02
R^2(model)				0.711	0.600

Total phenol content (TPC), total flavonoid content (TFC). Data expressed as means± SEM (n = 5). AA= Ascorbic acid, NC= negative control. *Ferricyanide reducing power (AAE 1mg/mL). *Values sharing the same letters in the same column means they are not significantly different from each other (P < 0.05) (Fisher LSD test).*

Reducing power was measured by donation of electrons in the reduction of Iron (III) to Iron(II) leading to the production of an intense Prussian blue Iron(II) hexacano complex at the absorbance measured at 750nm. The higher the absorbance, the stronger the reducing power, indicating higher antioxidant potential. The reducing power of BHT was highest followed by ascorbic acid owing to the higher absorbance (Fig. 4.6). The samples exhibited a concentration-dependent reducing power profile. At the lowest concentrations, the absorbance values were negative, implying weak reducing activity. Thus the ferric reduction by the tested samples was in the decreasing order: BHT > ascorbic acid > NK1F5 > F4 > NK1F4 > F1 > F3 > negative control > F2 > MeOH crude extract > F6, between 0.4mg/mL to about 1mg/mL. The reducing power effect was expressed as ascorbic acid equivalent (Table 4.11). The ferricyanide reducing power as equivalents of standard ascorbic acid was highest for F2, then MeOH crude extract, F3, NK1F4~F1, NK1F5 and finally F4. Thus deacetylviguiestenin (NK1F5) and 3-((4-*O*-(caffeoyloxy)methyl)-2-methyloxirane-2-carboxylic acid (NK1F4) were weakly active.

The samples exhibited a concentration-dependent reducing power profile (Fig.4.6). At the lowest concentrations, the absorbance values were negative, implying very minimal reducing activity. Results show that the ferricyanide reducing power expressed as equivalents of standard ascorbic acid was generally higher for fractions whose total phenol content (TPC) was higher (Table 4.11). The isolated compounds, NK1F5 (deacetylviguiestenin) and NK1F4 (3-((4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid) were weak reducing agents. The reducing power of fraction 4 (F4) was moderately higher among the *T. diversifolia* samples, even more than that of 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid probably due to synergistic effects of other dicaffeic moieties present in this fraction. The trend of the reducing power effect differs slightly from that of DPPH radical scavenging activity. For example, fraction F1 showed moderate reducing power but the DPPH radical scavenging activity was considerably high similar to that of F2. The reason for the different results is due to their different reaction mechanisms and measurements (Huang et al., 2005). No single method exceptionally estimates total anti-oxidant potential of extracts precisely.

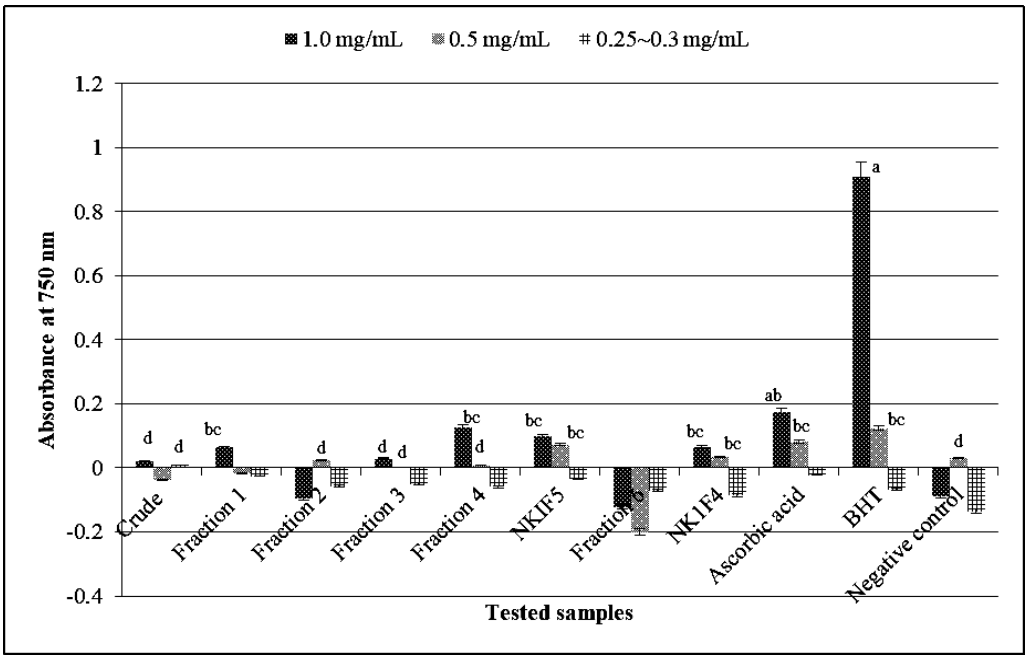


Figure 4.6: Average reducing power of *T. diversifolia* samples at 1.0, 0.5 and 0.3mg/mL

Data expressed as means±SE of 5 replicates of mean values of two independent experiments. Values sharing the same letters in the column means they are not significantly different from each other (P < 0.05) (Fisher LSD test).

In this study, the MeOH crude extract of *T. diversifolia* has shown TPC of 220.00±80.00 and that of TFC was 220.00±180.00 similar to the data put forward by Ojo et al., (2018). The authors reported TPC and TFC to be 251.63 ± 0.47 GAEmg/100 g) and 98.21 ± 0.08 (QEmg/100 g) respectively. The authors further reported high amounts of chlorogenic acid (7.95 ± 0.02 mg g^{-1}), gallic acid (1.28 ± 0.01 mg g^{-1}), p-coumaric acid (0.53 ± 0.01 mg g^{-1}), apigenin (5.09 ± 0.02 mg g^{-1}) and caffeic acid (5.18 ± 0.02 mg g^{-1}) in *T. diversifolia* (Ojo et al., 2018). The present findings show that phenolic content was moderately correlated with both DPPH radical scavenging effect (Spearman, r= 0.31, p=0.422) and reducing power (Spearman, r= 0.28, p=0.472) assays (Table 4.13). These results thus suggest that in the *T. diversifolia* samples analyzed in the current study, about 30% ability to scavenge DPPH radicals and reduce ferric cyanide was probably due to phenolic compounds. Among the phenolic compounds, the flavonoids contributed the largest radical scavenging effect (79%) (Spearman, r= 0.79, p<0.05).

4.3.2 Total phenol and total flavonoid content of *T. diversifolia*

Phenolics play a vital role in protecting humans from free radicals when they scavenge them through the donation of electrons or hydrogen to stabilize the radical (Gyamfi et al., 1999; Oboh et al., 200). Table 4.11 shows the results of total phenol content (TPC) and total flavonoid content (TFC) obtained in this study. The total phenol content (TPC) obtained in this study was in the decreasing order: F2 > F4 > F1 > MeOH crude extract > F6 ~ F3 > NK1F5 > NK1F4. Total flavonoid content (TFC) was in the decreasing order: MeOH crude extract > F2~ F1 > F3 > F4~ F6~ F5~ NK1F5 > NK1F4.

Many phenolic compounds including hydrocinimic acids or chlorogenic acids, and their derivatives, flavonoids and their derivatives, and polyphenols such as saponin and coumarins have been characterised from *T. diversifolia* in this study. In a similar study, an aqueous extract of *T. diversifolia* has previously been shown to possess phenolic acids (gallic acid, chlorogenic acid, caffeic acid and *p*-coumaric acid) and flavonoids (apigenin) (Ojo et al., 2018). The authors reported TPC and TFC to be 251.63 ± 0.47 GAEmg/100 g) and 98.21 ± 0.08 (QEmg/100 g) respectively.

Table 4.12: Correlations between the different assays used in this study.

Correlation	r(Pearson)	r(Spearman's)
DPPH-TPC	0.07	0.31
DPPH-TFC	0.09	0.42
DPPH-Reducing power	0.03	0.19
TPC-TFC	0.47	0.79
TPC-Reducing Power	0.39	0.28
TFC-Reducing power	0.36	0.36

Total flavonoid content (TFC). Total phenol content (TPC), Classification of associations: small = 0.1-0.29, moderate 0.3-0.49, large >0.5. Pearson is for parametric while Spearman's is for non parametric correlations. r= correlation coefficient. Correlation is significant at the 0.05 level (2-tailed) (see Appendix 11).

The present finding shows that phenolic content was moderately correlated with both DPPH radical scavenging effect (Spearman, r = 0.31, p = 0.422) and reducing power (Spearman, r = 0.28, p = 0.472) assays (Table 4.12). These results thus suggest that in the *T. diversifolia* samples analyzed in the current study, about 30% ability to scavenge DPPH radicals and reduce ferric

cyanide was probably due to phenolic compounds. Of the phenolic compounds, the flavonoids contribute the largest radical scavenging effect (79%) (Spearman, r= 0.79, $p<0.05$).

The moderate contribution of phenolic compounds to the total antioxidant potential in the current study is nearly similar to some results obtained in several earlier studies on different plant extracts (Dorman and Hiltunen., 2004; Dastmalchi et al., 2007; Pantoja Pulido et al., 2017). These earlier studies indicate weak associations between total phenol content and DPPH free radical scavenging ability. Velioglu et al., (1998), however, reported a strong correlation between phenolic content and antioxidant activity. In other investigations, authors have found no correlation at all (Kaehkoenen et al., 1999). All these findings reveal that the antioxidant potential of extracts or fractions cannot be predicted on the basis of their total phenolic content. This is because the anti-oxidant properties of phenolic compounds are limited by their chemical structures (Rice-Evans et al., 1996). Flavones and catechins are, in general, better anti-oxidants compared to other polyphenols (for example, lignans and coumarins) in this regard (Agati et al., 2011; Tattini et al., 2004). Additionally, the number of hydroxyl groups and the extent of glycosylation play an additional role in the anti-oxidant activity. Among flavonoids, the antioxidant properties have been enhanced by the presence of a 2, 3 double bond neighbouring the carbonyl group in the C ring, a free hydroxyl group at the 3 position on the C ring and the presence of hydroxyl groups in the 5 and 7 positions on the A ring (Rice-Evans et al., 1996; Pietta, 2000). Flavonoids with these 3-OH groups have displayed maximum radical scavenging activity. For example, quercetin 3-*O* glucoside has exhibited an IC_{50} (DPPH) of 39.3 µM while the IC_{50} of kaempferol 3-*O* glucoside is greater than 500 µM (Tattini et al., 2004). Some reports have indicated substantially higher anti-oxidant properties from resorcinol and phloroglucinol substituents compared to phenol (Rezk et al., 2002). Therefore there are two anti-oxidant pharmacophores recognized in flavonoids i.e. the catechol structure in ring B and ring A and C (Rezk et al., 2002). This is because phenolics with ortho-dihydroxy (catechol) structure are good hydrogen-donating antioxidants. As they are easily oxidized and therefore, are much better radical scavengers than monohydroxy phenolics (Rice-Evans et al., 1996).

Chapter Five

Future work and Conclusion

The oils of *T. vogelii* may be classified as chemotypes due to the significant variation in the concentration of their chemical compounds. Investigation of the chemotypes in the essential oils of *T. vogelii* samples from the eastern part of Uganda has revealed three chemotypes based on the profiles of farnesene compounds; one that possesses the farnesol, and the other that has the springene and *β*-farnesene type and a mixed chemotype of the two; all from the farnesene family. All the chemotypes of essential oils exhibited repellence and fumigant toxicities against S. *zeamais*. There were significant differences in the pesticidal potential of these chemotypes. Evaluation of the repellency effects on these chemical varieties of *T. vogelii* showed that chemotype 2 was the most active and closely followed by chemotype 1 and lastly chemotype 3 in terms of their potent against *S. zeamais*. The difference in the fumigant toxicity and repellency potential against *S. zeamais* could undermine the efficacy of this plant. Although farnesol has been individually tested against *Sitophilus* species (most often *S. oryzae*) and for this current study against *S. zeamais* for repellency, mortality, or both, it is important to establish whether individual compounds of farnesol and springene compounds differ in fumigant and repellency potential. In addition, their contact toxicity and that of the chemotypes needs to be investigated. The complementary part of all other compounds found in the same oils offers a big role in the overall repellent and insecticidal effect of this oil. However, more study is needed that aims to optimize and standardize the chemical varieties and harvesting period needed for recommendation to smallhold farmers especially under field conditions before it can be adopted more widely.

