Discovery of Novel Plant Based Compounds to Address the Drug Resistance Problem in Nematode Infested Ruminants

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Abstract

In this thesis, the discovery of novel compounds from plant extracts towards addressing the drug resistance issue in nematode infested ruminants is presented. The nematocidal efficacy of the plant extracts was tested against the L3 stage nematode larvae through bioassay-guided fractionation and chromatographic separation. Quebracho crude powder (QCP) is the only commercially available source of polyphenols. The efficacy of a separated fraction of the QCP was found to be higher ($P<0.05$) than the crude extract against batches of L3 larvae. However, the research with QCP encountered many challenges and attention was shifted towards medicinal plants found in New Zealand. From a screening study, the Māori plant *Piper excelsum* (Kawakawa) was found to be the most effective. Of its different components, the leaf component was found to have the highest nematocidal efficacy. Kawakawa (KK) leaf samples were collected over three seasons and the nematocidal efficacy of the leaf samples was found to be independent. From the bioassay-guided fractionation study, the Water and MeOH solvent fractions were found to be most effective, and they were subjected to further reverse-phase chromatographic separation. It was found that the separated fractions had better anthelmintic efficacy than the parent crude solvent fractions ($P<0.05$). An improvised separation technique named '*Hand Controlled Countercurrent Separation*' (HCCCS) based on the principle of CCS was developed. It was found that the fractions obtained from the HCCCS study had better anthelmintic efficacy than the parent MeOH-Fraction-4 and the anthelmintic ivermectin (IVM) ($P<0.05$). A series of combination formulations were made with the separated fractions and IVM. It was observed that these formulations had better efficacy than IVM and the individual HCCCS Fractions ($P<0.05$). The HCCCS Fractions were subjected to LC-MS/MS investigation and 34 compounds were identified. Of which, 8 were reported in the literature from published KK research. However, the anthelmintic properties of these compounds were not previously reported. The cytotoxicity evaluation of a series of fractions did not reveal any toxic effect to mammalian epithelial cells. Therefore, there is further potential towards isolating leaf fractions into pure isolates which may possess higher nematocidal efficacy compared to the fractions presented in this thesis.

Contribution

All the original research work presented in this thesis were solely performed by Gupta except:

1. The nematode L3 larval identification and quantification of the larval population were achieved with the help of Ms Barbara Adlington, senior parasitology technician, Massey University. The L3 larval development from the nematode infected ruminant faeces throughout the PhD research was performed by Ms Adlington.
2. The solvent systems for the Hand Controlled Countercurrent Chromatography Separation (HCCCS) analysis were designed with the contribution from Mr Wenliang Xu, PhD student, School of Food and Technology, Massey University.
3. The cytotoxicity evaluation of the Kawakawa leaf extracts and the standard anthelmintics was performed by Dr Paul Davis, Director of Research, Trinity Bioactives Ltd., New Zealand.

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Firstly, I want to thank my main PhD supervisor Prof David RK Harding. He went above and beyond the call of responsibility to ensure I had a trouble-free PhD journey. Prof Harding's selflessness, thorough guidance, tireless support in every step of the way including to make sure I am having a positive experience in a foreign country, and strong work ethics cannot be surpassed. I am grateful to have a terrific co-supervisory research team of Dr Preet Singh, Prof Bill Pomroy, and Dr Catherine Whitby. Without their constant help, supervision, and guidance throughout the PhD journey, I could not have achieved the things I achieved during my research. I want to profoundly thank Dr Singh for also providing me with the essential PhD scholarship. Together, these four compassionate and professional scientists shaped a great PhD journey for me. I am also thankful to my foreign co-supervisor, Prof Nadia Kandile of Ain Shams University, Cairo, Egypt for her support during this PhD.

There are many wonderful people who have greatly helped in my PhD journey. Ms Barbara Adlington, the senior technician of the parasitology lab where I spent many long hours, always made sure I had an abundant supply of nematode worms for my research. I was fortunate to have Mr Wenliang Xu as my lab-mate. His help throughout the research journey was extremely valuable. I am grateful for being able to call him a friend and a collaborator. I am thankful to Dr Matthew Perrott for allowing me to use the histology lab facility for microscopic work. I am grateful to A/Prof Vyacheslav Filichev and A/Prof Gareth Rowlands for granting me access to their flash chromatography facility for my chromatographic separation work. I am thankful to Mr Brian Best for letting me use the hot-air oven facility at AgHort C, which ensured the rapid drying of my plant samples. I want to thank Mr Trevor Loo for giving me access to the mass spectroscopy lab facility and the compound discoverer, the two essential tools of my PhD research. Mr David Lun, the chemistry research technician, helped me to run the countless LC-MS samples, including experimenting with multiple different columns and trying out different methods, always with a smile. I am grateful to Mr Paul Hocquard, the purchasing officer of School of Fundamental Sciences, for going the extra miles to ensure I had a fast supply of chemicals and other goods needed for the PhD research. I want to thank Dr Paul Davis, the Director of Research at Trinity Bioactives Ltd, for gleefully agreeing to collaborate with our research team and performing the essential research of evaluating the cytotoxicity of the research samples.

Being 12,000 km away from home and my family was not always an easy task. I am grateful to the people I have met during my PhD journey. These people provided one important thing that was essential for prodding though the ups and downs of the PhD life: friendship. I have been richly blessed with good friends: Sam, Zak, Cam, Taryn, Justin, Tommy, Lily, Jarred, Abby, Lydia, Bill, Maulik, Sarah, Charlotte, Will, Tash, Aden, Jayda, Dorian (and his dog Elijah), DK, and all the lovely humans of Palmerston North I met along the way.

Lastly but certainly not the least, I am grateful to my family for always being there for me. I dedicate the work I have done in this thesis to my mother, Mrs Anjushree Gupta, a son could not have asked for a more loving mother.

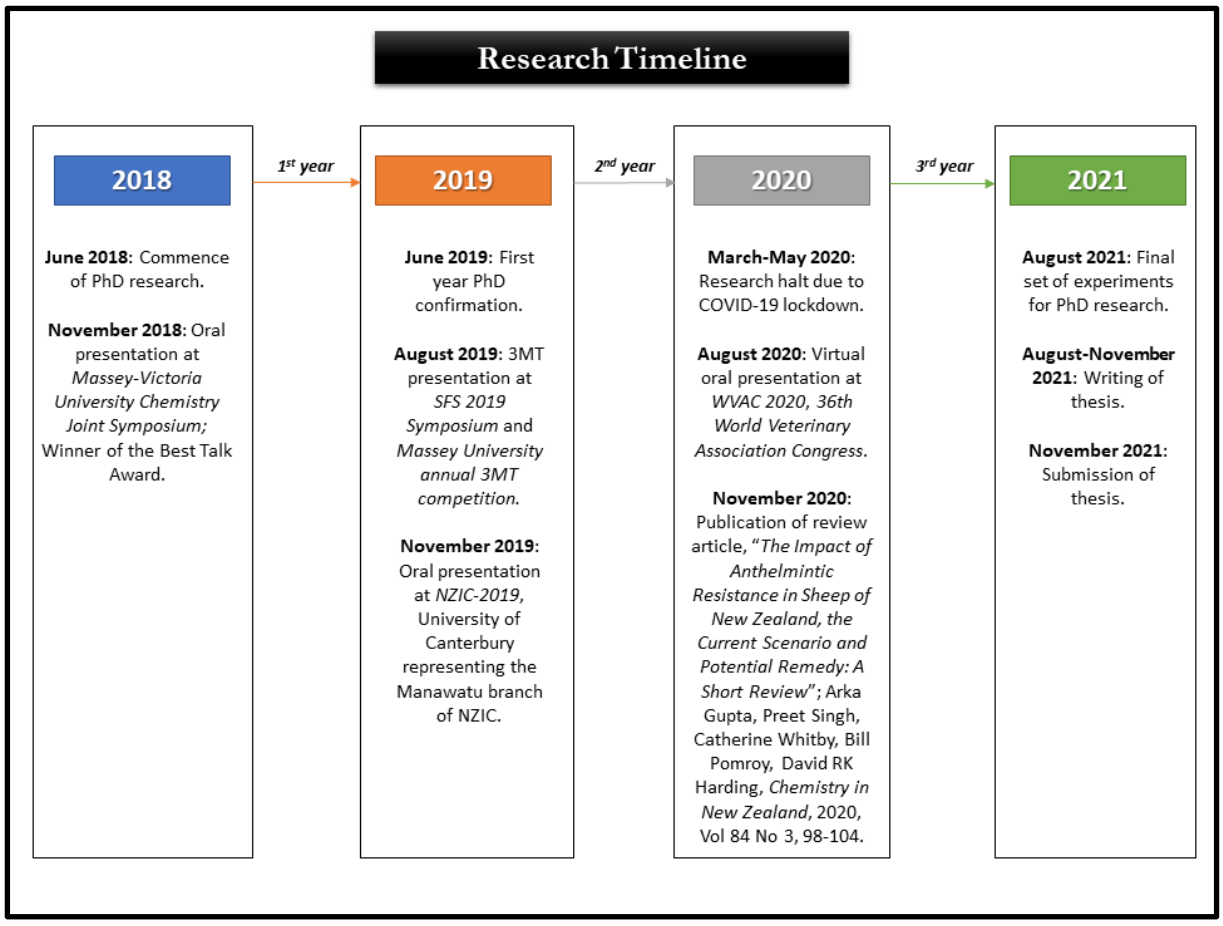
Preface

This thesis describes the research work towards the development of novel anthelmintics from plant extracts and overcoming the drug resistance problem. The thesis comprises of 12 discrete chapters. Each chapter is linked with the other. **Chapter 1** presents the literature reviews and the general introduction of the research topics undertaken in the thesis. **Chapter 2** presents all the materials used in the research and the methods followed except for *Chapter 8*, where a new separation method has been presented. **Chapter 3** describes the nematocidal studies conducted with a Quebracho extract through bioassay-guided fractionation and chromatographic separation. **Chapter 4** presents the studies of nematocidal efficacies of the various components from a series of medicinal plants found in NZ. In **Chapter 5**, the bioassay-guided fractionation and chromatographic separation of the Kawakawa fruit to achieve discrete fractions and a study of their nematocidal efficacy is presented, as KK was the most effective plant from the comparative studies conducted in *Chapter 4*. **Chapter 6** presents the assessment of nematocidal efficacy of the different plant components of Kawakawa. In **Chapter 7**, the bioassay-guided fractionation, chromatographic separation and nematocidal studies of the KK leaf is presented, as the leaf was the most effective component from *Chapter 6*. **Chapter 8** presents an improvised chromatographic separation method based on the countercurrent chromatography separation technique to better separate the fractions obtained from the KK leaf, after a chromatographic method described in *Chapter 7* was found to be inadequate. **Chapter 9** describes the qualitative chemical analysis of the crude and separated fractions of the KK leaf. While **Chapter 10** presents the compound identification and structure prediction of the active components present in the most effective fraction(s) based on liquid chromatography-mass spectroscopy/mass spectroscopy analysis. In **Chapter 11**, the cytotoxicity studies of the crude and effective fractions from *Chapter 7* and *Chapter 8* are reported. The final chapter, **Chapter 12** presents the general discussion of the studies presented in the previous chapters and the future direction of the research. In *Chapter 1*, three overall hypotheses taken in the PhD are presented. Additionally, a series of hypotheses are taken in the individual chapters and are numbered to the specific chapters. The Appendices section of this thesis are numbered accordingly to the chapters above.

COVID-19 had a strong impact on this PhD research with the following things:

1. The collection, chemical and nematocidal analysis of Autumn KK-leaf sample were not performed due to the university closure during March-May 2020.
2. LC-MS/MS instrumental facility closure during March-July 2020.
3. Delay of arrival of chemical goods (including columns) from outside of New Zealand.
4. Supply of nematode infected faeces.

The research timeline of this PhD highlighting the key events is presented below.