The results of the investigation of the comparative study of the insecticidal and repellent effects of the essential oils of *T. vogelii* with that of *T. diversifolia* show that, both possess considerable insecticidal potential. However, *T. vogelii* essential oil could be promoted more for contact toxicity effect especially when a mixed variety is used whereas *T. diversifolia* better suits fumigation effects against *S. zeamais*. A more satisfactory conclusion would work if pesticidal activities of all *T. vogelii* chemotypes are fully described and their potential compared with that of *T. diversifolia* both in the laboratory and under field conditions.

Investigation of the feeding deterrence of both volatile and non-volatile substances of *T. diversifolia* revealed that its essential oil did not show significant activity. The crude extract, some fractions and the isolated compounds demonstrated feeding deterrence activity against *S. zeamais*. Isolation of pure compounds from *T. diversifolia* could improve efficacy specificity except in circumstances where synergistism occurs. This was demonstrated when pure compounds could exhibit higher feeding deterrence efficacy against maize weevils than fractionated samples from which they were isolated. There were more active fractions in which bioactive compounds were not extensively isolated. Further study should target further isolation of bioactive compounds. The fractionation technique and subsequent identification could also be revised to come up with more bioactive compounds. Investigation of the anti-feedant activity involved gravimetric disc bioassay. Although this technique is believed to be highly accurate and could lead to reproducibility, use of other assays such as leaf disc assay, glass fibre assay, wafer disc e.t.c would generate comparative data and ascertain a high level of accuracy. In addition, bioassay used was a no-choice one implying that insects were forced to either eat the food or starve to death. In such circumstances, the extent of feeding deterrence of the diet against insects was compromised. Further work would also investigate the post-ingestion toxic effects of both volatile and non-volatile components of *T. diversifolia*.

The pesticidal and anti-oxidant activities reported from extracts, fractions and isolated compounds from *T. diversifolia* leaves necessitate the need to isolate and characterise many of its bioactive compounds. Due to its pesticidal properties, this plant can be utilised against agricultural pests. The UPLC-ESI-MS/MS enabled characterisation of many phenolic compounds among them were phenolic acids, caffeic acid, quercetin and kaempferol derivatives including seven new compounds. Some compounds with antioxidant activity such as caffeic acid derivatives could be used as a tyrosinase inhibitor. This makes it possible to avoid the use of synthetic chemical pesticides that pause hazardous and harmful effects on the ecosystem. Fractionation of crude extract helped to concentrate the phenolic compounds resulting in varying antioxidant potential between fractions compared to the crude extract. The isolated compounds (deacetylviguiestenin and 3-(4-*O*-(caffeoyloxy)methyl))-2-methyloxirane-2-carboxylic acid) both had weak antioxidant and antifeedant properties. The DPPH scavenging capacities and reducing power of *T. diversifolia* were partly attributed to its phenolic content from the tested samples in this study. There is a need to carry out comprehensive quantification of individual

phenolic and polyphenolic compounds using several standard compounds. To study the variation of biomolecules from different parts (leaves, stem, flower) of *T. diversifolia* and variation based on the methods of preparation and extraction is very enriching as far as healthy properties are concerned. Additionally, the analysis of compounds using LC-NMR and high resolution LC-MS, simultaneously would improve on the accuracy of identification and characterisation of phenolic compounds. A similar HPLC-MS/MS identification of sesquiterpene lactones could enrich the phytochemical knowledge of *T. diversifolia*. Another future work should target chemical studies on *T. diversifolia* to evaluate the bioactive compounds that help chelate metal complexes, activate antioxidant enzymes, reduce α-toxocopherol radicals and inhibit oxidases using metabolic profiling or metabolomics. And lastly, but not the least, proposed principal fragmentation and metabolic pathways for identified compounds for both phenolic compounds and sesquiterpene lactones is very essential in understanding the chemistry of biological activities of *T. diversifolia*.

Worthwhile to note is that the chemistry of *T. vogelii* is quite well known under certain circumstances as highlighted in the published review about *T. vogelii* and *T. diversifolia*. The chemistry of *T. diversifolia* is still much wanting as discussed in the preceding paragraphs and also in line with the pesticidal application against different pests. In general, specific compound efficacy for specific pest or biological activity would enable a better understanding of the biological activities of these plants.

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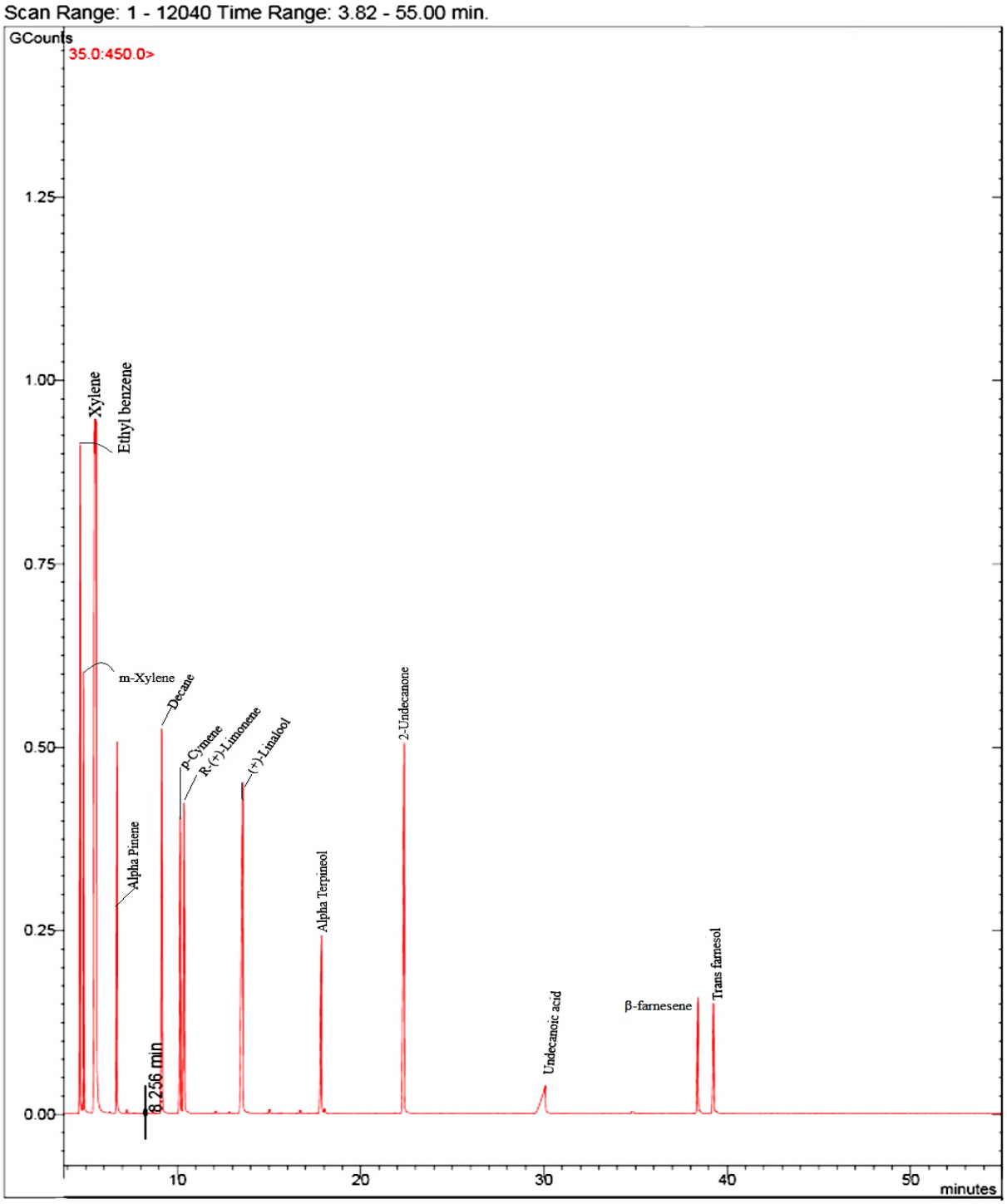
Appendix

Appendix 1*:* GC-MS chromatogram for the mixture of standard compounds



Appedix 2: Pearson correlations of major compounds identified from *T. vogelii* essential oil

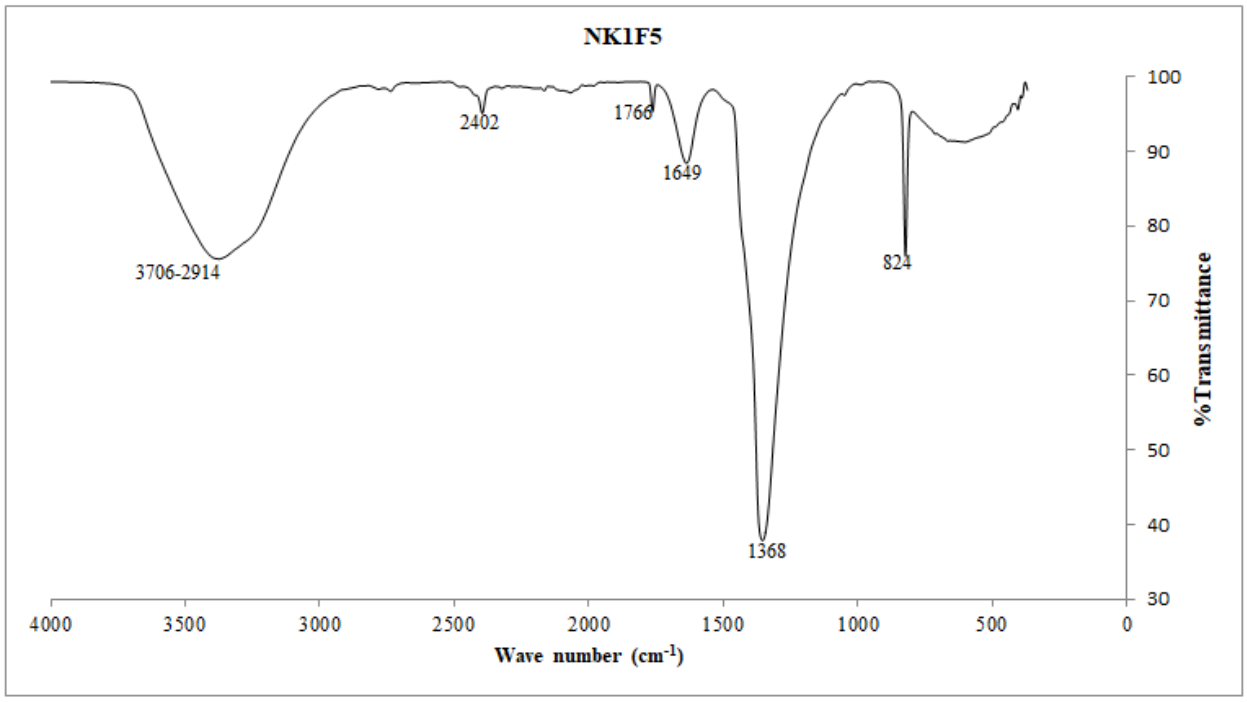
Pearson Correlations

		EB	OX	pX	mX	βF	βS	Fnso	αSp	FnsoEm
EB	Pearson Correlation	1	.974**	.251	.946**	-.215	-.053	.503*	.268	.060
	Sig. (1-tailed)		.000	.150	.000	.189	.415	.014	.134	.404
	N	19	19	19	19	19	19	19	19	19
OX	Pearson Correlation	.974**	1	.305	.925**	-.248	-.089	.587**	.322	.016
	Sig. (1-tailed)	.000		.102	.000	.153	.358	.004	.089	.474
	N	19	19	19	19	19	19	19	19	19
pX	Pearson Correlation	.251	.305	1	.009	-.107	-.224	.611**	.347	-.197
	Sig. (1-tailed)	.150	.102		.486	.331	.178	.003	.073	.210
	N	19	19	19	19	19	19	19	19	19
mX	Pearson Correlation	.946**	.925**	.009	1	-.247	-.110	.431*	.221	.070
	Sig. (1-tailed)	.000	.000	.486		.154	.327	.033	.181	.389
	N	19	19	19	19	19	19	19	19	19
βF	Pearson Correlation	-.215	-.248	-.107	-.247	1	.253	-.217	.078	-.072
	Sig. (1-tailed)	.189	.153	.331	.154		.148	.186	.375	.384
	N	19	19	19	19	19	19	19	19	19
βS	Pearson Correlation	-.053	-.089	-.224	-.110	.253	1	-.246	-.104	-.123
	Sig. (1-tailed)	.415	.358	.178	.327	.148		.155	.336	.307
	N	19	19	19	19	19	19	19	19	19
Fnso	Pearson Correlation	.503*	.587**	.611**	.431*	-.217	-.246	1	.403*	-.249
	Sig. (1-tailed)	.014	.004	.003	.033	.186	.155		.043	.152
	N	19	19	19	19	19	19	19	19	19
αSp	Pearson Correlation	.268	.322	.347	.221	.078	-.104	.403*	1	-.143
	Sig. (1-tailed)	.134	.089	.073	.181	.375	.336	.043		.280
	N	19	19	19	19	19	19	19	19	19
FnsoEm	Pearson Correlation	.060	.016	-.197	.070	-.072	-.123	-.249	-.143	1
	Sig. (1-tailed)	.404	.474	.210	.389	.384	.307	.152	.280	
	N	19	19	19	19	19	19	19	19	19

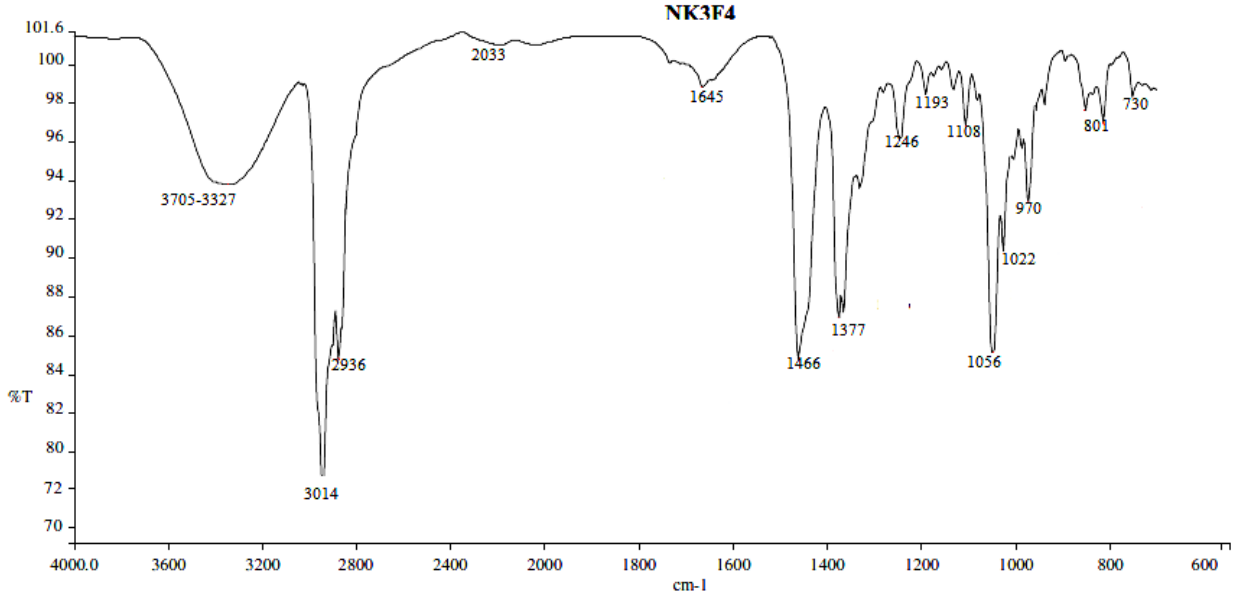
**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

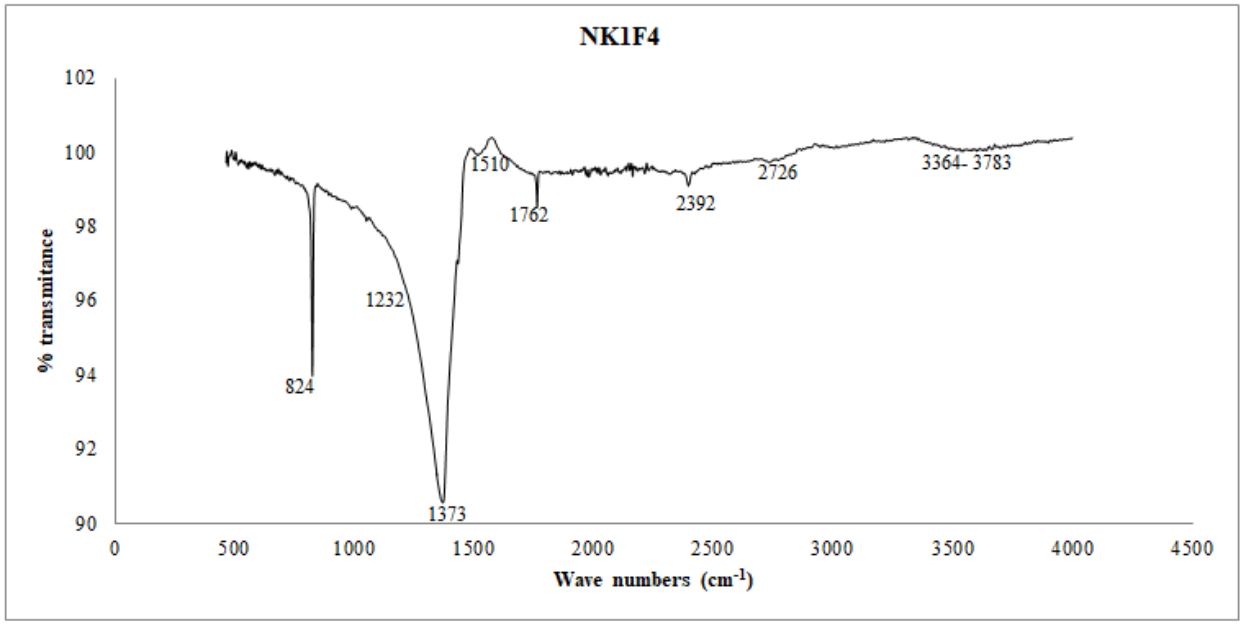
Appedix 3: Infra red spectra for isolated compounds