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List of Abbreviations

AAD	Amino-acetonitrile derivative
AB	Abamectin
ANOVA	Analysis of variants
AR	Anne Riddler farm
ARGL	AgResearch Grassland
BuOH	n-Butanol
BZ	Benzimidazole
CCS	Countercurrent Chromatography Separation
Contd.	Continued
CT	Condensed tannins
DCM	Dichloromethane
Dec	December
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
FC	Flash Chromatography
FEC	Faecal egg count
FOB	Freight on board
Fr	Fraction
GA	Gallic acid
GIN	Gastrointestinal nematode
HCCCS	Hand Controlled Countercurrent Chromatography Separation
ID	Identification
IVM	Ivermectin
KK	Kawakawa
LATU	Large Animal Teaching Unit, Massey University
LC_{50}	Lethal concentration with 50% mortality

LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectroscopy
LC-MS/MS	Liquid Chromatography Mass Spectroscopy/Mass Spectroscopy
LT	Long tails
Ltd	Limited
LDH	Lactate dehydrogenase
LV	Levamisole
MBT	Mercaptobenzothiazole
Me	Methanol
ML	Macrocyclic lactone
m/z	Mass-to-charge ratio
NLA	Nematocidal Lab Assay
NMR	Nuclear Magnetic Resonance
NP	Normal Phase
NP-FC	Normal Phase Flash Chromatography
NZ	New Zealand
Oct	October
PBS	Phosphate buffered saline
PBS-T	PBS with Tween-80
PC	Procyanidins
PD	Prodelphinidins
PSM	Plant secondary metabolite
PVC	Polyvinyl chloride
PrOH	n-Propanol
QBF	Quebracho Butanol Fraction
QCP	Quebracho crude powder
QDF	Quebracho DCM Fraction
QHF	Quebracho Hexane Fraction

QMe	Quebracho MeOH Extract
QMe-I	Insoluble sample from Quebracho MeOH extraction
QWF	Quebracho Water Fraction
RP	Reverse Phase
RP-FC	Reverse-Phase Flash Chromatography
RT	Room temperature
RT (min)	Retention time (minute)
SA	Sue Artner farm
SEM	Standard error of means
SI	Spiroindole
SLCC	Sephadex LH-20 Column Chromatography
Spp.	Species
T1-3	Test number 1-3
Temp	Temperature
TLC	Thin Layer Chromatography
UV	Ultraviolet
w/v	weight/volume

Chapter 1. General Introduction and Literature review

General Introduction and Literature review

1.1. Sheep industry in NZ

The sheep industry has been of paramount importance for the economic growth of New Zealand (NZ) since the early nineteenth century. With over 90% of sheep meat being exported, NZ is the largest lamb exporter in the world. The total sheep population of the country has seen a decrease from a peak of 70 million in the early 1980s to around 26 million as of 2020 (StatsNZ, 2021, Granwal, 2021). Significantly, between 2012 and 2017, the total sheep population has decreased by 12.45 percent (Industries, 2012, Granwal, 2021). However, declining sheep numbers have not impacted on lamb production which remains relatively constant. Even during to the recent COVID-19 impact, the demand of lamb export to foreign countries has been high with firm in-market prices generating a freight on board (FOB) of $3,507M in 2019-20 which dropped by only 14% in 2020-21 with a net FOB $3,304M (BeefLambNZ, 2021). The climate of NZ favours the growth of pasture which has been highly beneficial to sheep production as pasture provides over 95% of the diet of sheep (Hodgson et al., 2005).

1.2. Gastrointestinal Nematode Infection

Gastrointestinal nematode (GIN) infection represents a major threat to sheep production systems in the world (Vande Velde et al., 2018). The infection occurs mostly in young lambs when grazing nematode contaminated pastures, the primary source of feeding (Hodgson et al., 2005). The species of nematodes that are primarily responsible for producing the disease are dependent on the climate that determines which pathogens are of greatest importance and weather determines the epidemiology of transmission (Miller et al., 2012). GIN infection usually consists of a mixture of nematode species, which affects largely the abomasum and intestine in the host and is highly aggregated within the host population (McRae et al., 2015). Most nematodes of concern affect the abomasum or small intestine and cause the greatest levels of clinical disease in young, growing animals. Consequently, the susceptible host can give shelter to a large number of nematodes which then contaminate the pasture resulting in a cycle of the GIN infection affecting any sheep on that pasture (Roeber et al., 2013).

1.2.1. Nematode: A Brief Overview

The term nematode is a term to classify any helminth of the phylum *Nematoda*. Nematodes are among the most abundant species on earth, occurring as parasites in plants, animals and humans or as free-living forms in the environment (Scheffers et al., 2012). Nematodes can be found in soil, water, water-filled cracks deep within earth's crust and several unusual places like vinegar and in food additives (Basyoni and Rizk, 2016). About 20 thousand nematode species have been named so far but there is still a huge proportion of the free-living forms remaining to be identified (Blaxter and Koutsovoulos, 2015). Since the inception of their discovery, they have been a research interest as many of them have high medical, veterinary and medical importance and impact (Holden-Dye and Walker, 2014). GIN remain as one of the most researched and sought after biological species (Zajac and Garza, 2020).

1.2.2. Nematode Life Cycle in Sheep

All GIN in the Superfamily Strongyloidea have a direct life cycle with six phases which is broadly divided into three stages: host, dung and pasture stage and each is linked with the other (Karp et al., 2011). In the host stage, sexually dimorphic nematode adults are present in the digestive tract of the host where the female nematode produces eggs containing an embryo (Roeber et al., 2013). This is passed into the faeces which is the dung stage. The embryo develops and is followed by three larval stages which happen in the environment, L1 to L3 (Karp et al., 2011). The first larval stage, L1 develops in the egg. It hatches and feeds on bacteria in the faeces, and moults to the second larval stage, L2 (Karp et al., 2011) which feeds and grows as before. Moulting follows but is incomplete so that the third larval stage, L3 retains the L2 cuticle as a sheath around it (Roeber et al., 2013). The time from egg to L3 is about 4-10 days but can be longer (Karp et al., 2011, Scott et al., 2013). It is usually slower when colder and faster in warmer conditions in the general range of 10-30 °C with the optimum temperature range being 20-25 °C. During the pasture stage, the old L2 cuticle remaining around the L3 provides some protection from harsh environmental conditions (Karp et al., 2011). In the pasture stage, the cuticle protects L3 from harsh environmental conditions and it can survive cold reasonably well, surviving up to 5-6 months in the outside environment (Karp et al., 2011). This ensheathed L3 is the infective stage and wriggles freely

and randomly on the pasture being largely controlled by temperature as it is active above 10-15 °C and inactive below this temperature range. The level of activity is important as this stage cannot feed and is thus reliant on stored metabolites for nutrition. Once stored metabolites are exhausted the L3 will die. The L3 is ingested by the ruminant host while grazing pastures contaminated with the larvae and thus GIN infection of the host occurs. In the host stage, L3 exsheathes by discarding the L2 cuticle and moults to the L4 stage by entering the mucosal gland crypts. Exsheathment usually occurs in the region of the digestive tract immediately above its preferred site and is triggered by chemical stimuli provided by the digestive tract environment. L4 feeds and moults to the immature adult by emerging on to the surface of the mucosa. The immature adult feeds and grows to sexual maturity and the cycle is completed. Life cycle of GIN is illustrated in Figure 1.1.

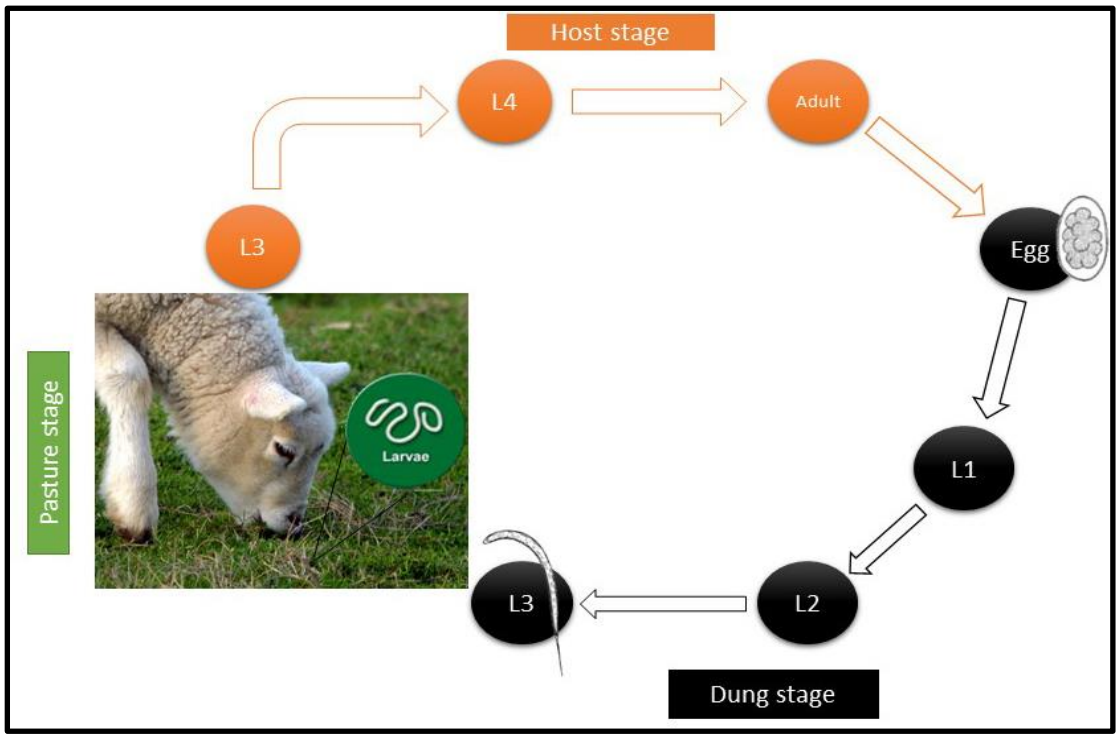


Figure 1.1. Life cycle of GIN.

1.2.3. Important GIN of Sheep

The most common and pathogenic GIN that infect sheep in NZ include *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Cooperia curticei* of the Trichostrongylidae family (Karp et al., 2011). As these species are all in the same family,

they share a lot of same characteristics. Morphology, pre-patent period (the time in a parasitic infection between when the parasite has invaded the host and the appearance of the products of reproduction e.g., eggs in faeces) and location of most commonly found GIN species of sheep in NZ are depicted in Table 1.1.

Species	***Morphology***		***Pre-patent period (days)***	***Location in the host***
	Length (mm)	***Characteristics***		
H. contortus	♂ 10-20 ♀ 18-30	Appearance of a barber's pole.	18-21	Abomasum
T. circumcincta	♂ 7-8	Small head and buccal cavity.	12-21	Abomasum
	♀ 10-12	Presence of a vulvar flap.		
T. colubriformis	♂ 4-8 ♀ 5-9	Triangular tip.	15-23	Small intestine
C. curticei	♂ 4-5	Transverse cuticle.	14-15	Small intestine
	♀ 5-6	Small cephalic vesicle and watch-spring-like posture.		

Table 1.1. Morphology, pre-patent days and location of most commonly found GIN species of sheep in NZ (Salisbury and Arundel, 1970, Levine, 1968, Anderson, 2000, Karp et al., 2011).

1.2.4. Consequences

GIN infestation may cause severe harm to the infected ruminants. It represents a major and severe threat to the health, welfare and productivity of the sheep population in NZ (Nieuwhof and Bishop, 2005). Young lambs are more vulnerable to the infection because of their less developed immune system. By comparison, ewes usually show better resistance although they will harbour small burdens and on occasions this will be sufficiently large to compromise productivity and even risk death. Infected lambs have a reduced ability to absorb nutrients from GI tract as nematodes feed and grow in the GI region and spread in the small intestine and abomasum region depending on the species (Alba-Hurtado and Muñoz-

Guzmán, 2013). Most species live in the mucus layer of the GI and ingest liquids from that site (Alba-Hurtado and Muñoz-Guzmán, 2013). Some nematodes, such as *H. contortus,* will also suck blood (Acevedo-Ramírez et al., 2019). Consequences range from poor feed conversion rates through to acute diarrhoea and death. In the older sheep, the infection also affects the milk production (Donald et al., 1978). GIN infection usually consists of a mixture of nematode species, which affects largely the abomasum and upper small intestine in the host and is highly aggregated within the host population (McRae et al., 2015). Each nematode will produce a large number of eggs which pass into the faeces and, if environmental conditions are suitable, a proportion of these will successfully develop into infective larvae that are able to re-infect more sheep. Consequently, a susceptible host can propagate a cycle of the GIN infection affecting a large number of sheep (Roeber et al., 2013). As a result, a sudden outbreak of diarrhoea in lambs occurs that results in weight reduction, loss of wool growth, and mortality.

1.3. Anthelmintic: The Solution

Anthelmintic or antihelminthic is classified as a group of antiparasitic drugs that can eradicate parasitic worms (helminths) including GIN (Zaman et al., 2017). They act in various ways within the host, killing the worms on contact or by stunning them, affecting the nervous system of the parasite and resulting in muscle paralysis, or by altering the permeability of their plasma membranes without doing any significant damage to the host. The paralysed or dead worms pass out of the host in the faeces.

1.3.1. Anthelmintic Groups

Attempts at management and eradication of GIN largely has been engineered through treatment with broad-spectrum anthelmintics belonging to five main chemical classes: 1-BZ benzimidazole, 2-LV imidazothazoles/tetrahydropyrimidines, and 3-ML macrocyclic lactone (Shoop et al., 1990). Two more classes of anthelmintics 4-AAD amino-acetonitrile derivative (von Samson-Himmelstjerna, 2006) and 5-SI which is a combination of Spiroindole and ML (Kaminsky et al., 2008) have been released in the last decade but as yet are only represented by single products commercially. However, there has been a major limitation in development of novel anthelmintic over the last two decades (Kaplan, 2004, Scott et al., 2013, Dobson et al., 2012). A feature of all anthelmintics has been the very slow rate of development of new

classes and members of existing classes in recent years, largely due to the expense involved. The classes of broad-spectrum anthelmintic, drug used, chemical structure and mode of action is depicted in Table 1.2.