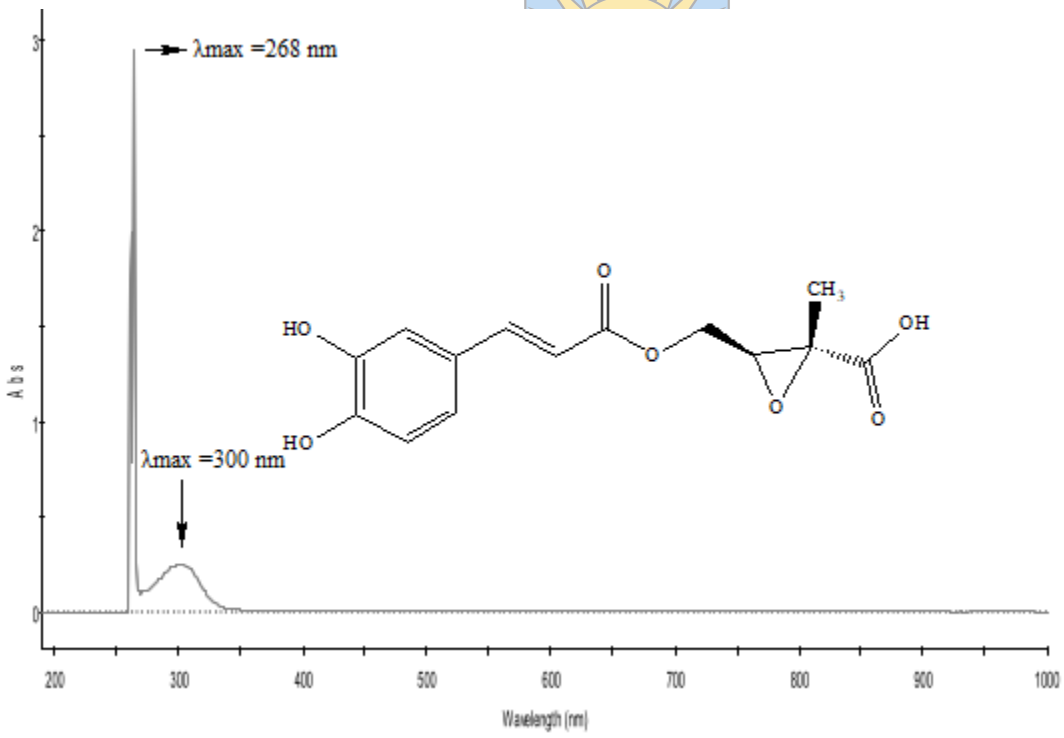
Infra-red spectrum for NK1F5



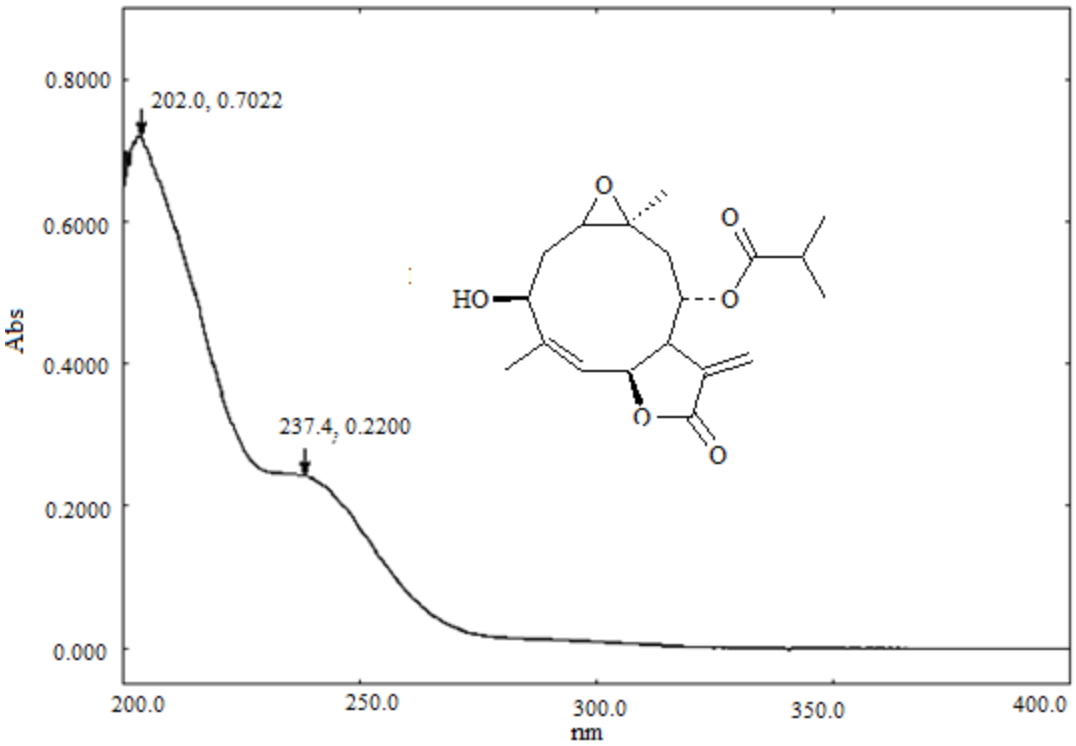
Infra-red spectrum for NK3F4



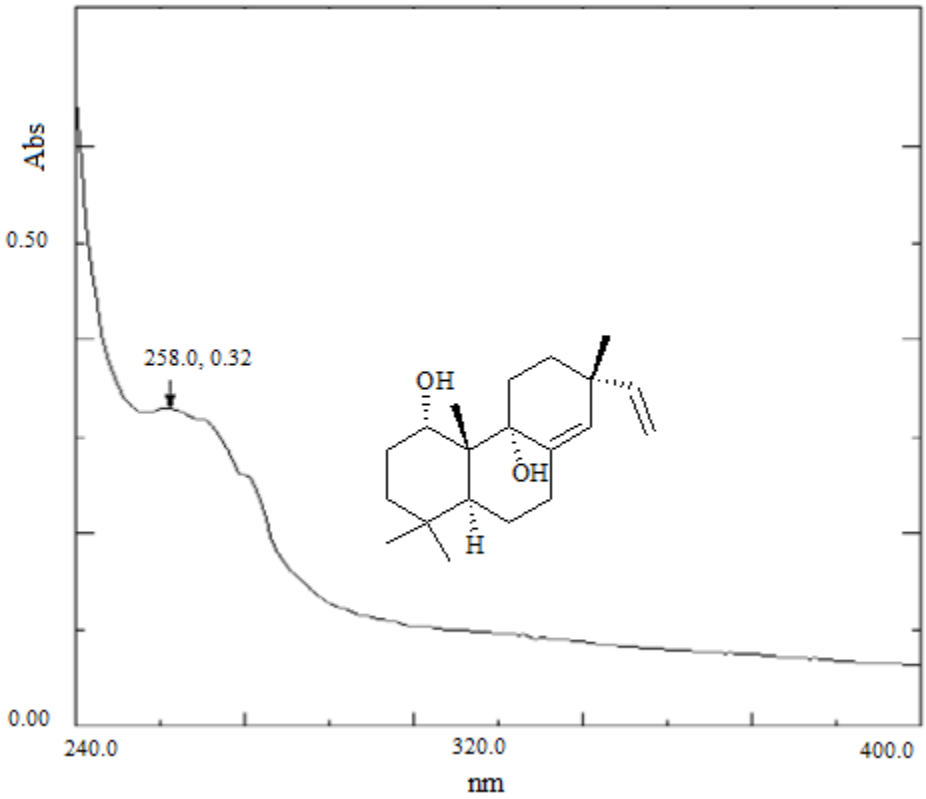
Infra-red spectrum for NK1F4



UV spectrum of NK1F4

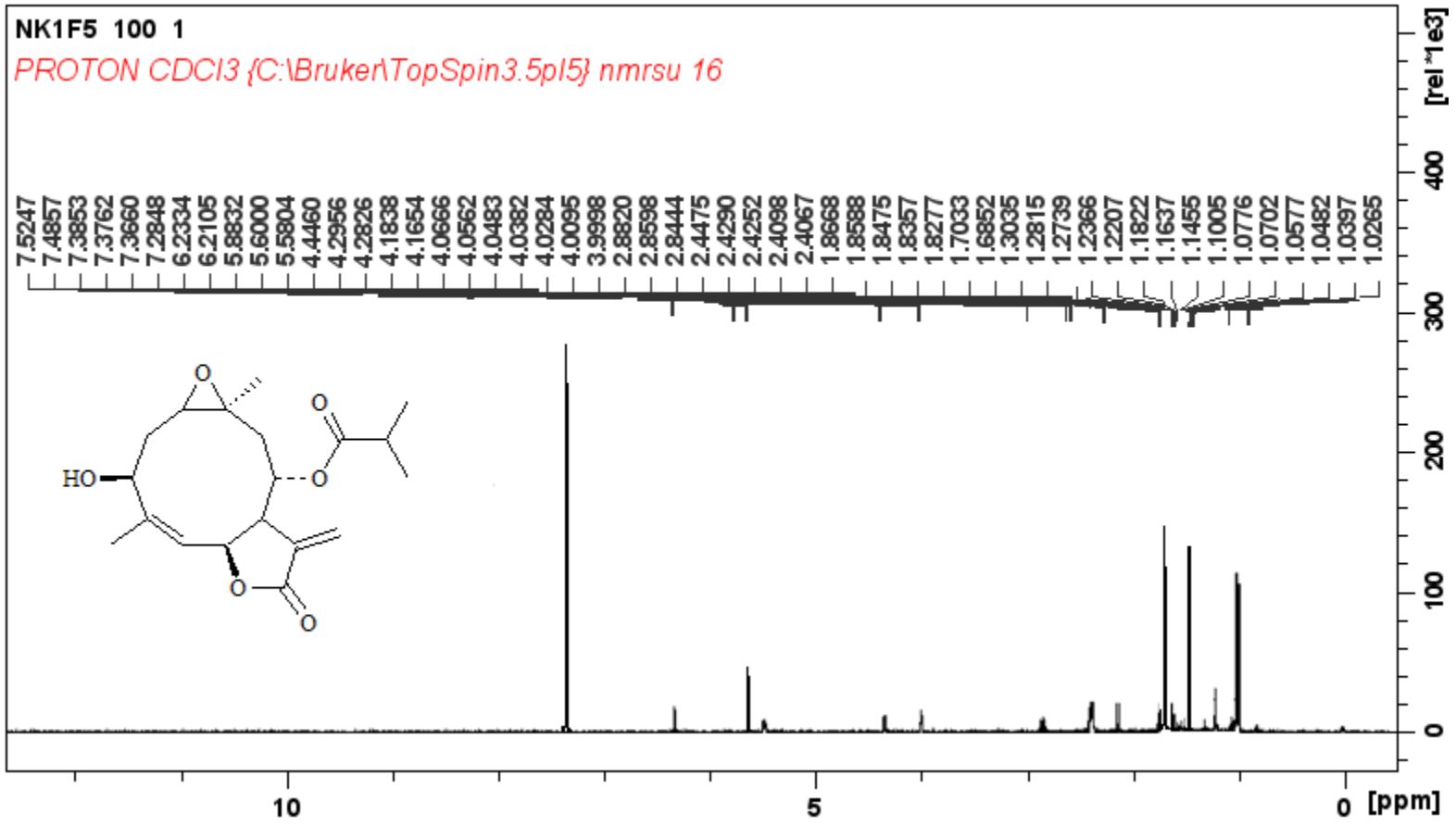


UV spectrum of NK1F5

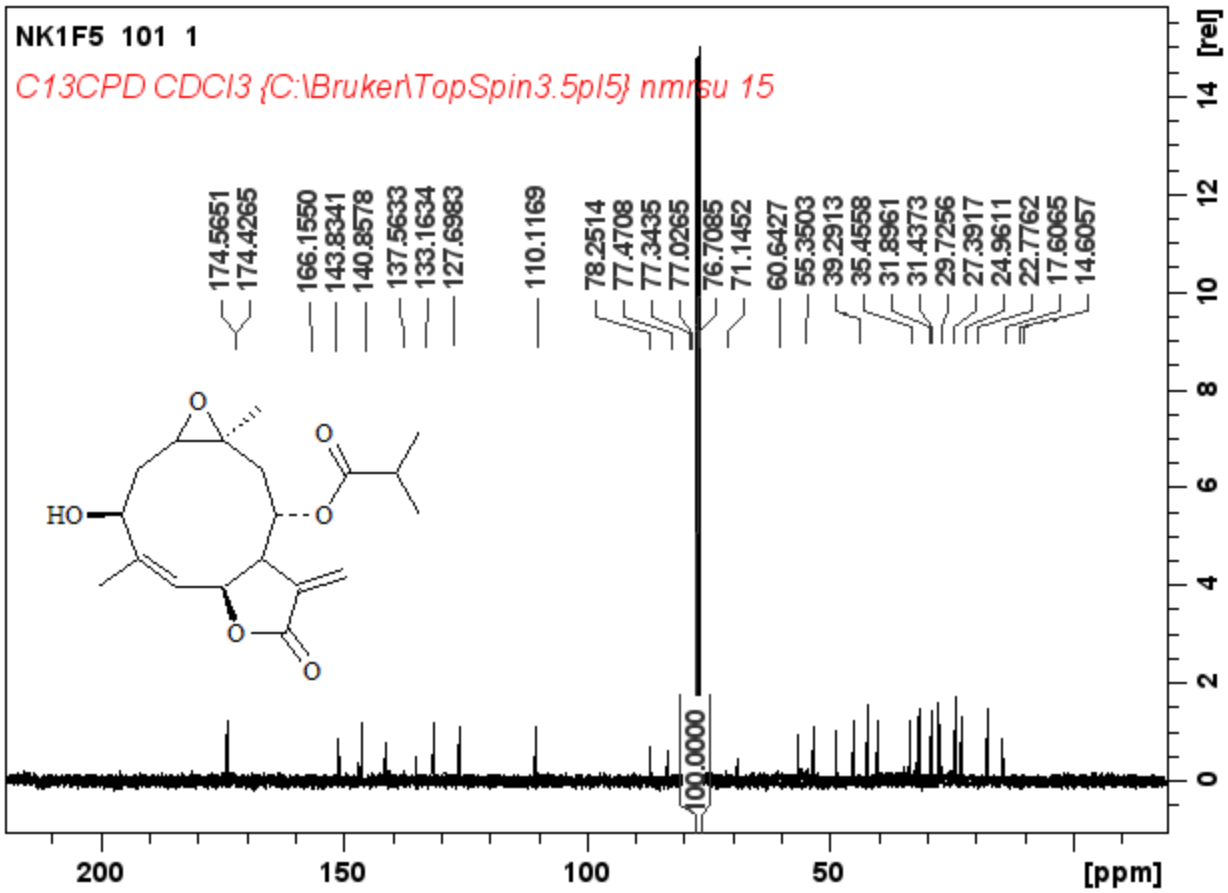


UV spectrum of NK3F4

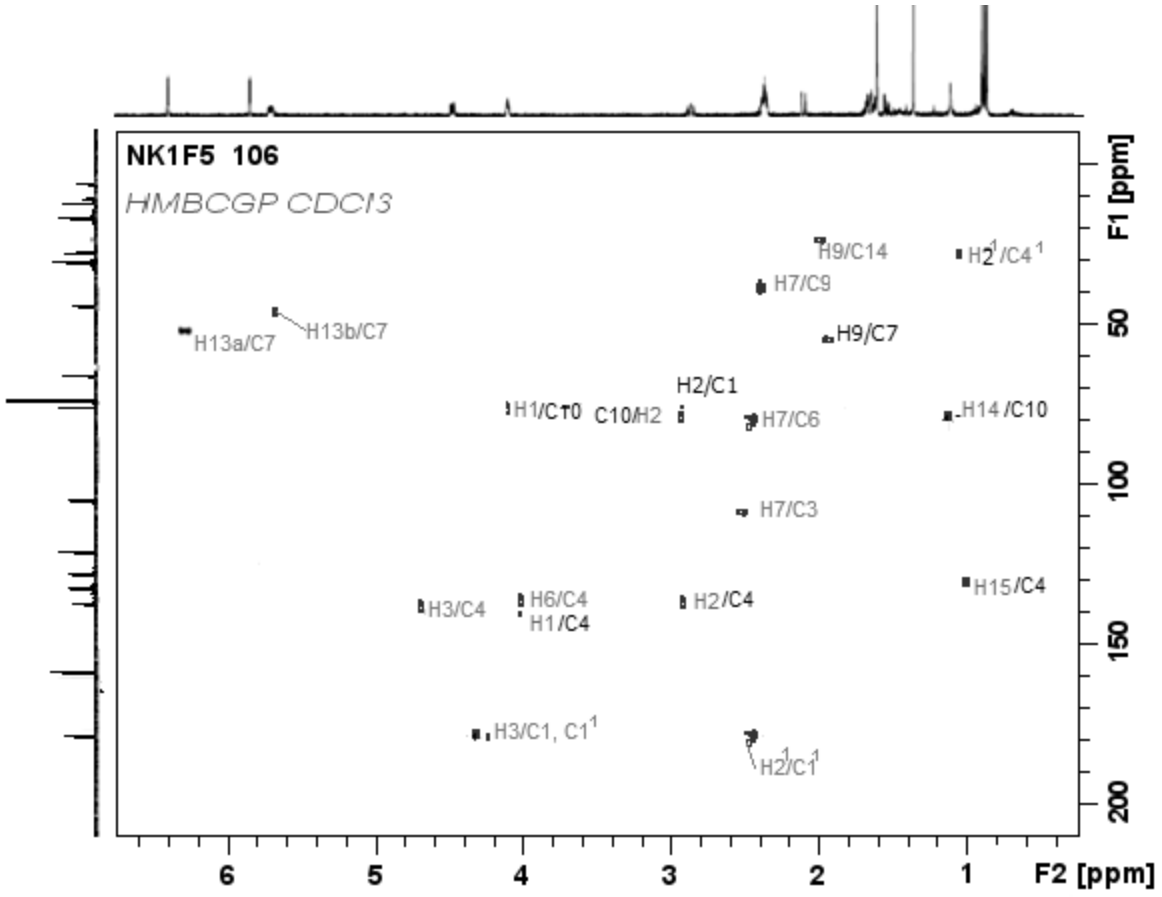
Appedix 4: NMR Spectra (^{1}H and ^{13}C NMR: 1D and 2D) for NK1F5



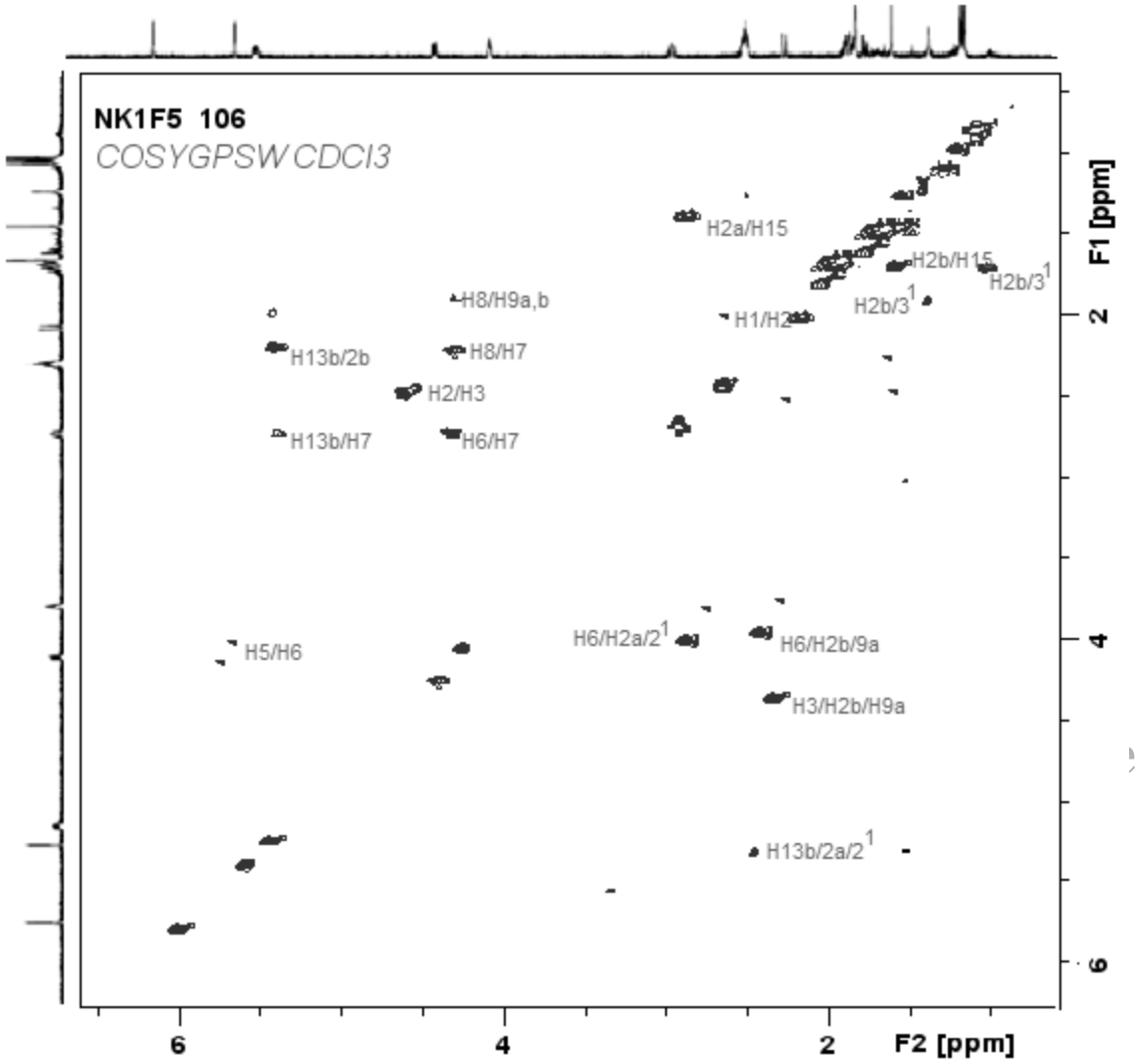
^{1}H NMR (400 MHz, $CDCl_3$) spectrum for NK1F5



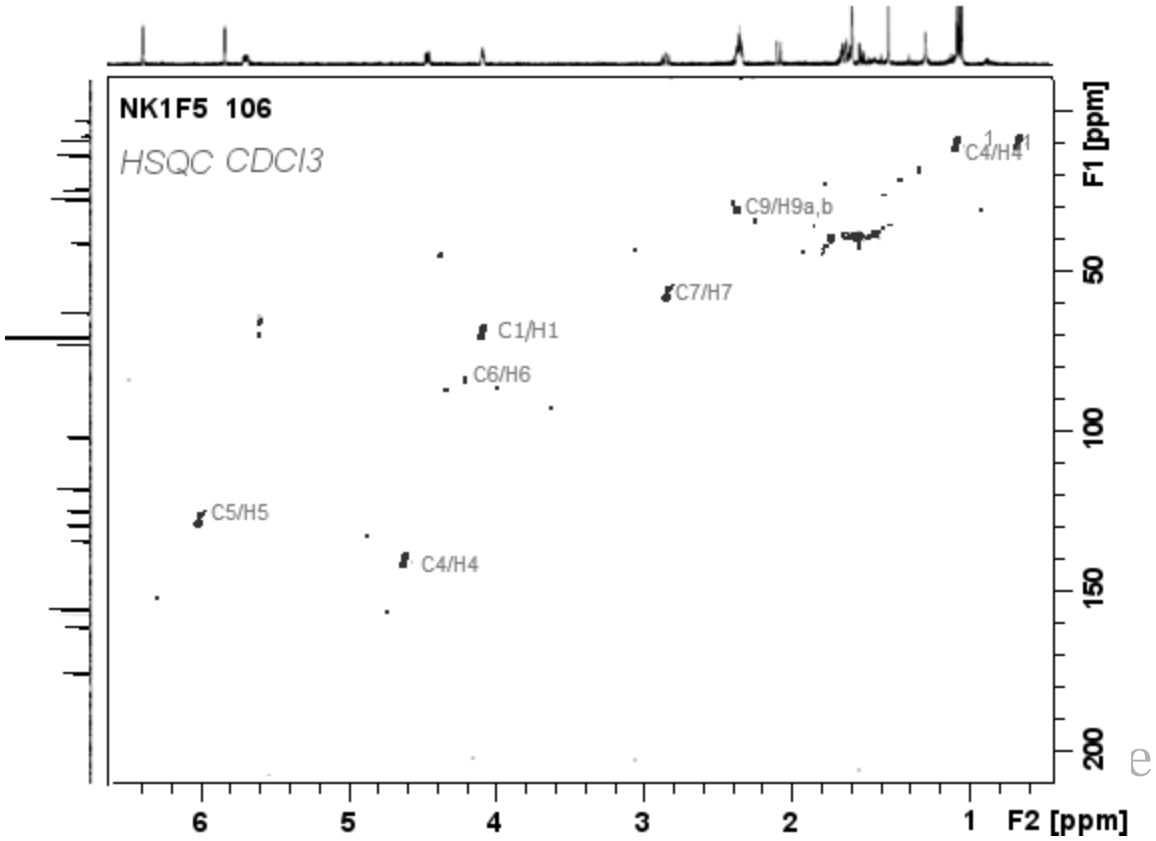
^{13}C NMR (151 MHz, $CDCl_3$) spectrum for NK1F5