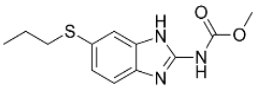
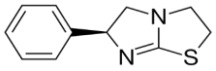
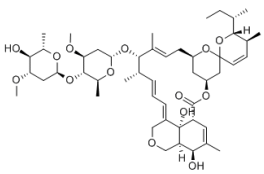
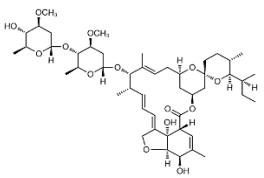
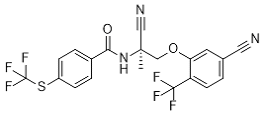
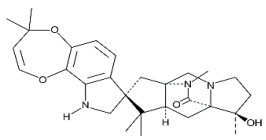
Anthelmintic class	*Example of drug (s)*	*Structure of drug(s)*	*Mode of operation*
1-BZ Benzimidazole	Albendazole Mebendazole Fenbendazole		Prevents the establishment of microtubules by binding to β-tubulin and preventing polymerisation into the formed microtubule. Starvation is achieved through inhibition of glucose uptake and protein secretion largely as a consequence of interrupted microtubules (Lacey and Gill, 1994, Martin et al., 2003).
2-LV Imidazothiazole	Levamisole Tetramisole		Cholinergic agonist which causes spastic paralysis of the parasite and they are eradicated by gut peristalsis which leads to rapid removal of the worms present (Martin and Robertson, 2007).
3-ML Macrocyclic lactone	Abamectin		Causes flaccid paralysis by creating an opening of glutamate-gated chloride channels which leads to an increased Cl-ion influx into nerve cell and subsequent hyperpolarisation of the cell which is then unable to transmit an action potential (Blackhall et al., 2003).
	Ivermectin		
4-AAD Amino-acetonitrile derivative	Monepantel		Cholinergic agonist as for levamisole but binds to a specific receptor which is unique to nematodes and is different to the receptors used by levamisole. Subsequently causes hypercontraction of the body wall leading to paralysis (von Samson-Himmelstjerna, 2006).
5-SI Spiroindole	Derquantel		Cholinergic antagonists which prevents acetyl choline from binding to its receptors. Causes hypercontraction of the body wall leading to paralysis (von Samson-Himmelstjerna, 2006).

Table 1.2. Anthelmintic classes, drugs used, the chemical structure and mode of operation.

1.3.2. Anthelmintic Resistance

Modern anthelmintics are expected to achieve in excess of 95% efficacy against GIN with most achieving >99% efficacy against most species of GIN (Bosco et al., 2020). The reliance on anthelmintics for controlling nematodes, often with excessive use of these chemicals has led to the widespread problem of anthelmintic resistance which has become a global phenomenon, especially in the GIN of small ruminants (Waller, 1994, Bosco et al., 2020, Vande Velde et al., 2018). Anthelmintic resistance results from a genetic change within the nematodes and is generally accepted to exist when the efficacy of an anthelmintic has declined to <95% (Shalaby, 2013). The anthelmintic in question at this level, is not able to avoid subclinical problems of parasitism (Bosco et al., 2020, Ikurior et al., 2020). Anthelmintic resistance is now so widespread that resistance to all classes of broad-spectrum treatments has reached a serious level in NZ and worldwide for the important GIN found in sheep, especially for the Classes 1-3 listed above which have now been around for more than forty years (Ikurior et al., 2020, Arsenopoulos et al., 2017, Sepúlveda-Vázquez et al., 2021). Unfortunately, there are also reports of resistance to more recently introduced Class 4 and 5 anthelmintics (Crook et al., 2016, Cerutti et al., 2018, Hodgson and Mulvaney, 2017). A parasite having a resistance to one drug from a certain anthelmintic class is resistant to other products from the same class (Shalaby, 2013). Additionally, multi-drug resistance to several anthelmintics from different classes has been well documented both in NZ and elsewhere (Kerboeuf et al., 2003, Demeler, 2005, Puspitasari et al., 2016, Scott et al., 2013, Sales and Love, 2016, Hodgson and Mulvaney, 2017).

Different mechanisms apply to different anthelmintic groups but in general all involve gene mutation causing a gain-of-function, which commonly leads to a more rapid removal of the drug or an inability to bind to its receptor and thus it can no longer produce an effective response (Kerboeuf et al., 2003, Demeler, 2005). However, the actual chemistry is still unclear for many if not all drugs (Roeber et al., 2013).

1.3.3. Combination Strategy and the Current Picture

The widespread development of resistance in GIN of sheep in NZ has required new approaches to be taken for their control. One effective way to tackle this resistance problem have been achieved by treating simultaneously with a combination of 2 or more drugs from different anthelmintic classes (Hodgson and Mulvaney, 2017, Dobson et al., 2012). Thus, contrary to individual drug effects, anthelmintics of different classes administered together has resulted in better efficacy (Puspitasari et al., 2016, Le Jambre et al., 2010). This effect is most pronounced when the level of resistance of a particular anthelmintic class is low. For example, monepantel (4-AAD) was first introduced in 2009 (Lecova et al., 2014) and the resistance was first evidenced in 2012 (Scott et al., 2013). It has been found that it takes about 15-20 years to develop appreciable resistance to a class of drug but once resistance alleles accrue in GIN population, this combination strategy will not work (Shalaby, 2013). In 2016, there has already been a report of reduced efficacy of the combination of derquantel and abamectin (Sales and Love, 2016). It is highly probable that the current combination of monepantel and abamectin will not be as effective in coming years as there are already reports of resistance to either component when used separately. Hence, there is an urgent need to introduce a new class of anthelmintic in the market.

1.4. Bioactive Plant Extracts: The Possible Answer

Plants have survived and thrived for over 400 million years on this planet (Wink, 2015). As a tool for plants to shield themselves against the challenging factors such as bacteria, fungi, viruses and parasites, thousands of structurally differing secondary metabolites have evolved during plant development (Schmeller et al., 1997, Wink et al., 1998, Wink, 2015, Seigler, 1995). From the point of view of evolutionary pharmacology, plant secondary metabolites represent an exciting library of bioactive compounds filtered by natural selection, which have been used to treat infections and many health disorders of humans and animals alike (Russo, 2001, Nichols, 1997, Wink, 2008). Drug discovery from medicinal plants rich in secondary metabolites has continued to be an effective source of new drugs and drug leads (Newman and Cragg, 2016). Many drugs, such as galantamine, tiotropium, artemether and artemisinin which are all derived from plants have been brought forward into the market over the years (Balunas and Kinghorn, 2005, Tu, 2016).

A number of secondary metabolites derived from bioactive medicinal plants have been widely reported to be effective anthelmintic drug candidates (Githiori et al., 2005, Panda et al., 2019). In addition, the use of the bioactive plants themselves have been found to be effective against parasites at various stages of their life cycle (Rahmann and Seip, 2007). A recent study by Romero-Benavides et al. (2017) has reported that many plant extracts have demonstrated promising anthelmintic properties. However, the number of isolated identified compounds from these bioactive plants was not high (Romero-Benavides et al., 2017).

1.4.1. Secondary Metabolites Derived from Plants Active Against GIN

In this section, the discovery of compounds derived from bioactive plants with anthelmintic activity against GIN is summarised. An emphasis on tannins and alkaloids is highlighted in the next two subsections. The two classes of compounds possessing anthelmintic properties being the most researched are reported in more detail.

In Table 1.3, a literature summary of a few active compounds derived from various medicinal plants and their activity against species of parasite(s) is presented.

Active compounds	***Source***	***Parasite***	***Activity***
β-sitosterol	*Mentha cordifolia* (Spearmint)	*Ascaris suum*	60 mM induced paralysis of worm in 1 hr (Villaseñor et al., 2002).
trans-cinnamaldehyde	*Cinnamomum verum* (Cinnamon)		98% L3 larval death at 25.6 µg/mL(Williams et al., 2015).
12-amino-7, 17-dioxo-2-oxa-8,16-diazatricylo [14.2.2.2 3, 6] tetraicosa-1 (20),3,5,18,21,23-hexaene-12-carboxylic acid	*Acacia oxyphylla* (Acacias)	*Ascaridia galli*	L3 death after 15 hr at 1 mg/mL (Roy et al., 2012)
Condensed tannins (CT)	*Onobrychis vicifolia* (Sainfoin)	*H. contortus*	L3 inhibition LC_{50} = 1.2 mg/mL (Barrau et al., 2005)
(S)-dicentrine and (S)-neolitsine	*Cissampelos capensis* (Dawidjieswortel)		Free-living stage inhibition. EC_{90}= 6.3 and 6.4 µg/mL (Ayers et al., 2007)
Caffeoyl and coumaroyl derivatives	*Acacia cochliacantha* (Huinolo)		98% L3 inhibition at 1 mg/mL (Castillo-Mitre et al., 2017)
4,5-di-O-caffeoylquinic acid	*Baccharis conferta*		Ovicidal activity with IC_{50} = 80 µg/mL. 100% egg hatching inhibition at 3 mg/mL (Cortes-Morales et al., 2019)
Terpinene-4-ol	*Melaleuca alternifolia* (Tea tree)		85.7% larval inhibition at 56 mg/mL and 82.4% at 3.5 mg/mL (Grando et al., 2016)
Thymol	*Thymus vulgaris* (Thyme)		100% egg hatch inhibition at 20 mg/mL (Ferreira et al., 2016)
Cysteine cellulose	*Ficus benjamina* (Weeping fig)		L3 inhibition. LC_{50} of 260 and 790 µg/mL (Wanderley et al., 2018)
Chlorogenic acid	*Tagetes filifolia* (Irish lace)		L3 inhibition. LC50 = 248 µg/mL (Jasso Díaz et al., 2017)
Gallic acid derivatives	*Caesalpinia coriaria* (Divi-divi)	*Cooperia* spp., *H. contortus*, *Teladorsagia* spp., *Trichostrongylus* spp.	100% in-vitro ovicidal activity at 1000 µg/mL (García-Hernández et al., 2019)

Table 1.3. Active compounds from various medicinal plants and their anthelmintic efficacies.

However, the application of all these active compounds were with the in-vitro studies of the GIN. From a recent literature review (2020) on development of novel anthelmintics from plant based natural products by Liu et al. reported that since 2002, 34 anthelmintic compounds from bioactive plants were isolated and identified against GIN. Nevertheless, only five compounds were researched for the in-vivo anthelmintic activity in animal models, mostly in rodents. These compounds are presented in Table 1.4.

Plant	***Active compounds***	***In-vivo anthelmintic activity***
Cissampelos capensis (Dawidjieswortel)	(*S*)-Dicentrine	25 mg/kg dosed orally resulted in 67 % reduction of worm counts in a mouse model infected by *Heligmosomoides polygyru* (Ayers et al., 2007).
Cinnamomum verum (Cinnamon)	trans-Cinnamaldehyde	Infection was not significantly decreased by daily administration in the diet (1000 mg/d) or as a targeted, encapsulated dose (1000 mg, twice daily) in a pig model (Williams et al., 2015).
Ajania nubigena (Cloud sugarbush)	Luteolin	A single oral dose of 100 mg/kg induced a 27.6% reduction of worm burden in a mouse model (Wangchuk et al., 2016).
Avena sativa (Oat)	Avenacoside	Avenacosides reduced the infectivity of *H. bakeri* larvae in mouse model by 30% (Doligalska et al., 2017).
Gliricidia sepium (Gliricadia)	Kaempferol 3-O-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside-7-O-rhamnopyranoside	Fully inhibited the *C. punctata* exsheathment process at 2400 µg/mL in calves (von Son-de Fernex et al., 2018).

Table 1.4. In-vivo anthelmintic efficacy of the active compounds isolated from bioactive plants.

Therefore, the active compounds detected in the extract of the medicinal plant having anthelmintic properties was not widely reported in the literature. Most of the research with

medicinal plant extracts against GIN are with its crude methanolic or other solvent extracts against the L3 stage of the larvae under in-vitro conditions (Liu et al., 2020).

1.4.1.1. Tannins in Management of GIN

Bioactive substances from tannin rich plants have attracted significant research attention for their effect on GIN management in ruminants. They have direct antiparasitic activity as well as direct or indirect effect on increasing host resistance (Hoste et al., 2006, Mupeyo et al., 2011, Athanasiadou et al., 2021, Maestrini et al., 2021, Pech-Cervantes et al., 2021). Tannins are secondary plant polyphenols with a high affinity for proteins and polysaccharides (Pandey and Rizvi, 2009). They can be classified into two categories depending on their chemical structure; 1) hydrolysable and 2) condensed tannins (Waterman, 1999, Waghorn and McNabb, 2003). Hydrolysable tannins are gallic or ellagic esters of sugars and are degraded into gallic acid and readily absorbed in the digestive tract upon being consumed by ruminants (Bruneton, 1999). Condensed tannins are polyphenols of higher molecular weight and upon consumption, they are metabolised to mainly cyanidin or delphinidin and hence have been classified as procyanidins (PC) or prodelphinidins (PD) (Bruneton, 1999). Condensed tannins are not as readily absorbed in the digestive tract and they form soluble and insoluble complexes with macromolecules, such as proteins, fibre and starch with a particular affinity for protein (Waterman, 1999). This topic is further discussed in the following section. There have been some research gone into the beneficial effect of tannin rich plants on livestock production and GIN management (Hoste et al., 2006, Maestrini et al., 2021, Mupeyo et al., 2011).

1.4.1.1.1. Effect of Condensed Tannins on GIN

It has been found that condensed tannins (CT) rich forages grown (sainfoin, chicory, big trefoil) under temperate condition can reduce the worm burdens in small ruminants (Marley et al., 2003). Direct interaction of CT with surfaces of nematode larvae is well evidenced. CT can disrupt the life cycle and other physiological functions of GIN in-vivo by slowing down the hatching process as well as preventing the hatched larvae from attaining full development to infective larvae (Molan et al., 2002, Mupeyo et al., 2011). This may reduce or prevent pasture contamination with infective larvae. In-vitro studies have shown that CT can destroy the cuticle and kill L3 stage larvae which is the infective stage of the larvae

(Williams et al., 2014). The purified CT extracts have anticoccidial activity as they have the ability to significantly decrease the sporulation of the parasite oocysts (Molan et al., 2009).