HMBC spectrum for NK1F5

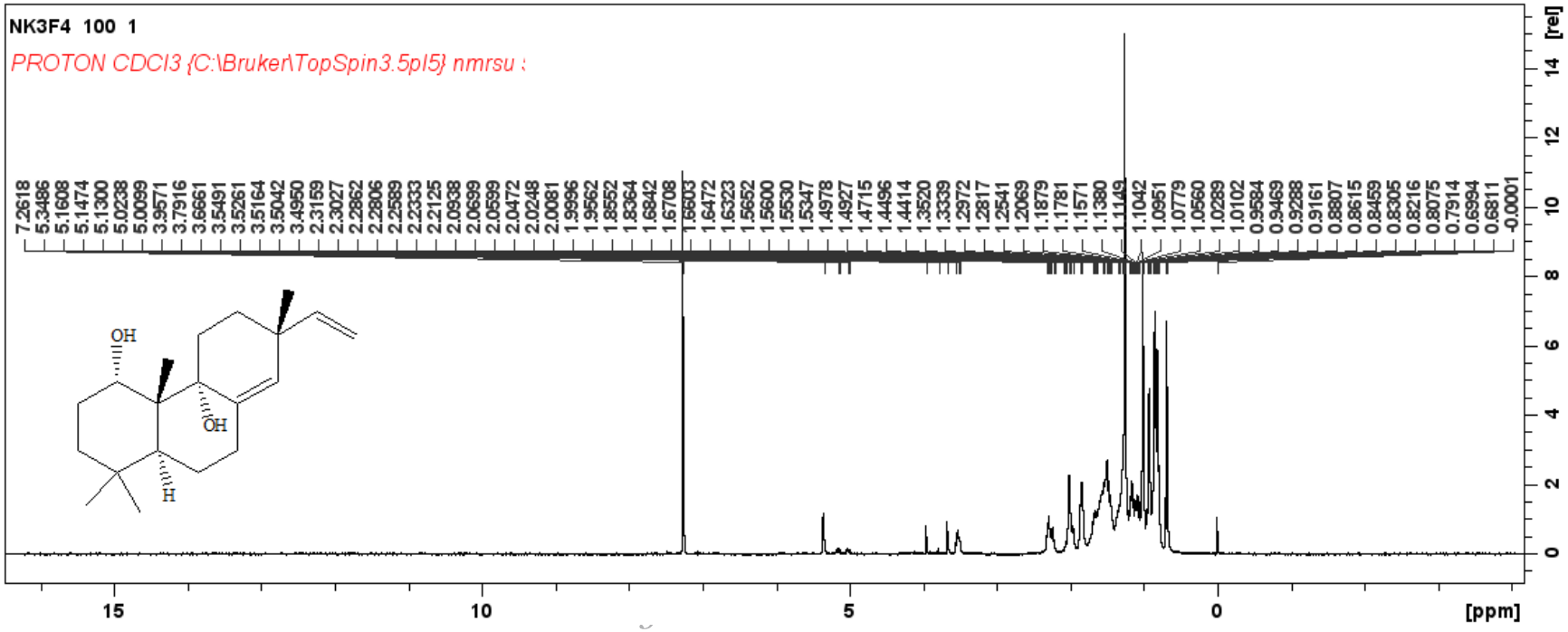


^{1}H-^{1}H Cosy spectrum for NK1F5

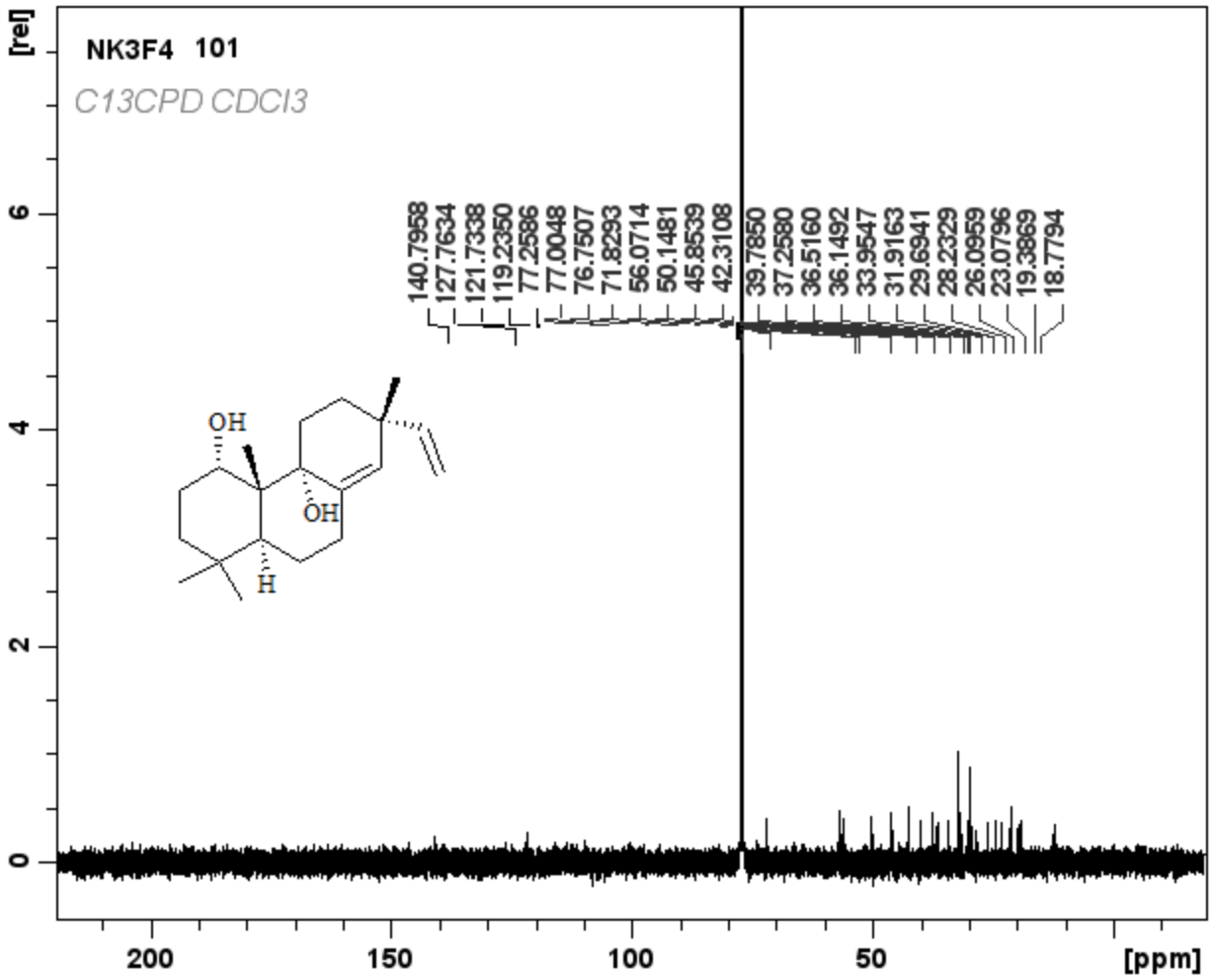


HSQC spectrum for NK1F5

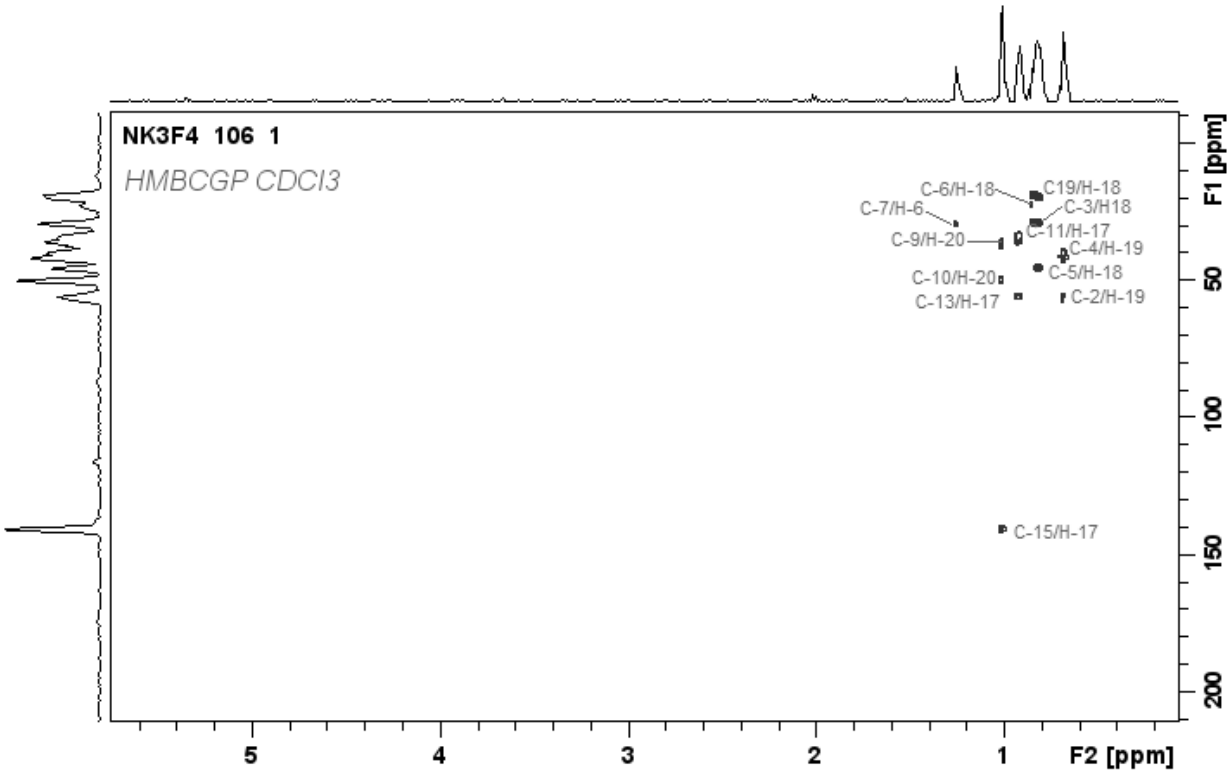
Appendix 5: NMR Spectra (1H and 13C NMR: 1D and 2D) for NK3F4



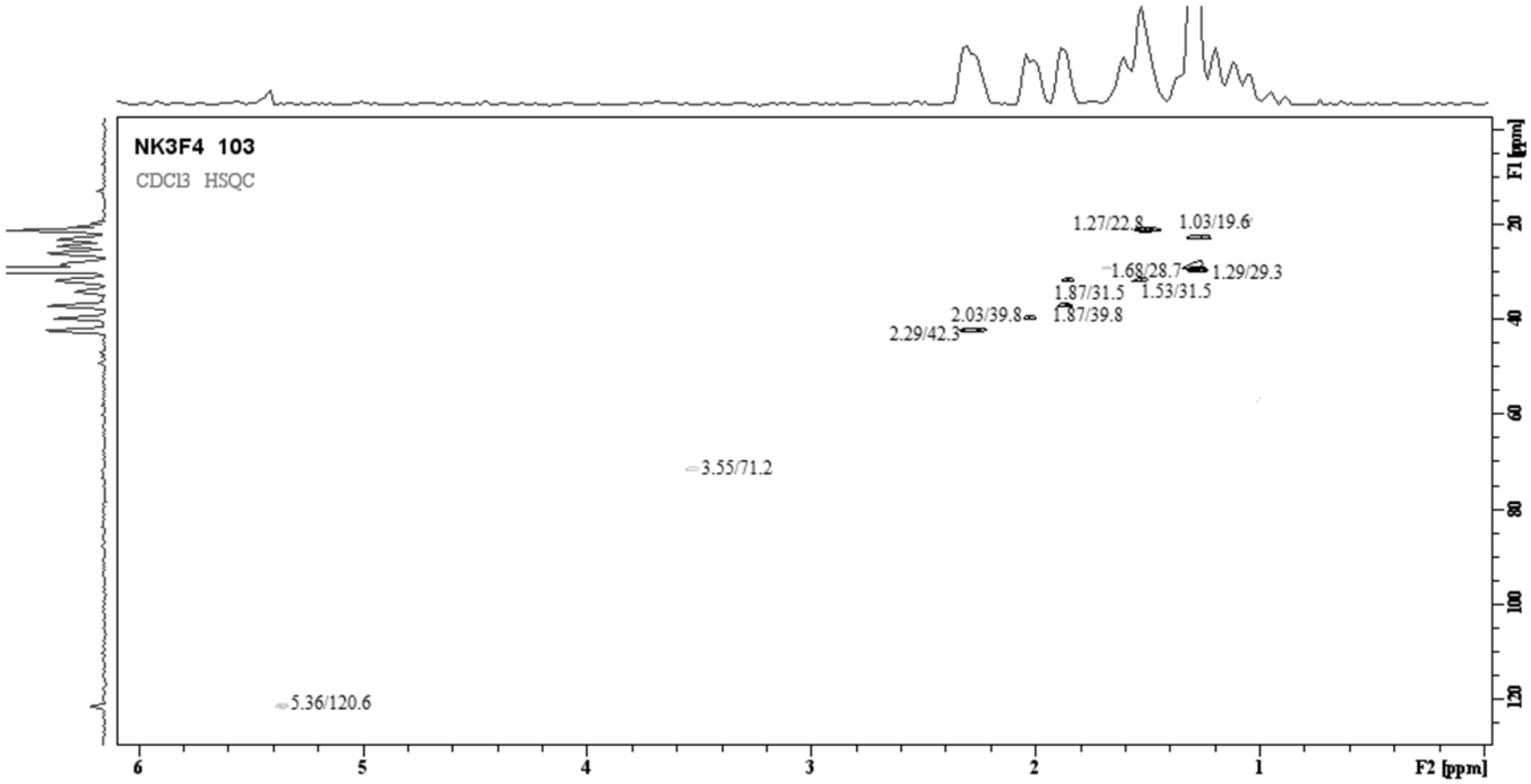
^{1}H NMR (400 MHz, CDCl3) spectrum for NK3F4



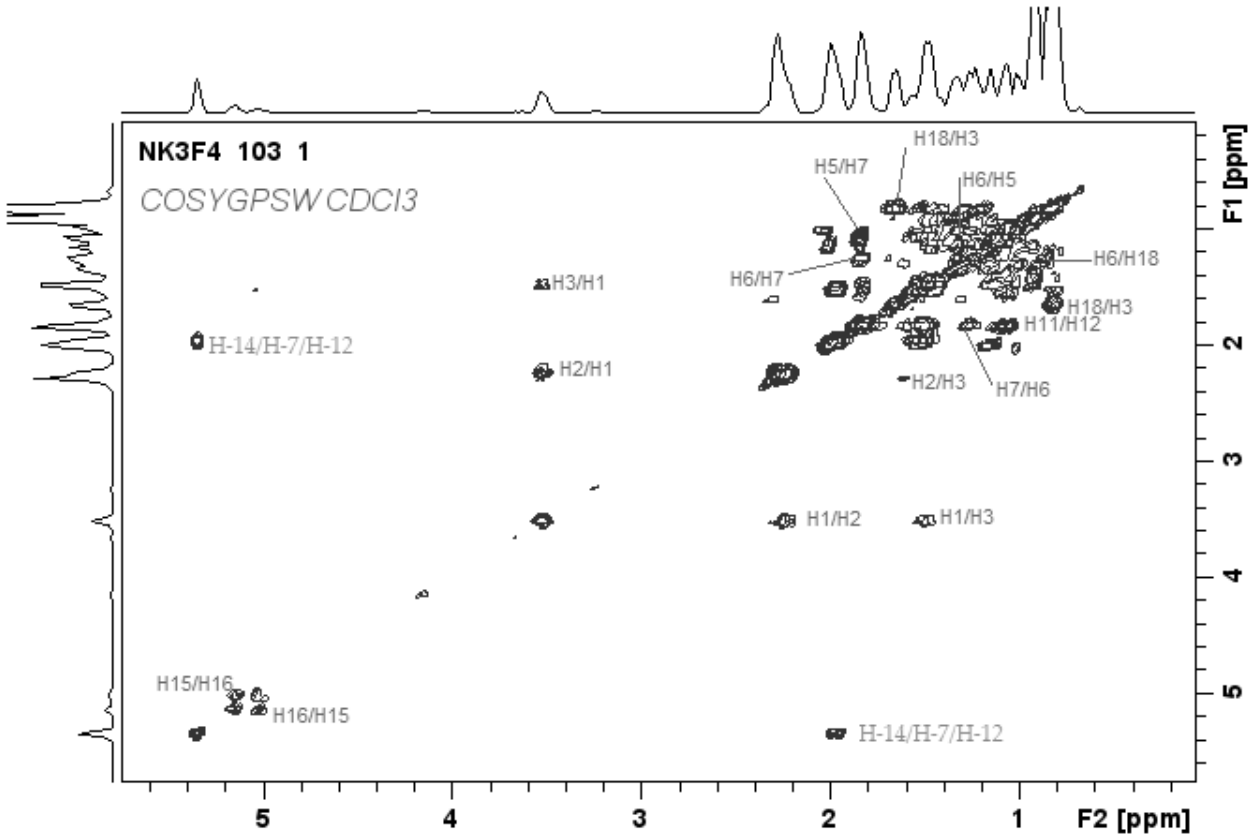
^{13}C NMR (151 MHz, CDCl3) spectrum for NK3F4