1.4.1.1.2. Biological Activity of CT

The biological activity of CT depends on the mean degree of polymerisation, polydispersity, cis/trans ratio and PC:PD ratio (Zhou et al., 2014). The characteristics of CT are defined by their strong affinity for proteins. It has been found that CT with higher proportions of PD reacts more strongly with proteins because of the higher -OH content in the heterocyclic B-ring (Theodoridou et al., 2011). CT affect GIN by protecting dietary proteins from microbial degradation during passage through the rumen (J Aerts et al., 1999). Protein and CT interact during harvesting, grinding, extraction, mastication and digestion through H-bonding and hydrophobic interactions (Hanlin et al., 2010). CT are known to dissociate from proteins in the abomasum (pH 3.5) and in the small intestine (pH 7.0) (Frutos et al., 2004). This increases the absorption of amino acids which in turn enhances the ruminant's resistance power to GIN. The affinity of proteins to CT is influenced by the high molecular weight, the open and the flexible tertiary structures of the protein (Hofmann et al., 2006). CT do not get absorbed in the GI tracts of ruminants, they remain intact and get eliminated with the faecal matter (McMahon et al., 2000).

1.4.1.1.3. Anthelmintic Efficiency of Quebracho Extract

Quebracho extract (originating from the bark of the tree *Schinopsis balansae*) is a commercially available rich source of polyphenols. This brown coloured fine powder contains 73% of CT, 19% of simple phenolics and 8% water (Athanasiadou et al., 2000b). Quebracho extracts have been used in previous studies to observe their feeding effect on parasitised sheep (Athanasiadou et al., 2000b, Athanasiadou et al., 2000a). Studies conducted by Athansasiadou et al. (2000) on the short and long-term feeding effects of Quebracho extract towards parasitised sheep with *T. colubriformis* have shown the reduced worm burdens and fecundity on the infected sheep. The direct anthelmintic effect of CT from Quebracho extracts has been attributed to the reduction in the faecal egg count, FEC observed during a 10-week experimental period (Athanasiadou et al., 2000b, Athanasiadou et al., 2000a). Hence, CT from the Quebracho extracts could be a potential answer for tackling the current nematode situation. However, all the nematocidal research performed with the Quebracho extract were

with its crude aqueous extract. The further separation of the crude extract towards obtaining CT isolates was not reported in the literature.

1.4.1.1.4. Limitations

As discussed in the previous section, the beneficial effects upon the consumption of CT are due to their protein binding ability, as they prevent the degradation of dietary proteins in the rumen and increases the protein availability in the lower digestive tract. However, there is a number of detrimental effects associated with the consumption of a high concentration of CT (Hoste et al., 2006), such as growth inhibition in young lambs, and reduction in food intake. Also they can interfere with the morphology and proteolytic activity of ruminal microbes (Hoste et al., 2006). Hence, this protein-binding feature of CT has some adverse effect on livestock production.

1.4.1.2. Alkaloids in Management of Nematodes

Many naturally occurring alkaloids as discussed in the previous section, may be extracted from plant sources. Many possess remarkable biochemical effects and pharmacological properties such as antitumor, antithrombotic, antiviral and antiparasitic activities (Havlasova et al., 2014). The nematocidal activity of naturally occurring alkaloids has been well founded (Onda et al., 1970, Singh, 2006, Chandravadana et al., 1994, Kusano et al., 2000, Matsuda et al., 1991, Thoden et al., 2009, Wuyts et al., 2006). However, most of these reported studies were with plant-parasitic and free-living parasites. In Table 1.5, the alkaloids reported in the literature to demonstrate anthelmintic activity against ruminant GIN are presented.

Alkaloids	***Source***	***GIN Parasite***	***Activity***
Berberine	Chemically sourced	*H. contortus*	Inhibition of larval motility of 98% at 1 mg/mL (da Silva et al., 2021)
Naturally occurring alkaloids mixture containing 1.33 g/kg DM of Protoberberine-type alkaloids	Mixed formulation: Stems of *Artemisia absinthium L.* (1%), *Malva sylvestris L.* (12.4%), *Achillea milefolium L.* (12.4%), *Cichorium intybus L.* (12.4%), *Hypericum perforatum L*. (12.4%) and *Urtica dioica L.* (12.4%). Flowers of *Matricaria chamomilla L.* (12.4%), *Fumaria officinalis L*. (12.4%) and *Calendula officinalis L.* (12.4%)		Supplementation of herbal mixtures to the diets of GIN parasite infected-lambs decreased medium chain fatty acids and increased long chain fatty acids in liver and meat, and decreased lipid oxidation in meat due to their inhibitory effects on the ruminal biohydrogenation (Szulc et al., 2020)
Lupin alkaloids mixture: 13-tigloyloxy-lupanine, 17-oxoisosparteine, 7-hydroxysparteine, N-Methyldehydroalbine	*Lupinus spp.* (Lupin)		Total paralysis of L3 larvae at 150–250 µg/mL (Dubois et al., 2019)
Basic alkaloids	*Tabernaemontana citrifolia* (Milkwood)		L3 inhibition with 49.4% efficacy at 1 mg/mL of methanolic extract (Marie-Magdeleine et al., 2010)
Convolutamine H and Convolutindole A	*Amathia convolute*		L3 growth inhibition. LD_{99} = 0.20 µg/mL (Narkowicz et al., 2002)
Atanine (3-dimethylallyl-4-methoxy-2-quinolone)	*Evodia rutaecarpa* (Evodia)	*T. circumcincta*	L4 death (Perrett and Whitfield, 1995)

Table 1.5. Anthelmintic activity of a few selective alkaloids against ruminant GIN reported in the literature.

The effect of the alkaloids has been reported to inhibit various phases of the respective nematode life cycle. However, most of the above reported studies were with the in-vitro research with nematode parasites. Assessment of the anthelmintic efficacy of the alkaloids as pure drug form has not been reported. Furthermore, none of these alkaloids were extracted from the native plants found in NZ. The NZ native medicinal plants containing alkaloids could potentially be an ideal candidate for anthelmintic research.

1.4.2. Limitations of Secondary Metabolites of Bioactive Plants as usage of Anthelmintics

From the literature, none of the active compounds derived from medicinal plants appear to have been developed or administrated as a clinical anthelmintic yet. The principal reason is the lack of information and sufficient research to qualify the isolated compounds as novel anthelmintics. This prevents them from becoming substitutes to what currently is available in the market and attracting industrial interest.

Most current anthelmintics, as discussed in section 1.3.1, cause motor paralysis to the worm, but the mechanism of action or mode of operation for the secondary metabolites derived from bioactive plants has not been properly elucidated in the literature (Faizi et al., 2011, Dubois et al., 2019, Liu et al., 2018). This is understandable as most research has been carried out in parasitology labs. The larval motility inhibition test against L3 stage of the worm appears to be the most popular test, but there is a lack of reports as to whether the secondary metabolites are effective in all stages of the larval development as well as adult phase (Holden-Dye and Walker, 2014, Liu et al., 2020). A partially understood mechanism of action has been mitochondrial inhibition without exerting the toxic effect to the host (Sakai et al., 2012). In addition, it is not fully understood whether the bioactive compounds exert a transient or a permanent effect when interacting with the worm.

Synergistic effects have been reported to be common for many bioactive plants when administered against GIN (Klongsiriwet et al., 2015) which explains the lower potency of individual secondary metabolites (Wagner and Ulrich-Merzenich, 2009). Nevertheless, the synergistic effect has the advantage of exerting lower risk of resistance against GIN, especially if the mechanism of action of the individual components differs. However, a very important point to note here is that synergy of plant secondary metabolites with commercially available anthelmintics has hardly ever been tested (Hu et al., 2010). The useful combination formulas

of secondary metabolites derived from plant extracts with known anthelmintics have the potential to be successful, which might overcome resistance, and this can also be commercially patented.

Most reported studies of active compounds from bioactive plants use only a single target organism, mostly *C. elegans*, which is not a GIN. It is imperative that the suitable parasite or a mixture of parasites is tested against the plant derived secondary metabolites for progressing towards further development as an anthelmintic. The currently available anthelmintics are effective on a wide range of GIN, which is essential for livestock management where mixed infections are common. Hence, to become a suitable candidate as an anthelmintic, the secondary metabolite derived from the bioactive plant should be tested on a mixture of GIN originating in ruminants. Toxicity of the anthelmintic compounds have been seldomly reported (Martin and Robertson, 2010). For the clinical development potential assessment, this is essential.

As discussed in section 1.4.1, only a handful of studies have assessed the in-vivo efficacy of the active anthelmintic compounds. Most of them have used a lab animal model, which is significantly more cumbersome and expensive (Giordani et al., 2016), but nevertheless essential for progressing towards clinical development. In order to be a successful anthelmintic, proper trial studies with live ruminants affected with GIN need to be assessed.

A wide range of larval bioassays has been reported such as motility, egg laying, egg hatching, and development to assess the anthelmintic efficacy of the plant secondary metabolites. However, there is an absence of a fixed consensus on which of these demonstrate the best value to further develop as a clinical anthelmintic. The potency of the reported active compounds ranges in a wide order of magnitudes, spanning anywhere from 1 µg/mL to around 1 mg/mL. The differences in bioassay type and measured parameters, in conjunction with species difference, contribute to this wide order of magnitudes. It should be noted that for even one species (*C. elegans*), the potency values cover a very wide range (Mathew et al., 2016). It is worth noting that a few active compounds overlap in its lower range with IC_{50} for some clinically used anthelmintics (Liu et al., 2020). This is due to the fact that traditionally many purified compounds have been used to treat GIN.

The question now arises as to how this scenario can potentially be improved. The answer lies in thorough chemical investigation of the active compounds, which can be obtained by chemical synthesis of analogues of the active compounds. Achieving this, it may also permit filing composition of matter patents, which in turn will increase the industrial opportunities. Most academic parasitology labs do not have the necessary means or expertise to obtain this. Therefore, chemically focused research of bioactive compounds performed by a chemistry group may provide the necessary expertise.

1.5. Medicinal Plants Found in NZ of Interest

1.5.1. Kawakawa: Endemic Māori Medicinal Plant

Macro excelsum, also called *Piper excelsum*, commonly known as Kawakawa (KK), or NZ pepper tree, belongs to the *Piperaceae* family. It is an endemically found shrub which can grow up to six meters and is widespread in NZ. Occurrence and distribution *of M. excelsum* is predominant on the North Island of NZ and reaches as far as to the northern warmer part of the South Island (John Dawson, 2019). Māori have deep heritage associated with this plant and it has huge cultural significance. Kawakawa leaves have been used as a food ingredient since the mid 1800's, and is generally based on flavouring or the consumption of the sweet peppery fruit (Riley, 1994). Māori medicinal uses of kawakawa include a range of ailments (eczema, boils, rheumatism, toothache), as a general tonic and for the treatment of various genito-urinary, dermatological, gastrointestinal and respiratory complaints (Riley, 1994). Specimen of *M. excelsum* and its distribution in NZ, its fruits and leaf are presented in Figure 1.2.

(a)

(b)

(c)

(d)

Figure 1.2. (a) Specimen of M. excelsum; (b) Distribution of M. excelsum in NZ, illustrated in green (John Dawson, 2019), (c) M. excelsum ripe and unripe fruits, (d) M. excelsum leaf.

The aromatic heart shaped leaves are 6 to 12 cm long. The fruit, orange when ripe and green when unripe, look like a miniature sweet corn cob. The pulp of the fruit is consumed by Māori while rejecting the seeds (Landcare-Research, 2021). The leaves are traditionally used as tea, as a spice for many dishes, or to prepare the popular 'Ti-toki' liquor (Venell, 2019). The leaves in general have been considered as less powerful than the seeds traditionally (Baber, 1887). However, the extracts of leaves have shown antimicrobial activity (Calder et al., 1986). The roots have also been regarded to have medicinal properties (Landcare-Research, 2021). Māori and early settlers in NZ regarded *M. excelsum* to possess aphrodisiac properties and stimulating effects on the bowels, kidneys, and salivary glands (Baber, 1887).

It is important to note here that a highly specialised and distinctive native caterpillar, *Cleora scriptaria*, also known as the KK looper moth of the family *Geometridae* endemic to NZ, feed on the leaves which causes the characteristic holes and riddled look of *M. Excelsum* (John Dawson, 2019). However, this damage does not contribute to the production of the specific defence compounds repelling *C. scriptaria* and the leaf shedding is not affected in response to the damage and also the content of the phytochemicals present (Hodge et al., 2007). It has been hypothesised that the tolerance against the defence mechanism of the host had been obtained over wide period of time (Hodge et al., 2000, Hodge et al., 2007). The picture of the dried *M. excelsum* leaf and the caterpillar is illustrated in Figure 1.3.

(a) (b)

Figure 1.3. (a) Dried M. excelsum leaf with holes caused by C. scriptaria and a fresh undamaged leaf; (b) C. scriptaria feeding on M. excelsum.

Kawakawa has been found to be rich in piperine as well as phenylpropanoid myristicin, an essential oil and lignans, terpenoids and amides (Cambie, 1976). In the essential oil obtained via steam-distillation of the leaves and terminal branches, myristicin was identified as the major volatile component (Briggs, 1941),and phenyl propanoids, mono- and sesquiterpenes (Briggs LH, 1975). In addition to these compounds, α-pinene, aromadendrene, γ-cadinene, (-)-cadinene dihydrochloride, palmitic acid, and elemicin have been reported and identified (Briggs LH, 1975). Lignans have been reported to have insecticidal properties and amides are responsible for the chemesthetic effect of *M. excelsum* leaves and fruit (Butts et al., 2019b, Briggs, 1941). (+)-Excelsin, (+)-epiexcelsin, (+)-demethoxyexcelsin, and sesangolin have been previously isolated from the leaves (Russell and Fenemore, 1973, Briggs LH, 1968) and (+)-diayangambin, which is also known as lirioresinol-C dimethyl ether is found in both the wood and leaves (Briggs LH, 1968, Russell and Fenemore, 1973). This lignan is known for its anti-inflammatory effect in-vivo (De León et al., 2002). In addition, lirioresinol-B dimethyl ether has been identified and found in the wood (Briggs LH, 1968). The structures of the lignans are described in Figure 1.4.

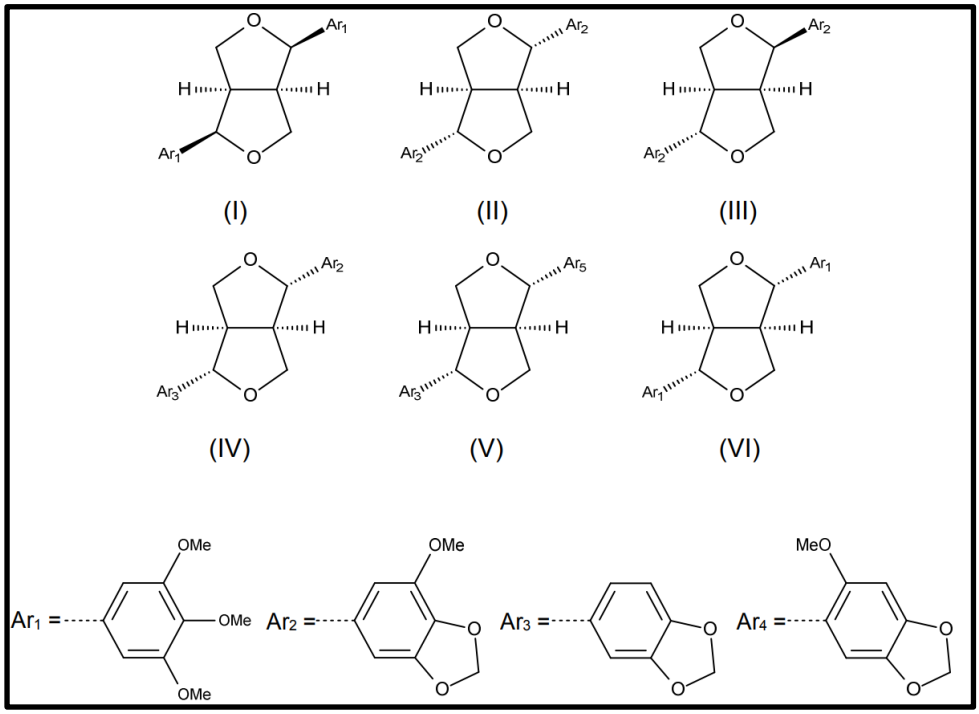


Figure 1.4. Structures of the lignans (+)-diayangambin (I), (+)-excelsin (II), (+)-epiexcelsin (III), (+)-demethoxyexcelsin (IV), sesangolin (V), and lirioresinol-B (VI) found in M. excelsum(Briggs LH, 1968, Russell and Fenemore, 1973).

Recently, Lei et al. have reported the identification of 17 amides from *M. excelsum* fruits, which included piperchabamide and the discovery of two new amide variants (4 & 5)(Lei et al., 2015). The amides are illustrated in Figure 1.5.

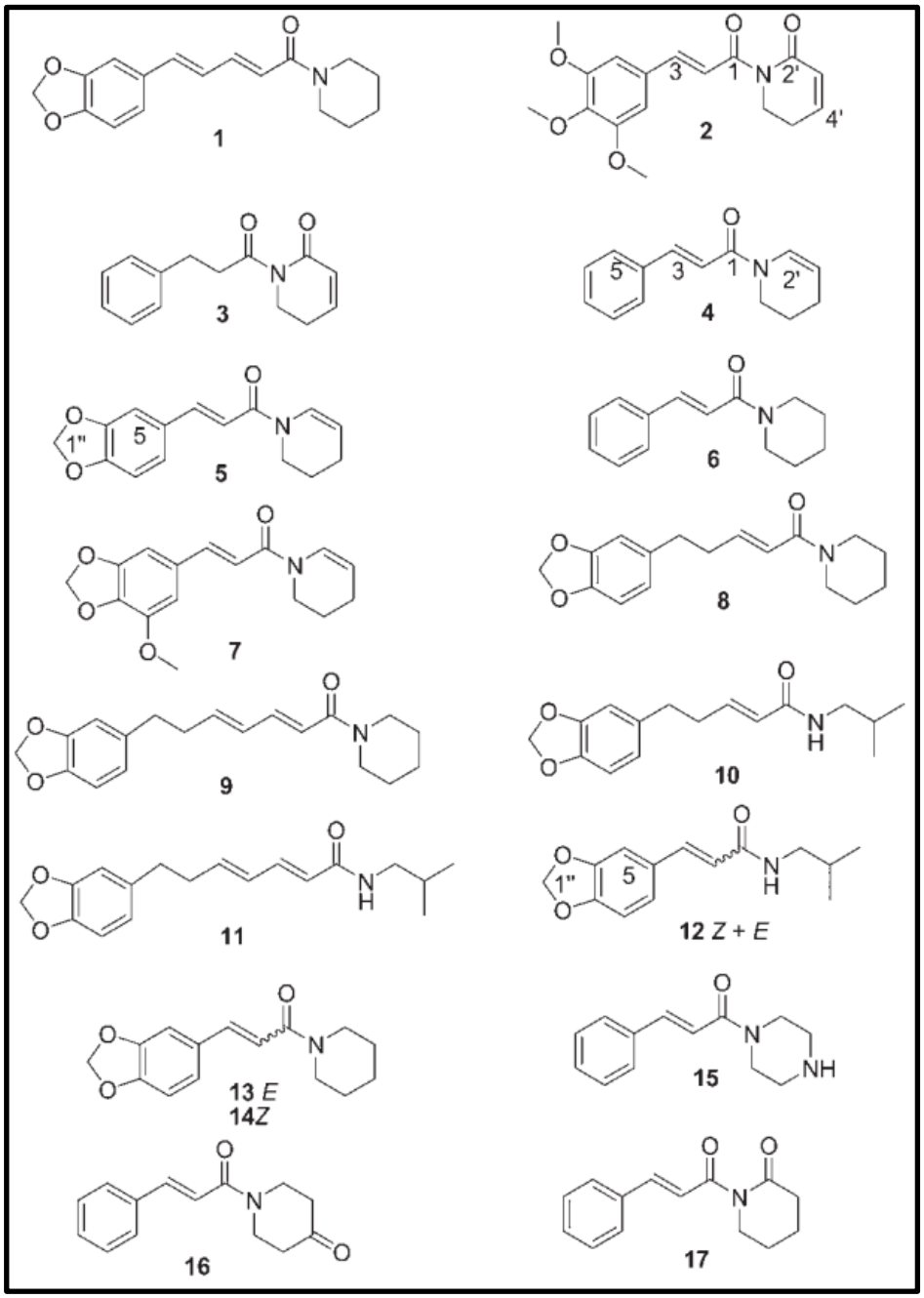


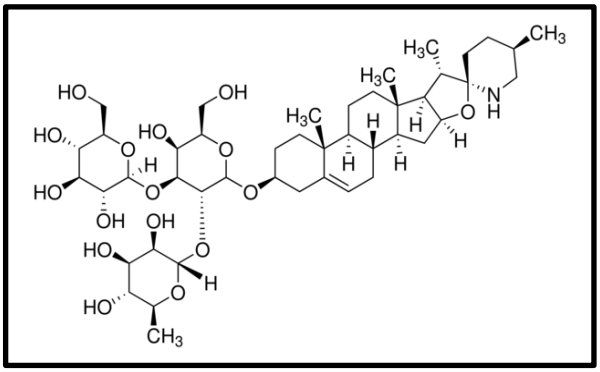
Figure 1.5. Kawakawa amides; 1. Piperine, 3. Piperchabamide A, 4. Piperideide, 5. Piperideide, 8. Piperanine, 9. Piperdardine, 12. Fagaramide (Lei et al., 2015).

It should be noted here that direct anthelmintic efficiency of *M. excelsum*, extracts of leaves, fruits, or total plant or the compounds separated has not been studied or reported in the literature previously. This thesis delves deep into the phytochemical separation of different components of kawakawa, collected from the local Bledisloe Park, Palmerston North and their nematocidal efficacy against GIN. The information provided and the research described relating to kawakawa in this thesis remains confidential and sensitive knowledge.

1.5.2. Other Medicinal Plants Found in NZ of Interest

Solanum aviculare, commonly known as Poroporo, is a NZ endemic plant of the family Solanaceae. The berries, and fruits of this plant have had traditional Māori use as a food. Most importantly, its use has been noted as a topical anti-inflammatory in the treatment of eczema, an inflammatory skin condition (Alspach et al., 1983). The leaves, including juice, inner skin and the underside were pulped and used as a poultice for skin irritations, ulcers, established sores, eruptions, and scabies. The plant has been found to contain the steroidal saponin solasonine, which is speculated to be responsible for the healing activity of this plant (Mann, 1979, Alspach et al., 1983). Solasonine, is used commercially as a precursor for the production of complex steroidal compounds in Europe. It is believed to either interact directly with steroid receptors on the outside of cells, or act as a precursor for the body's own production of anti-inflammatory steroid hormones within the body (Alspach et al., 1983). However, the nematocidal effect of this plant extract against important GIN found in NZ sheep is yet to be reported. A photo of a specimen of the plant and the structure α-Solasonine found in the extract are depicted in Figures 1.6.

(a)



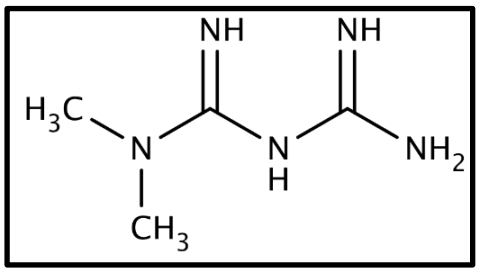
(b)

Figure 1.6. (a) Solanum aviculare (Poroporo), (b) α-Solasonine found in the extract of S. aviculare.

Goat's rue (*Galega officinalis*) is a leguminous plant native to Eastern North America. It is found abundantly in NZ growing in dry sandy woods, openings, fields, and roadsides. Goat's rue is used as a diuretic and it has diaphoretic properties in malignant fevers and the plague (Cornara et al., 2015). Several phytochemicals are found in goat's rue such as galegine, vasicinone, luteolin, saponins, flavonoids, and tannins (Phytochemicals, 2006). The active ingredients in goat's rue that lower of blood glucose levels were shown to be galegine or isoamylene guanidine (Witters, 2001). However, guanidine itself and certain direct derivatives are too toxic for the treatment of diabetes mellitus. Nevertheless, the biguanides (two linked guanidines) have proven to be useful (Bailey, 2017). The widely used hypoglycemic drug, metformin, came from the traditional approach of using this plant (Bailey, 2017). Goat's rue can be poisonous to hungry travelling livestock (especially sheep) when they are exposed to large amounts of the plant (AgPest-Agresearch, 2011). However, the nematocidal effect of this plant extracts against important GIN found in NZ sheep has yet to be seen. A photo of a

specimen of the plant and the structure of key metabolites and the drug metformin found in the extract are given in Figure 1.7.

(a)

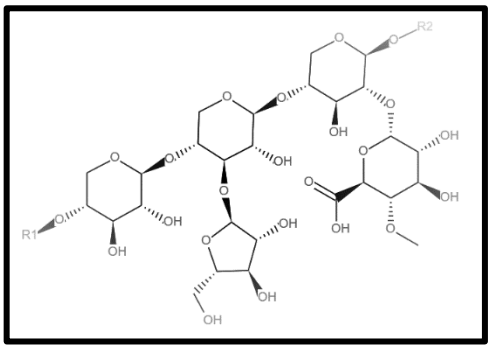


(b)

Figure 1.7. (a) Galega officinalis (Goat's Rue); (b) Metformin derived for the plant extract.

Harakeke, *Phormium tenax,* or most commonly known as NZ flax, is an evergreen plant endemic to NZ of the family *Asphodelaceae*. The medicinal use of this plant has been widely documented (Whenua, 2015). The Māori traditional uses of this plant include using the sticky sap or gum that the flax produces are applied to boils and wounds and also used for toothache. Flax leaves were used in binding broken bones and matted leaves were used as dressings. Flax root juice was routinely applied to wounds as a disinfectant. The reddish juice from the lower flax leaves was rubbed for the treatment of rheumatism or sciatica for pain relief and the decoction as a very mild laxative and for gonorrhoea (Bell, 1890, Richard, 1870). The decoction has also been documented for use as anthelmintic to treat roundworm diseases (Bell, 1890). Due to its well documented medicinal properties, it would be interesting to see whether it has any effect on infective stage of GIN. The mucilage isolated from the leaf base contains mostly xylose, arabinose, and glucuronic acid, with considerable variation observed in the proportions of xylose and arabinose and glucuronoarabinoxylan, a highly branched, high molecular weight xylan (Tauwhare et al., 2006, Sims and Newman, 2006). A photo of a specimen of the plant and the structure of Glucuronoarabinoxylan found in the mucilage of *P. tenax* are given in Figure 1.8.