HMBC spectrum for NK3F4

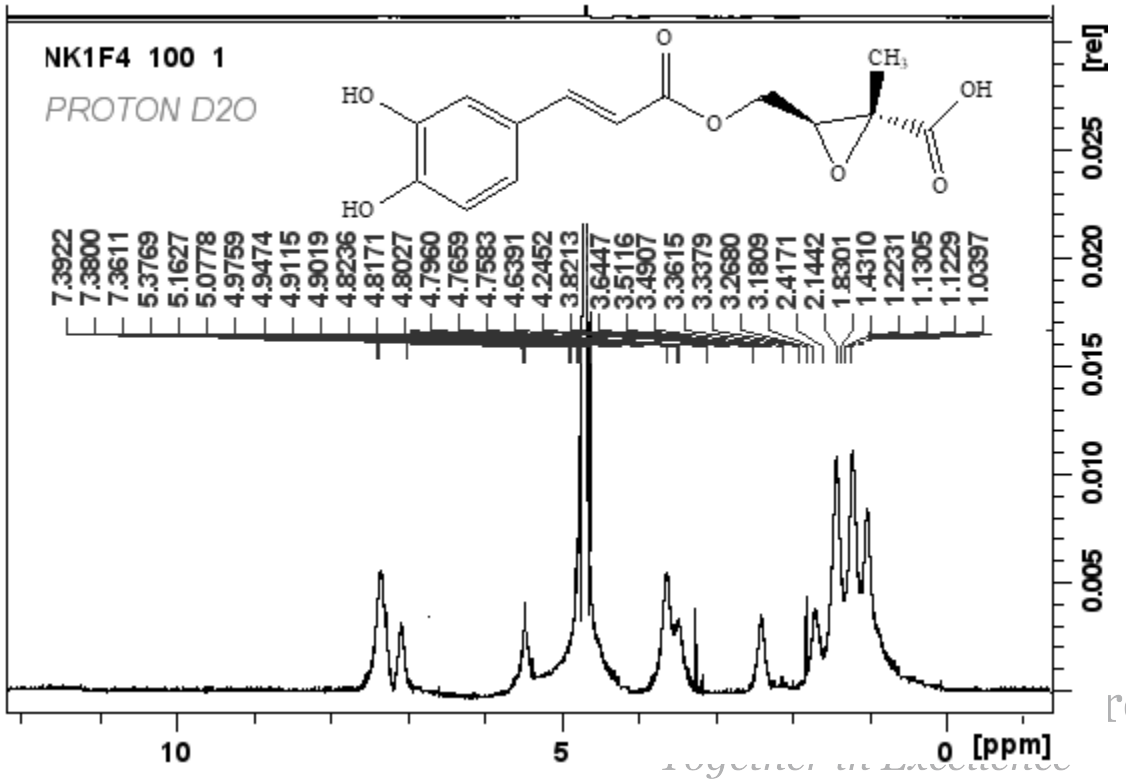


HSQC spectrum for NK3F4

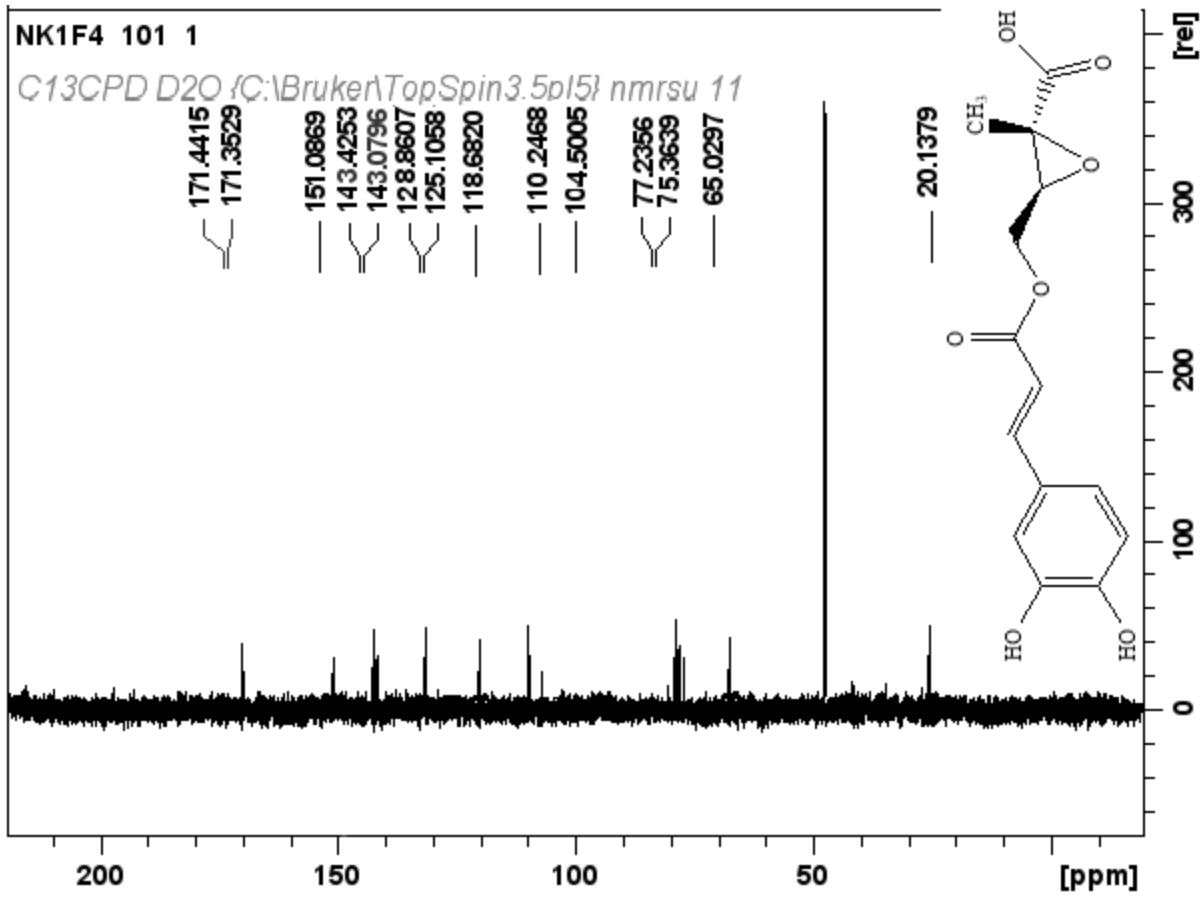


^{1}H-^{1}H Cosy spectrum for NK3F4

Appedix 6: NMR Spectra (^{1}H and ^{13}C NMR) for NK1F4

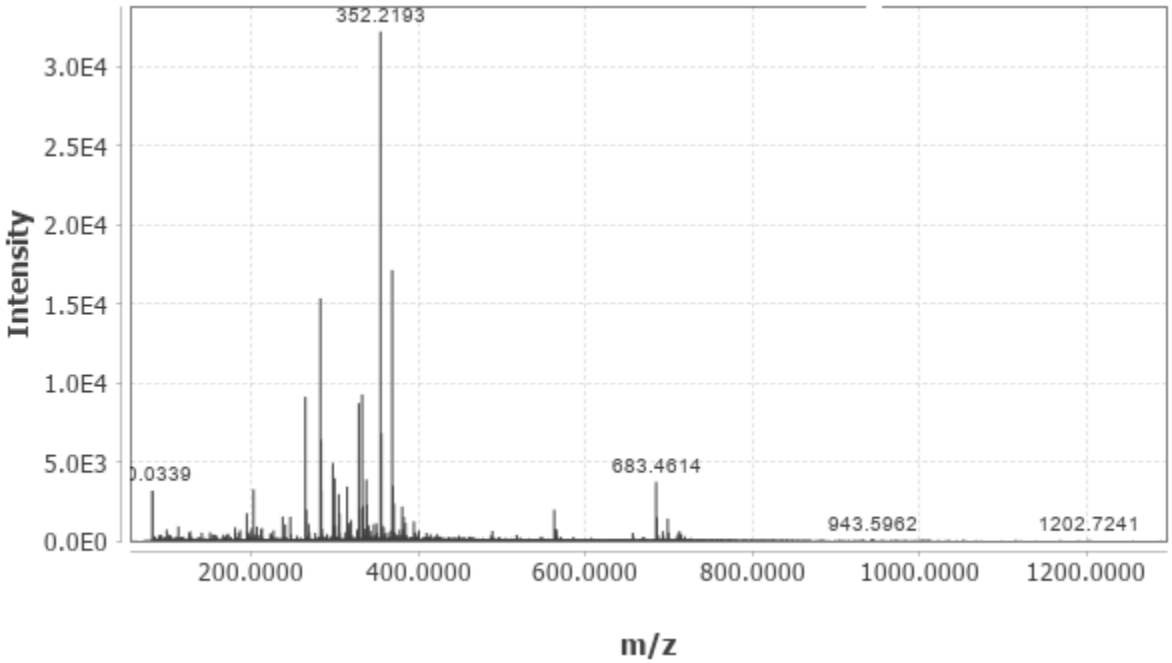


^{1}H-NMR spectrum for NK1F4

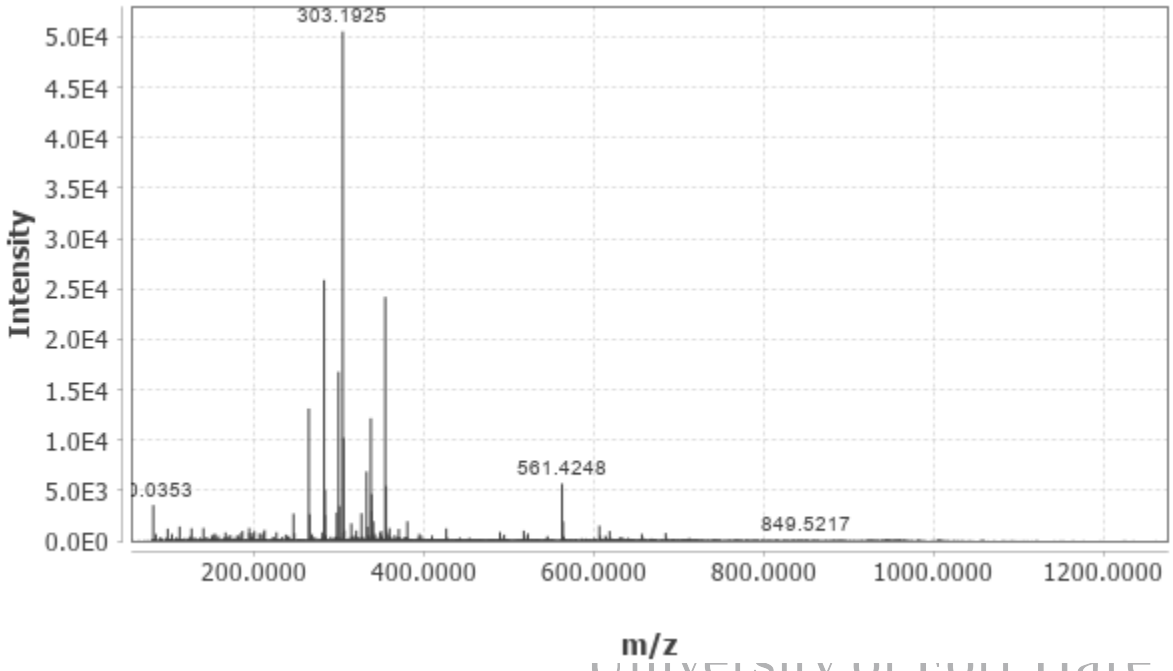


^{13}C NMR (100 MHz, CD_3OD) spectrum for NK1F4

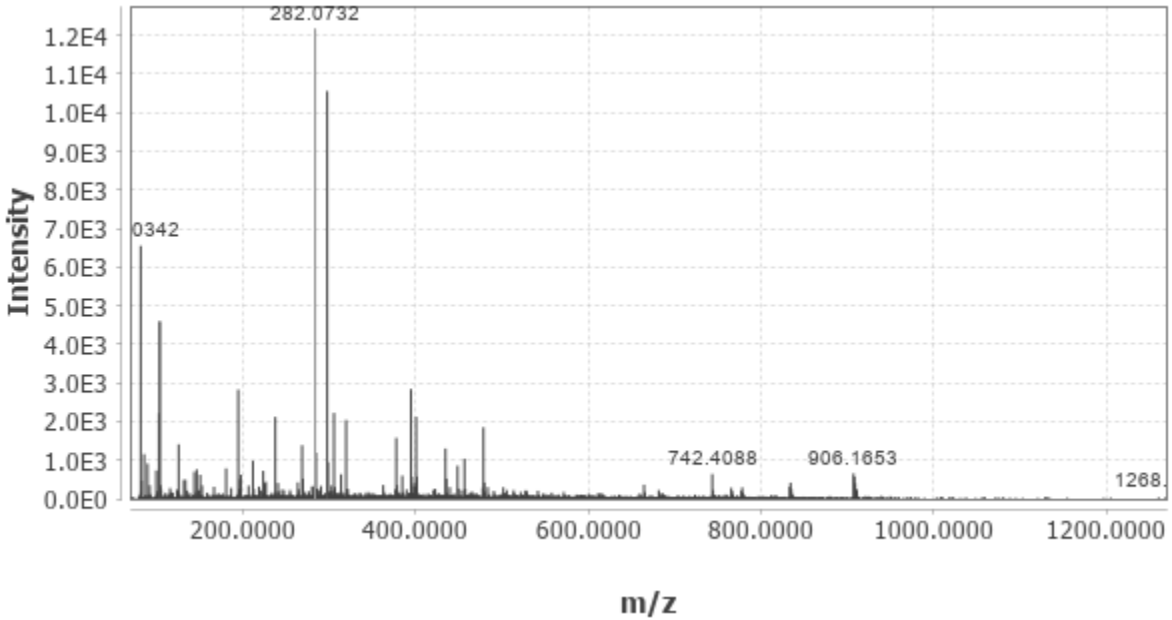
Appedix 7: LC-MS spectrum for NK1F5

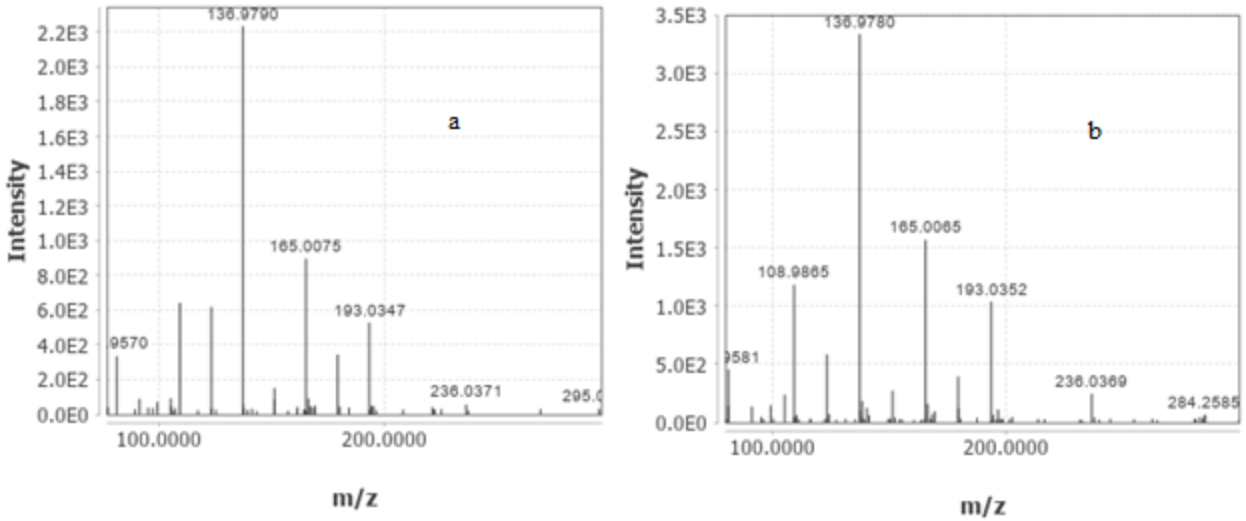


Appedix 8: LC-MS for the NK3F4



Appedix 9: LC-MS and MS/MS spectra for the NK1F4



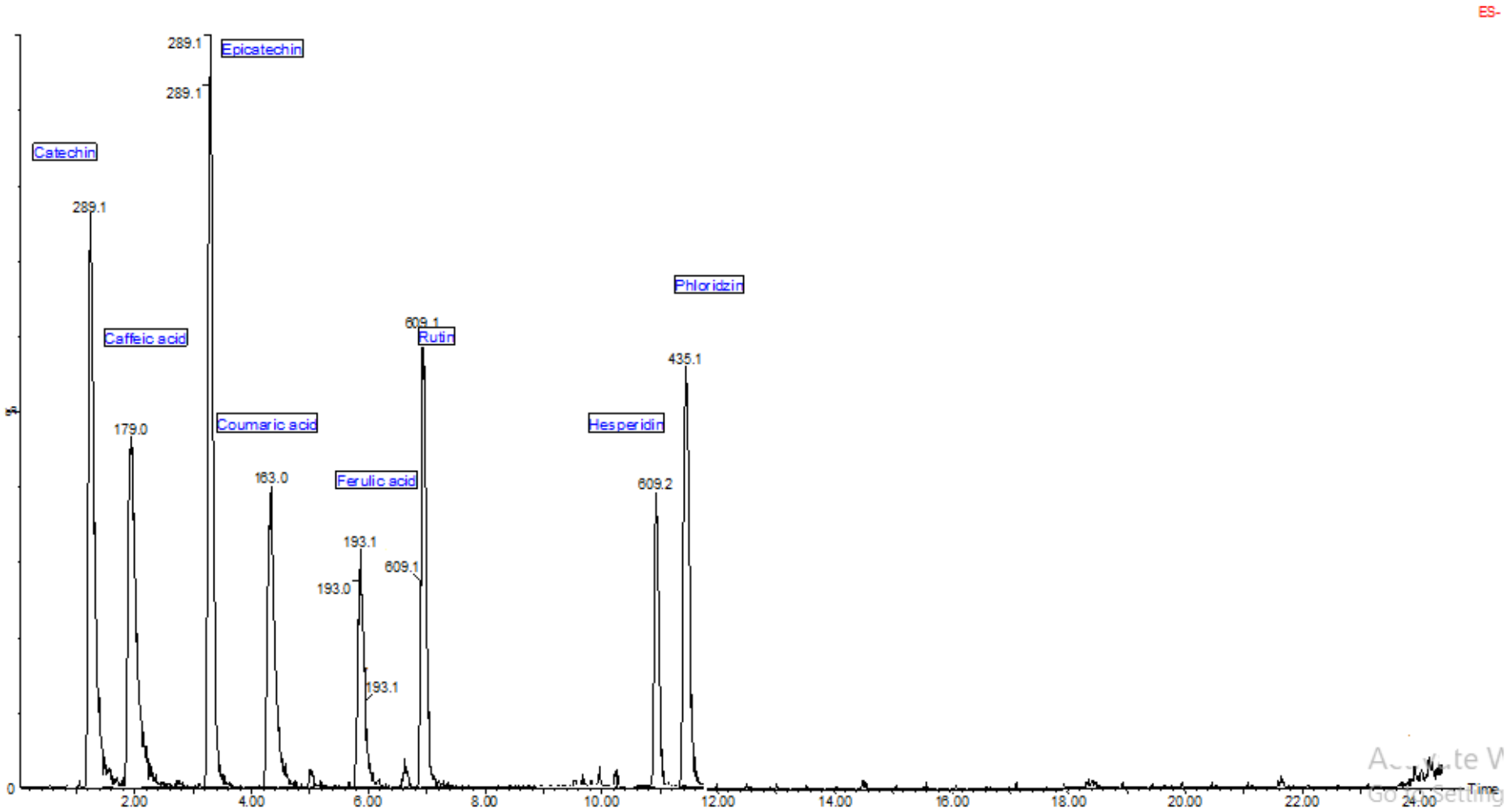


ESIMS/MS spectra for *m/z* 296 $(M + 2H]^+$ (a) and 282 $(M + 2H\text{-}CH_3]^+$ (b) adducts for NK1F4

Appedix 10: Objects and variables used during PCA analysis

Objects			Variables																				
	EB	oX	pX	mX	αP	Dl	E2B	Linl	Icrpn	59UD	EN	βF	SP	zMD	14Dm	26Do	3ccd	Hxd	Isamdn	βS	Fnso	αSp	FnsoEm
TV1kya	0,229	1,096	0,941	0,334	0,657	0,000	0,264	0,000	0,000	0,000	0,000	0,336	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
TV1kyb	3,978	29,425	0,000	24,970	1,716	0,159	2,701	1,765	0,000	0,836	0,195	0,000	0,198	0,000	0,229	0,000	0,000	0,523	0,189	0,000	1,807	0,192	0,000
TV1kyc	0,556	3,187	1,609	3,609	0,744	0,184	3,353	0,000	0,000	0,858	0,000	0,000	0,236	0,000	0,386	0,000	0,000	0,000	0,000	0,000	2,156	0,181	0,000
TV2kya	0,358	2,105	0,000	1,020	0,510	0,755	0,589	0,000	0,000	1,186	0,000	0,000	0,272	0,000	0,000	0,000	0,000	0,000	0,000	0,650	0,000	0,000	0,000
TV2kyb	1,411	8,966	0,000	5,128	1,013	0,188	1,001	0,000	0,000	1,277	0,240	0,258	0,264	0,000	1,392	0,000	2,878	0,000	0,000	5,726	0,000	0,000	0,000
TV2kyc	0,214	1,068	0,000	0,305	0,000	0,000	0,226	0,000	0,000	0,250	0,187	0,793	0,193	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
TV2kyd	0,460	3,416	1,451	2,095	0,478	0,132	2,945	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,270	0,000	0,000
TV3kya	0,709	6,712	0,000	3,330	0,000	0,000	1,085	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,239	0,295	0,408	0,000	0,000
TV4kya	0,000	0,000	0,000	0,000	0,418	0,000	0,000	0,000	0,000	0,158	0,900	0,000	0,207	0,000	0,000	0,223	0,000	0,000	0,281	0,000	0,000	0,000	0,000
Tv4kyb	0,000	0,000	0,000	0,000	0,948	0,148	0,000	0,000	0,000	0,528	0,000	0,000	0,000	0,000	1,076	0,612	0,000	0,000	0,337	0,000	0,000	0,000	0,970
Tv4kyc	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,214	1,059	0,000	0,000	0,213	1,238	0,948	0,483	0,000	0,000	0,705	0,000	2,890	0,189	0,000