(a)

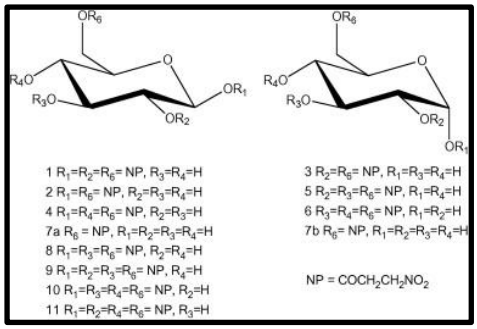


(b)

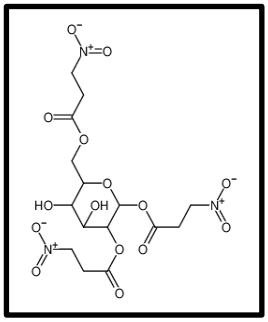
Figure 1.8. (a) Phormium tenax (NZ flax or harakeke); (b) Glucuronoarabinoxylan found in the mucilage of P. tenax.

Karaka, *Corynocarpus laevigatus*, is an endemic plant to NZ of the family *Corynocarpaceae*. Māori traditional uses of this plant included the consumption of fruit and karaka kernels which is of great importance, second only to kumara (Shaw and Billing, 2006, Tiritiri-Matangi, 2016). However, due to the presence of the alkaloid toxin karakin, the fruits have been considered as deadly poisonous if not consumed without removing all the traces of the alkaloid (Slaughter et al., 2012, MacAskill et al., 2015). The Karaka nuts were a highly valuable food source. Māori people stored and ate them during winter when other foods were scarce (Tiritiri-Matangi, 2016). Karaka is also important in traditional ceremonies, banquets, funerals and in formal exchanges between tribes (Klinac, 2009). It would be interesting to see whether the extracts have any anthelmintic properties against GIN as it has not been researched yet. A picture of the plant and key metabolites found in the extract are given in Figure 1.9.

(a)



(b)



(c)

Figure 1.9. (a) Corynocarpus laevigatus with its berry (Karaka); (b) Nitropropanoyl esters of d-glucopyranose found in Karaka drupes (Majak and Benn, 1994); (c) the toxin alkaloid karakin.

1.6. Aims of this PhD

As discussed throughout the previous sections, tannins, alkaloids, and other secondary plant metabolites from plant extracts have been reported to possess anthelmintic properties. In this PhD, the anthelmintic properties of the commercially available Quebracho extract and the medicinal plants discussed in Section 1.5 were evaluated against ruminant nematode L3 stage larvae under in-vitro conditions. These larvae were cultured in-lab from ruminant faeces infected with GIN procured from local farms in the Manawatu-Wanganui region.

The anthelmintic efficacy of the crude plant extracts and their separated fractions were compared with a series of chemical anthelmintics. A few combination formulations were designed with the plant fractions and the chemical anthelmintic to observe any potential synergistic effect and whether the efficacy` of the anthelmintic was improved in the combination. The toxicity of the most effective plant fraction was assessed.

These aims were addressed through a series of hypotheses and objectives as presented below.

1.6.1. Overall Hypothesis

Following were the three main hypotheses taken in this PhD.

1. The bioassay-guided fractionation and chromatographic separation of a crude plant extract will result in a series of fractions distinct from one another.
2. The separated fractions will possess higher anthelmintic efficacy than their parent crude plant extract. The major active phytochemical groups isolated from the active fractions may belong to plant secondary metabolites known for their anthelmintic activity, such as tannins, and alkaloids.
3. The efficacy of a sample will not vary depending on the larval population i.e., when tested with two or more batches of larvae having different larval populations.

1.6.2. Specific Objectives

Following were the specific objectives of the research conducted in this PhD.

1. To conduct larval motility assay of the plant samples with the larvae cultured from the GIN infected faeces to assess their nematocidal efficacy.
2. To study the nematocidal efficacy of the commercially available Quebracho extract and its fractions obtained through bioassay-guided fractionation and chromatographic separation such as reverse phase flash chromatography and Sephadex LH-20 column chromatography.
3. To collect various components of the NZ medicinal plants discussed in Section 1.5 from the local region and conduct their nematocidal efficacies to find the most effective.
4. To separate the crude plant extract through various chromatographic separations such as, silica-gel chromatography, normal phase and reverse phase flash chromatography, Sephadex LH-20 column chromatography, countercurrent chromatography, in order to obtain discrete and purified fractions.
5. To design combination formulations of the purified plant fractions and commercially available chemical anthelmintic to assess their nematocidal efficacy.
6. To characterise the plant samples using liquid chromatography-mass spectroscopy analysis.

7. To qualitatively detect the phytoconstituents present in the active pool of fractions of the most effective plant and its crude extract.
8. To understand and determine the structure of the active compound(s) present in the most effective purified fraction(s) using liquid chromatography-mass spectroscopy/mass spectroscopy analysis.
9. To evaluate the cytotoxicity of the effective plant samples with mammalian epithelial cells using the standard methods of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Lactate Dehydrogenase (LDH) assays to evaluate cell cytotoxicity.

Chapter 2. Materials and Methods

Materials and Methods

2.1. Introduction

In this chapter, the list of solvents, chemicals, apparatus, equipment, chemical and nematocidal methodology that were used in this PhD research are presented with two exceptions. An improvised version of an established chromatographic separation was developed during the course of this PhD. The methodology of this separation is described in Chapter 8. The toxicity assessment of Kawakawa leaf samples used a methodology exclusive to this research. This methodology of this is presented in Chapter 11.

This chapter is divided into 3 individual sections. The first section (2.2) contains the list of solvents, chemicals and equipment. The second section (2.3) depicts all the chemical methods used in the research and the third section (2.4) presents the nematocidal culture assay methods, calculation and statistics used in the nematocidal experiments.

2.2. List of Solvents, Chemicals and Equipment

2.2.1. List of Solvents

The following solvents were used for plant extracts preparation, fractionations, and chromatographic separation: distilled water (H_2O), hexane, dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH), ethanol (EtOH), butanol (BuOH), chloroform ($CHCl_3$), propanol (PrOH), and glacial acetic acid (AcOH). Analytical grade solvents were obtained from Fisher scientific (*Fisher Scientific New Zealand Company, Auckland, NZ*).

2.2.2. List of Chemicals, Reagents, Indicators, and Pharmaceuticals Compounds

The following list of chemicals, reagents, indicators, and pharmaceutical compounds was used throughout the plant extraction process, for preparation of staining reagents, identification of phytoconstituents, and in-vitro nematocidal analysis: acetonitrile (CH_3CN), ammonia solution, ammonium formate (NH_4COOH), Benedict's reagent, benzene, copper sulphate ($CuSO_4$), diethylamine, Dragendorff's reagent, Fehling's A and B solutions, ferric chloride ($FeCl_3$), formic acid (HCOOH), gallic acid (GA), glyoxylic acid, hydrochloric acid (HCl), iodine crystal, lead acetate ($Pb(OAc)_2$), Mayer's reagent, Millon's reagent, ninhydrin, nitric acid (HNO_3), picric acid, potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide (NaOH), concentrated sulfuric

acid (H_2SO_4), tannic acid (TA), and vanillin. Reference anthelmintics, abamectin (AB), benzimidazole (BZ) and ivermectin (IVM), were used as positive controls for nematocidal experiments. All the analytical grade chemicals and reagents were purchased from either Fisher Scientific or Sigma-Aldrich (*Sigma-Aldrich New Zealand Company, Auckland, NZ*).

2.2.3. List of Apparatus and Equipment

The equipment used for plant material processing, fractionation, extraction, and separation steps and in-vitro nematocidal analysis were as follows: Milli-Q water purification system (*Merk-Millipore Milli-Q Reference Water Purification System C79625, Merck KGaA, Darmstadt, Germany*), digital analytical balances (*Mettler-Toledo ML204T/00, Mettler AE200, Mettler PE12; Mettler-Toledo, LLC, Columbus, USA*), hot-air oven, high-speed multifunction grinder (*Wuyi Haina Electric Appliance, China*), electric magnetic stirrer (*IKA RCT Basic; IKA Works (Asia) Sdn Bhd, Malaysia*), heating mantle (*Electromantle Electrothermal EM2000/CEMK5*), rotary evaporator (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland*), heating gun (*Makita HG1100CS/662Q; Makita Inc., CA, USA*), blow dryer (*Sunbeam HD4400*), UV lamp (*ZF-1 Three UV Analyser*), centrifuge (*Eppendorf Minispin, Eppendorf AG, UK; Thermoscientific Megafuge 40, Thermofisher Scientific, Massachusetts, USA*), ultrasonic cleaner (*Cole-Palmer 8891; Cole-Palmer, IL, USA*), flash chromatographic system (*BÜCHI C-620*), freeze dryer (*FTS Systems Flexi-Dry; Stoneridge, NY, USA*), refrigerator (*Fisher and Paykel Active Smart*), incubator (*BINDER APT. Line KBF Climatic Chamber; BINDER GmbH, Tuttlingen*), and microscope (*Leica EZ4W with ICC50W camera attachment, Leica AG, Wetzlar, Germany; Olympus CX41 with DP22 camera attachment, Olympus Corp., Tokyo, Japan*). The following apparatus were used throughout the various steps of the chemical process and in-vitro nematocidal analysis: beakers, conical flasks, round bottom flasks, Soxhlet extractor with glass thimble, filter papers, separatory funnel, reflux condenser, test tubes, micropipettes (*Gilson pipetman* 2-20 µL, 50-200 µL, 100-1000 µL), graduated pipettes with pipette bulb, vacuum desiccator, syringes, syringe filters, capillary tubes, glass columns, sieves, 48 well tissue culture plates, microscopic slides, microscopic coverslips, and PVC tubes.

2.3. Methodology of Preparation, Extraction and Separation of Plant Materials

2.3.1. Selection of Plants

The Quebracho extract was selected because of its documented use as an anthelmintic reagent (Athanasiadou et al., 2000b) which was previously described in Section 1.4.1.1.4. It is a commercially found source of polyphenols. The other four plants researched in this PhD were selected based on their medicinal usage in traditional Māori healings against gastrointestinal nematode infections and for other medicinal properties as described in Chapter 1. These plants were: Harakeke (*Phormium tenax*), Karaka (*Corynocarpus laevigatus*), Kawakawa (*Piper Excelsum*) and Poroporo (*Solanum aviculare*). All the aforementioned plants are natively found in NZ. A fifth plant, Goat's Rue (*Galega officinalis*), was collected and studied for its anthelmintic property. This plant is not native to NZ. However, it is endemically found in NZ.

2.3.2. Collection of Quebracho Extract and Plants

Quebracho extract was purchased in bulk (40 kg) from INDUNOR S.A, Cerrito 1136, Buenos Aires – Argentina. For the series of plants selected, the whole plant or a component of the plant was collected where the metabolites of interest were perceived to be accumulated (Gajbhiye et al., 2015). Hence, as documented in the traditional Māori medicinal usage, a series of components of plants were selected. The components that were selected are presented below in Table 2.1.

Plant	***Component(s) collected***
Kawakawa (*Piper Excelsum*)	Fruit, leaf, stem, root
Poroporo (*Solanum aviculare*)	Leaf
Karaka (*Corynocarpus laevigatus*)	Leaf
Harakeke (*Phormium tenax*)	Leaf
Goat's Rue (*Galega officinalis*)	Leaf

Table 2.1. List of plants collected, and components selected.

These plants were identified by Gupta at Bledisloe Park adjacent to Massey University, and other areas around the Massey University campus. Fresh components of each

abovementioned plants were gently plucked without doing any damage to the plant. The components were collected in polyethylene bags, tied with rubber bands, and transported to School of Fundamental Sciences, Massey University on the same day and immediately processed for drying.

2.3.3. Drying and Grinding of Plant Components

After collection, the plant components were thoroughly brushed to remove any kind of debris and cut into small pieces. The cut pieces were placed on clean a plastic tray and evenly distributed to facilitate homogenous drying. For the initial studies, the trays were placed outside and dried under sunlight during daytime and then taken inside in a room with adequate ventilation overnight. This drying procedure took 4-5 days. For later studies, the KK leaf component was subjected to drying inside a hot-air oven set at 65 °C for a period of 11-12 hrs. After drying, a high-speed mechanical grinder was used to shred the plant component to fine, evenly sized particles (~1 mm) which increased the surface area and enabled better solvent extraction.

2.3.4. Preparation of Crude Plant Extracts

Fine powder (20 g) of each plant component was suspended in 200 mL MeOH in a 500 mL conical flask designated for the respective plant components. The suspension was stirred using an electric stirrer for a period of 24 hr for extract preparation. Supernatants were collected by carefully decantation followed by filtration using Schleicher & Schuell filter paper (No 595, ø 270 mm). They were concentrated to dryness by rotary evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland*) and transferred to a round bottom flask. The flask was subjected to freeze drying in a freeze-drier (*FTS Systems Flexi-Dry; Stoneridge, NY, USA*) to remove any trace of moisture and residual solvent by covering the flask mouth with Kimwipe tied with a rubber band to avoid any spillage. The dried plant extract in the round bottom flask was weighed and stored in a vacuum desiccator connected to the freeze drier awaiting further use.

2.3.5. Soxhlet Extraction of Plant Components

Dried fine powder of the selected plant component was subjected to Soxhlet extraction. Methanol (1500 mL) was placed in a 3 L round bottom flask placed over the

concave heating mantle and was used as the extraction solvent. Crude dried fine powder of the plant component (250 g) was added to the sample thimble chamber (75 x 180 mm) of the Soxhlet extractor. A reflux condenser was set on top of the extractor and the temperature of the heating mantle was set to 150 °C. The extraction temp in the round bottom flask was found to be ~70 °C. The Soxhlet extraction was run for 9 hrs. After extraction, the non-soluble portion of the extracted plant sample remained in the thimble and the solvent in the flask contained all the soluble components of the sample.

2.3.6. Solvent Partitions Using Liquid-Liquid Extraction

The MeOH extract of the plant component obtained from Soxhlet extraction was concentrated by rotary evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland*) until 80% of its volume was removed. It was then diluted with water to make the final volume to 500 mL. This was the parent Water-Methanol Fraction. This solution was extracted first with 500 mL of hexane and followed with the same volume of ethyl acetate (EtOAc). The Water-MeOH Fraction and the respective solvent were mixed using vigorous shaking for 5 mins in a separatory funnel and the layers were then allowed to settle for 45-60 mins. The individual layers then collected in respective round bottom flasks. These solvents were concentrated to dryness by rotary evaporation followed by freeze drying to remove any residual solvent as previously described in Section 2.2.4. The dry matter yield of each solvent fraction was recorded. The samples were stored in a vacuum desiccator (273 x 240 mm; *Sigma Aldrich NZ*) connected to the freeze drier (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* until further use.

The Water-MeOH Fraction was further separated into Water and MeOH Fractions. The Water-MeOH Fraction was dissolved in 200 mL MeOH taken in a conical flask. The supernatant was collected by filtration (Schleicher & Schuell filter paper (No 595, ø 270 mm)) in a round-bottomed flask. It was concentrated to dryness by rotary-evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland)* and freeze-dried (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* to obtain MeOH Fraction. The yield of this fraction was recorded. The sample was stored in a vacuum desiccator (273 x 240 mm; *Sigma Aldrich NZ*) connected to freeze drier (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* until further use.

The undissolved solid matter which remained in the conical flask was further treated with 100 mL water. The filter paper was washed off with 100 mL water to collect any insoluble matter that was accumulated on the filter paper from MeOH extraction. The solution was then concentrated to dryness by rotary-evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland)* and freeze-dried (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* to obtain the Water Fraction. The yield of this fraction was recorded. The sample was stored in a vacuum desiccator (273 x 240 mm; *Sigma Aldrich NZ*) connected to freeze drier (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* until further use.

2.3.7. Thin Layer Chromatography Study of the Fractions of Plant Component

The solvent fractions of various plant extracts were subjected to thin layer chromatography (TLC) study using silica-gel TLC plate with F_{254} fluorescent indicator. The solvent fractions were dissolved in either DCM or MeOH depending on their solubility. The solution was loaded in microcapillary tubes and carefully spotted 4-5 times onto a TLC plate around 1 cm above the bottom of the plate. The plate was then dried using a blow dryer on its minimum heat setting. The mobile phase of the TLC study for the Hexane and Ethyl Acetate Fractions was hexane:acetone = 70:30. For Water-Methanol Fraction, it was dichloromethane:methanol = 20:80. Diethylamine (1%) was added in the mobile phase to prevent any tailing and to achieve good separation. The mobile phase was carefully poured into the chamber and a filter paper (Whatman No 1) moistened with the mobile phase solvent gradient was placed inside the chamber wall to maintain even humidity. The TLC plate with dried spots was carefully placed in the chamber ensuring the spots on the plate were not dipped but stayed above the mobile phase line enabling its migration to the spots. The mouth of the TLC chamber was covered with a glass plate. The plate was allowed to develop until the mobile phase travelled to about half a cm below the top of the plate. The plate was then removed from the chamber and the solvent front was marked with a pencil. The coloured spots were marked with a pencil and viewed inside the UV lamp chamber to identify the UV active spots. Iodine vapour and vanillin were used for staining.

2.3.7.1. Staining with Iodine Vapour

Iodine vapour is a universal reagent which is used for its reversible reactions with a wide range of organic lipophilic molecules. Hydrocarbons, fats, waxes, some fatty acids and

esters, steroids, antioxidants, detergents, emulsifiers, antibiotics and many miscellaneous organic substances (Joshi, 2012) form yellow-brown spots upon interaction with iodine vapour (De et al., 2010). If lipophilic constituents are present on the chromatogram, the iodine molecules concentrate with the substance spots and produce brown chromatographic zones on a yellow background (Joshi, 2012). Iodine crystals (1.5 g) were placed in a TLC chamber and the treated plates were stained with iodine vapour.

2.3.7.2. Staining with Vanillin Reagent

Vanillin in sulfuric acid is a universal reagent that irreversibly stains a range of organic compounds such as plant secondary metabolites containing nucleophile group which react with the aldehyde functional group in vanillin (Dykes and Rooney, 2006). TLC plates were stained by spraying 2% (w/v) of vanillin in ethanol containing 10% concentrated H_2SO_4 (2 g Vanillin 100 mL EtOH containing 10 mL H_2SO_4). After staining, the plates were heated using a heat gun and observed for coloured spots.

2.3.8. Separation of Plant Fractions by Normal Phase Silica Gel Gravity Chromatography

Normal phase silica gel chromatography has been widely used to separate and purify active and pure compounds from plant extracts (Chen and Zhang, 2017, Zerargui et al., 2020, Ahuchaogu and Echeme, 2019, Widyowati et al., 2020). The dried plant crude solvent fraction (5 g) was subjected to gravity chromatography on a glass column (30x400 mm) loaded with silica gel (230-400 mesh) using dry-column method. The plant fraction was first mixed homogenously with silica gel to form a mixture. The mixture was then loaded on top of the silica gel bed present in the column. Elution was carried out using 500 mL hexane: dichloromethane with increasing amounts of dichloromethane (100:0 (E1), 98:2 (E2), 90:10 (E3), 70:30 (E4)) followed by hexane: acetone = 70:30 (E5), dichloromethane: acetone = 70:30 (E6) and dichloromethane: methanol = 0:100 (E7). TLC of initial fractions obtained from each eluent was carried out following the method described in Section 2.2.7. The initial fractions were combined with one another on the basis of the TLC result to get the final fractions.

2.3.9. Separation and Purification of Plant Extracts by High-Performance Flash Chromatography

In the flash chromatography techniques, compressed air pressure is used to push the mobile phase through the stationary phase packed in a tightly closed plastic column or prepacked cartridges (Cvetković et al., 2020). Flash chromatography has been widely used in recent years for rapid and faster separation and purification of plant metabolites (Soerensen et al., 2021, Kapoor et al., 2020). For the flash chromatography study, the flash chromatography apparatus (BÜCHI C-620) of the *Filichev and Rowlands* group at Massey University was used. A picture of the flash chromatography apparatus in Massey University is presented in Figure 2.1 and the procedure is described below.

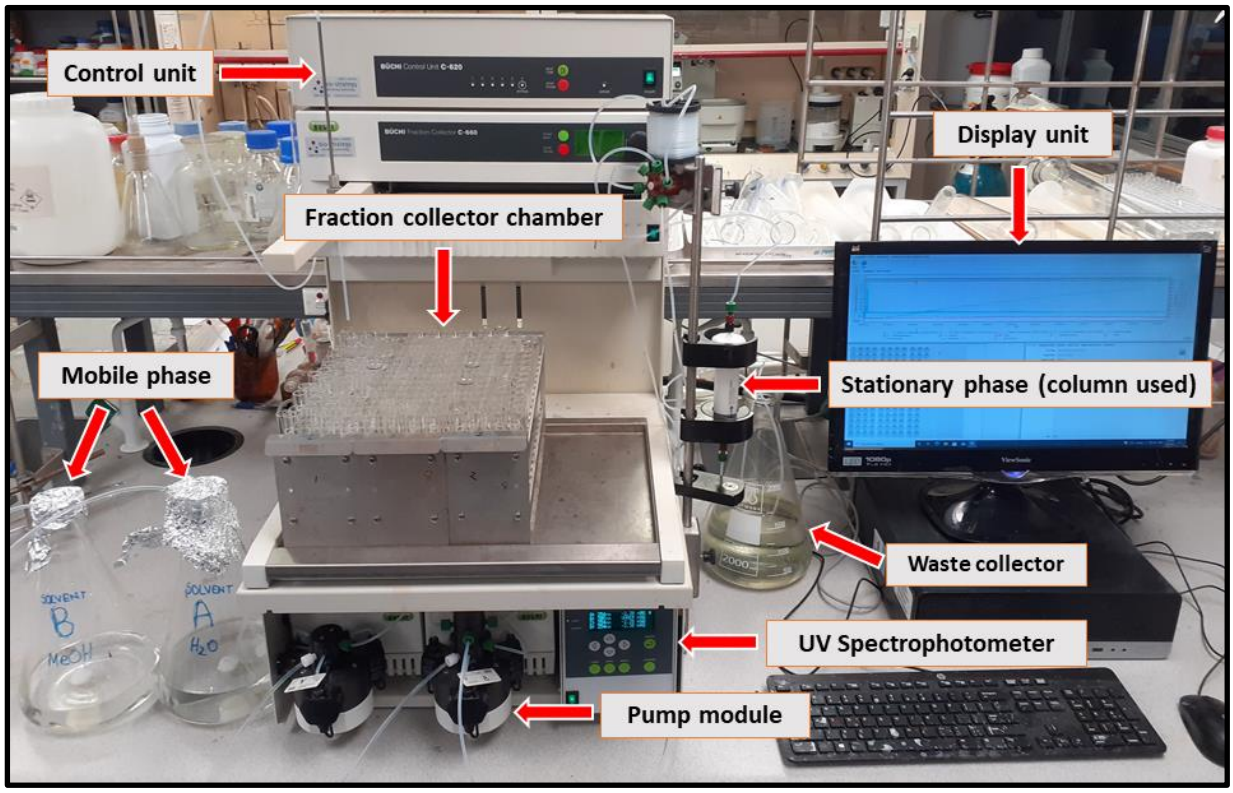


Figure 2.1. Flash Chromatography setup in Massey University and schematic description of each unit.

In the flash chromatography apparatus, the control unit BÜCHI C-620 is connected to BÜCHI Fraction Collector C-660 and BÜCHI Pump Module C-605. A UV spectrophotometer, BÜCHI UV Photometer C-640, with the function to choose 4 different wavelengths is connected to the control unit. A gradient of two solvents is used for the mobile phase.

Solvents A and B are present in respective flasks, which get directed to the stationary phase by the pump module consisting of two pumps (radially arranged 3-piston pump). Pump A is connected to the flask containing solvent A and pump B is connected to the flask containing solvent B. Two types of columns are generally used for the stationary phase; 1) An empty cartridge which can be filled with column packing material of choice by user, 2) A pre-packed cartridge filled with column packing material from the manufacturer. Both are designed specifically for flash chromatography. The sample is loaded using either dry (for an empty cartridge) or wet (for a pre-packed cartridge) method into the column. The separated fractions get directed to the tubes present in the fraction collector chamber. The tubes are test tubes present in a rack designed to fit into the fraction collector chamber. Any solvent waste gets directed to the flask present in the waste collector. This process is fully automated and controlled with a computer using *SepacoreControl - Chromatography Systems* software connected to the control unit. The progress of the separation and chromatogram can be viewed in the display unit.

2.3.9.1. Separation of Kawakawa Fruit and Leaf Fractions Using Normal Phase Flash Chromatography

The KK-Fruit-Hexane-Fraction-5, KK-May20-leaf-Hexane Fraction, and KK-May20-leaf-ETOAc Fraction were subjected to normal phase flash chromatography (NP-FC). The dried fraction (1.5 g) was dry-loaded (method described in Section 2.3.8) onto a flash chromatography cartridge (Cartridge PP 40/150) loaded with silica-gel (230-400 mesh). A gradient of hexane (solvent A) and acetone (solvent B) was used set to a flow rate of 100 mL/min. In the fraction collector chamber, a rack of 30 tubes (FC30; 30x60 mL) was positioned. The collection volume was 45 mL for the fraction collector tubes. UV detectors were set at 205 nm, 227 nm, 254 nm, and 280 nm respectively. The running method of flash chromatography of the KK-Fruit-Hexane-Fraction-5 is presented in Table 2.2, and the KK-May20-leaf-Hexane-Fraction, KK-May20-leaf-EtOAc-Fraction are presented in Table 2.3.

Time Interval	***% Of solvent B (Acetone)***
2 min	0%
20 min	0-15%
4 min	15-25%
5 min	25-40%

Table 2.2. Running method of NP-FC separation of the KK-Fruit-Hexane-Fraction-5 using Normal Phase Flash Chromatography.

Time Interval	***% Of solvent B (Acetone)***
5 min	0%
25 min	0-30%
15 min	30-85%

Table 2.3. Running method of NP-FC separation of the KK-May20-leaf-Hexane and KK-May20-leaf-EtOAc-*Fractions using Normal Phase Flash Chromatography.*

TLC of each fraction was carried out using the technique described in Section 2.3.7. Vanillin-H_2SO_4 was used as a staining reagent. The mobile phases used in the TLC study of the different fractions obtained from the NP-FC separation of the KK-Fruit-Hexane-Fraction 5 are presented in Table 2.4, KK-May20-leaf-Hexane are presented in Table 2.5 and the KK-May20-leaf-EtOAc are presented in Table 2.6.

Fractions	***Mobile phase for TLC study***
3-6	100% Hexane
7-25	10% DCM in hexane
26-50	20% DCM in hexane
51-70	30% DCM in hexane

Table 2.4. Mobile phase for TLC study of the different initial fractions obtained from NP-FC run of the KK-Fruit-Hexane-Fraction-5 using Normal Phase Flash Chromatography.