TV1muya	2,669	17,615	0,000	14,393	1,210	0,161	0,000	0,000	0,000	0,730	0,000	0,000	0,231	0,000	1,084	0,000	0,000	0,181	0,000	0,000	0,000	0,000	0,784
TV2muya	2,394	22,125	0,000	18,083	1,602	0,251	2,239	1,513	0,000	0,644	0,287	0,000	0,323	0,000	2,288	0,000	0,000	0,534	0,000	0,201	6,054	0,000	0,000
TV3muya	0,000	0,000	0,925	0,934	0,000	0,000	0,333	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	O.00	0,000	0,192	0,000	0,000	0,000
TV3muyb	1,698	8,556	3,119	5,346	1,511	0,197	3,710	0,000	0,000	0,000	0,194	0,219	0,223	1,597	0,000	0,000	0,000	0,294	0,801	0,199	4,535	0,000	0,000
TV3muyc	2,324	23,386	5,228	6,713	1,825	0,134	2,713	0,000	0,000	0,000	0,000	0,000	0,262	0,000	0,000	0,000	3,445	0,000	0,000	0,000	5,936	0,221	0,000
TV4muya	0,000	0,000	0,000	0,000	0,368	0,000	0,000	0,000	0,258	0,694	0,000	0,000	0,000	0,000	0,000	0,417	0,000	0,000	0,344	2,007	0,000	0,000	0,000
TV4muyb	0,000	0,000	0,000	0,000	0,620	0,000	0,000	0,000	0,224	0,981	0,000	0,000	0,247	0,000	1,282	0,601	0,000	0,000	0,000	0,851	1,224	0,000	0,000
Tv4muyc	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,552	0,208	0,809	0,000	1,057	0,000	0,000	0,000	0,000	0,000	2,047	0,206	0,193	0,189

Appedix 11: Chromatogram of the standard mix of phenolic compounds



Appedix 12: Correlations between total antioxidant capacity with total phenol and total flavonoid content

Parametric Correlations

Correlations

		TPC	TFC	IC50	AAE
TPC	Pearson Correlation	1	.466	-.067	-.388
	Sig. (2-tailed)		.206	.865	.302
	N	9	9	9	9
TFC	Pearson Correlation	.466	1	-.087	-.359
	Sig. (2-tailed)	.206		.824	.343
	N	9	9	9	9
IC50	Pearson Correlation	-.067	-.087	1	.026
	Sig. (2-tailed)	.865	.824		.944
	N	9	9	10	10
AAE	Pearson Correlation	-.388	-.359	.026	1
	Sig. (2-tailed)	.302	.343	.944	
	N	9	9	10	11

Correlation is significant at the 0.05 level (2-tailed).

Nonparametric Correlations

Correlations

			TPC	TFC	IC50	AAE
Spearman's rho	TPC	Correlation Coefficient	1.000	.790*	-.307	-.276
		Sig. (2-tailed)	.	.011	.422	.472
		N	9	9	9	9
	TFC	Correlation Coefficient	.790*	1.000	-.420	-.360
		Sig. (2-tailed)	.011	.	.260	.342
		N	9	9	9	9
	IC50	Correlation Coefficient	-.307	-.420	1.000	.188
		Sig. (2-tailed)	.422	.260	.	.602
		N	9	9	10	10
	AAE	Correlation Coefficient	-.276	-.360	.188	1.000
		Sig. (2-tailed)	.472	.342	.602	.
		N	9	9	10	11

*. Correlation is significant at the 0.05 level (2-tailed).