Fractions	*Mobile phase of TLC study*
1-5	100% hexane
6-10	10% DCM in hexane
11-15	20% DCM in hexane
16-20	30% DCM in hexane
21-22	50% DCM in hexane
23-25	20% hexane in DCM
26-42	5% hexane in DCM

Table 2.5. Mobile phase of TLC study of different fractions obtained from the separation of the KK-May20-leaf-Hexane-Fraction.

Fractions	*Mobile phase of TLC study*
1-5	100% hexane
6-18	70% DCM in hexane
19-21	80% DCM in hexane
22-27	100% DCM
27-31	90% DCM in acetone
32-37	20% DCM in acetone
38-62	100% acetone

Table 2.6. Mobile phase of TLC study of different fractions obtained from the separation of KK-May20-leaf-EtOAc-Fraction.

The fractions obtained from the separation were combined on basis of the TLC results to obtain final fractions. The final fractions were then concentrated by rotary evaporation and freeze-dried to remove any residual solvent. The dry matter yield of each fraction was recorded.

2.3.9.2. Separation of Kawakawa Leaf Fractions Using Reverse Phase Flash Chromatography

Reverse phase (RP) flash chromatography (FC) with C18 column has been used for the separation of mixtures of water-soluble hydrophilic fragments such as amino acids, tannins, glycosides, sulfonic acid (Hobbs and Young, 2013, Blunt et al., 1987, Zhong et al., 2014). The

RPHC technique has been used to facilitate efficient, conventional, and scalable separation and purification of fragments and drug like molecules, for which other chromatographic techniques often fail (Hobbs and Young, 2013). In this study, RP-FC separation was performed using four pre-packed C18 reverse phase columns. These columns were: SEPACORE Reverse Phase C18 4g column (12.3 x 60 mm, *BÜCHI Labortechnik AG*), TELOS Reverse Phase C18 23 g column (20 x 130 mm*, Kinesis Australia Pty Ltd*.), and TELOS Reverse Phase C18 4 g column (12.1 x 59 mm, *Kinesis Australia Pty Ltd*.). The columns used for the RP-FC separation of the different KK leaf fractions are presented in Table 2.7.

Kawakawa leaf fraction	***Column used***
KK-May20-leaf-Water	SEPACORE 4 g
KK-May20-leaf-MeOH	
KK-Oct20-leaf-Water	
KK-Oct20-leaf-MeOH	
KK-Oct20-leaf-MeOH-Fraction-1	
KK-Oct20-leaf-MeOH-Fraction-1-2	
KK-Dec20-leaf-MeOH (RUN 1)	TELOS 23 g
KK-Dec20-leaf-Water-Fraction-1-1	
KK-Dec20-leaf-Water (RUN 1)	TELOS 4 g
KK-Dec20-leaf-Water (RUN 2)	
KK-Dec20-leaf-MeOH (RUN 2)	
KK-Dec20-leaf-MeOH (RUN 3)	
KK-Dec20-leaf-Water-Fraction-1	

Table 2.7. Different kinds of columns used for the RP-FC separation of the different Kawakawa leaf fractions.

The sample quantity loaded to the pre-packed column was variable for each sample, depending on the quantity of the sample available. Usually, the sample was dissolved in water at 1 mg/mL concentration and injected carefully in the pre-packed column avoiding the formation of bubbles. In the fraction collector chamber, a rack of 60 tubes (FC60; 60x20 mL) was positioned. A gradient of water (solvent A) and MeOH (solvent B) was used set to a flow rate of 15 mL/min for the 4 g columns and a flow rate of 40 mL/min was selected for the 23 g

column. UV detectors were set at 208 nm, 254 nm, 325 nm, and 365 nm respectively. The threshold of detector signal for UV was set to 1.0 [Au, V]. Total runtime for each sample was set to ~70 minutes. The running method of the KK-May20-leaf-Water, KK-May20-leaf-MeOH and the KK-Oct20-leaf-MeOH Fractions are presented in Table 2.8; KK-Oct20-leaf-Water, KK-Dec20-leaf-Water (RUN 1 and 2), KK-Dec20-leaf-MeOH (RUN 2 and 3) Fractions are presented in Table 2.9; KK-Oct20-MeOH-Fraction-1, KK-Oct20-MeOH-Fraction-1-2, and KK-Dec20-leaf-Water-Fraction-1 are presented in Table 2.10; KK-Dec20-leaf-MeOH (Run 1) is presented in Table 2.11.

Time Interval	***% Of solvent B (MeOH)***	***Collection volume for the fraction collector tube and fraction size between peaks***
10 min	0%	9 mL
25 min	0-25%	
5 min	25%	
10 min	25-35%	
20 min	35-100%	

Table 2.8. Running method of the KK-May20-Water, KK-May20-leaf-MeOH and KK-Oct20-leaf-MeOH separation by RP-FC with % of solvent B (MeOH) applied at different time interval, and collection volume for the fraction collector tube and fraction size between peak.

Time Interval	***% Of solvent B (MeOH)***	***Collection volume for the fraction collector tube and fraction size between peaks***
6 min	0%	9 mL
12 min	0-20%	
4 min	20-35%	
18 min	35-55%	
15 min	55-90%	
15 min	90-100%	

Table 2.9. Running method of the KK-Oct20-leaf-Water, KK-Dec20-leaf-Water (RUN 1 and 2) and the *KK-Dec20-leaf-MeOH (RUN 2 and 3) separation by RP-FC with % of solvent B (MeOH)*

applied at different time interval, and collection volume for the fraction collector tube and fraction size between peak.

Time Interval	***% Of solvent B (MeOH)***	***Collection volume for the fraction collector tube and fraction size between peaks***
5 min	0%	7 mL
15 min	0-55%	
10 min	55-65%	
5 min	65-90%	
5 min	90-100%	

Table 2.10. Running method of the KK-Oct20-leaf-MeOH-Fraction-1, KK-Oct20-leaf-MeOH-Fraction-1-2, and the KK-Dec20-leaf-Water-Fraction-1 separation by RP-FC with % of solvent B (MeOH) applied at different time interval, and collection volume for the fraction collector tube and fraction size between peak.

Time Interval	***% Of solvent B (MeOH)***	***Collection volume for the fraction collector tube and fraction size between peaks***
5 min	0%	12 mL
30 min	0-25%	
50 min	25-100%	

Table 2.11. Running method of KK-Dec20-MeOH Fraction separation by RP-FC (RUN 1) with % of solvent B (MeOH) applied at different time interval, and collection volume for the fraction collector tube and fraction size between peak.

For the RP-FC separation of the KK-Dec20-leaf-Water-Fraction-1, the dried sample (305 mg) was dissolved in 0.3 mL water and carefully injected into a pre-packed TELOS RP C18 23 g column. The collection volume for the fraction collector tube and fraction size between peaks were set to 5 mL. The flow rate of the eluent was set to variable and gradually increased. The flow rate of eluent is presented in Table 2.12. The running method of the separation is presented in Table 2.13. All the other parameters were kept the same.

Tube present in fraction collector chamber	***Flow rate of solvent gradient (mL/min)***
1-3	3-4.5
4	7
5	7.5
6	8
7	8
8	8.5
9-15	9
16-20	12
21-23	15
23-27	15.5
28-36	19
37-end	22

Table 2.12. Flow rate of the solvent gradient (mL/min) set for respective tube(s) for the RP-FC separation of the KK-Dec20-leaf-Water-Fraction-1.

Time Interval	***% Of solvent B (MeOH)***
20 min	0%
25 min	0-20%
30 min	20-80%
15 min	80-100%
2 min	100%

Table 2.13. Running method of the KK-Dec20-leaf-Water-Fraction-1-1 separation by RP-FC with % of solvent B (MeOH) applied at different time interval.

The fractions obtained from the RP-FC were subjected to TLC to identify the identical fraction having the similar TLC profile. TLC profiling of each fraction obtained from the RP-FC separation of the KK-May20-leaf-Water Fraction was performed using a RP-18 TLC plate (*TLC Silica Gel 60 RP-18 F_{254} Aluminium Sheet 20x20 cm; Merck Limited*). The mobile phase of TLC study is presented below in Table 2.14. Vanillin-H_2SO_4 was used as a staining reagent.

Fractions	*Mobile phase of TLC study*
1-30	60% MeOH in Water
	70% MeOH in Water
	90% MeOH in Water
	EtOAc:AcOH:H_2O = 18:1:1
	IPA:AcOH:H_2O = 7:1:2
	BuOH:AcOH:H_2O = 5:1:4
31-50	70% MeOH in Water
	90% MeOH in Water

Table 2.14. Mobile phase of TLC study of different fractions obtained from the separation of the KK-May20-leaf-Water-Fraction.

The fractions were combined based on the peak area found in the flash chromatogram. The methodology is described in the next section.

2.3.9.3. Combination of Fractions Based on Peak Areas in the Flash Chromatogram

The fractions obtained from the RP-FC separation were combined based on peak areas obtained in the flash chromatogram. A series of hypotheses were used to combine the content of the tubes which were the fractions present in the fraction collector chamber. The hypotheses were:

1st Hypothesis: If a single peak was obtained which corresponded to the content of a single tube, there might be presence of a single fraction. The single peak might be resultant of a single isolate or a mixture of isolates.

2nd Hypothesis: Not all the components present in a fraction or in a group of simultaneous fractions were UV absorbent. There might be presence of a component which was not UV absorbent. Hence, it did not have a peak in the chromatogram.

3rd Hypothesis: The components that were present in a fraction or in a series of fractions, not all of them were UV absorbent, which is why small peak areas were obtained.

4th Hypothesis: If a fraction or a group of simultaneous fractions had the occurrence of a small peak area in the chromatogram, there might be presence of an UV-absorbent component in low quantity from a previous fraction which was responsible for this peak.

The fractions were combined using these hypotheses. Thus, the initial combined fractions obtained from the separation were then subjected to LC-MS analysis (full method presented in Section 2.3.11). An aliquot (1.2 mL) from each combined fraction was filtered using a syringe filter (ø 15 mm, pore size 0.2 µm; *Corning syringe filters*; *Sigma-Aldrich New Zealand Company, Auckland, NZ*) and transferred to a 2 mL short thread vial (32x11.6 mm, clear glass, wide opening with thread cap). It was subjected to LC-MS analysis using a Thermofisher Hypersil Gold (100x2.1 (mm) 1.9 µm particle size (THC25002-102130)) column following the method described in Section 2.3.11.2. The fractions having the same LC-MS trace were then combined. The mixtures were concentrated to dryness through rotary-evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland*) and freeze-dried (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* to obtain the final fractions. The dry matter yield of each fraction was recorded.

2.3.9.4. Separation of Quebracho Fractions Using Reverse Phase Flash Chromatography

The Quebracho Water and Butanol Fractions were subjected to RP-FC using a SEPACORE 25g column (20 x 130 mm, *BÜCHI Labortechnik AG*). The sample (1 g) was dissolved in water (1 mL) and loaded onto a pre-packed column. A gradient of water (solvent A) and MeOH (solvent B) was used set to a flow rate of 20 ml/min. The running method is described in Table 2.15. In the fraction collector chamber, a rack of 30 tubes (FC30; 30x60 mL) was positioned. The collection volume was 40 ml for the fraction collector tube. UV detectors were set at 208 nm, 254 nm, 280 nm, and 325 nm.

Time Interval	***% Of solvent B (MeOH)***
10 min	5%
40 min	5-25%
10 min	25-80%

Table 2.15. Running method of the Quebracho Water and Butanol Fractions separation by RP-FC with % of solvent B (MeOH) applied at different time interval.

The fractions obtained from the RP-FC run were combined with one another based on the method described in Section 2.2.9.3. The mixtures were concentrated to dryness by rotary-evaporation and freeze-dried.

2.3.10. Separation of Plant Extracts Using Sephadex LH-20 Column Chromatography

Sephadex LH-20 (Sigma-Aldrich) has a bead size of 25-100 µm that operates over 2-13 pH ranges. It is the alkylated derivative of hydroxyl groups of Sephadex G-25. The derivatisation adds lipophilic characteristic which preserves the hydrophilicity of the gel. Sephadex LH-20 swells with the presence of polar solvent such as methanol and water. A solvent mixture of highly polar solvents is used with Sephadex LH-20 and compounds are separated based on their difference in affinity for stationary or mobile phases (Ghisalbert, 2008).

The Quebracho Water Fraction, and the KK-May20-leaf-Water Extract were subjected to Sephadex LH-20 column chromatography. In this study, a slurry of Sephadex LH-20 was prepared in water as per the manufacturer's recommendation (1 g of dry Sephadex LH-20 in 4 mL water) and was packed in a glass column (15x450 mm) with slurry of 80 mL bed volume. The column was conditioned by passing ~300 mL of solvent for 12 hrs. The samples (2 mL with 1 g/mL of sample concentration in water) were then loaded onto the column. Elution was carried out with a gradient of 200 mL water: MeOH with increasing amounts of MeOH, 100:0 (E1), 80:20 (E2), 40:60 (E3), 30:70 (E4). The fractions were collected in 10 mL test tubes with around 6 mL of interval between tubes. The sample loaded onto the column was coloured and the solvent gradients (E1 to E4) were changed based on the progression of the analyte in the column. To elucidate, the elution started with E1 and when it was not able to move the sample analyte present in the column, the solvent gradient E2 was used which had a higher quantity of MeOH and this procedure was followed until the total passing of the sample analyte through the column using E4.

2.3.11. LC-MS/MS Analysis Method

Liquid Chromatography Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS) analysis is a widely used technique for plant extracts analysis and identification of separated

phytoconstituents (Wan et al., 2021, Jansons et al., 2021, Krasevec and Prosen, 2021). A picture of the LC-MS/MS setup in Massey University is presented in Figure 2.2.

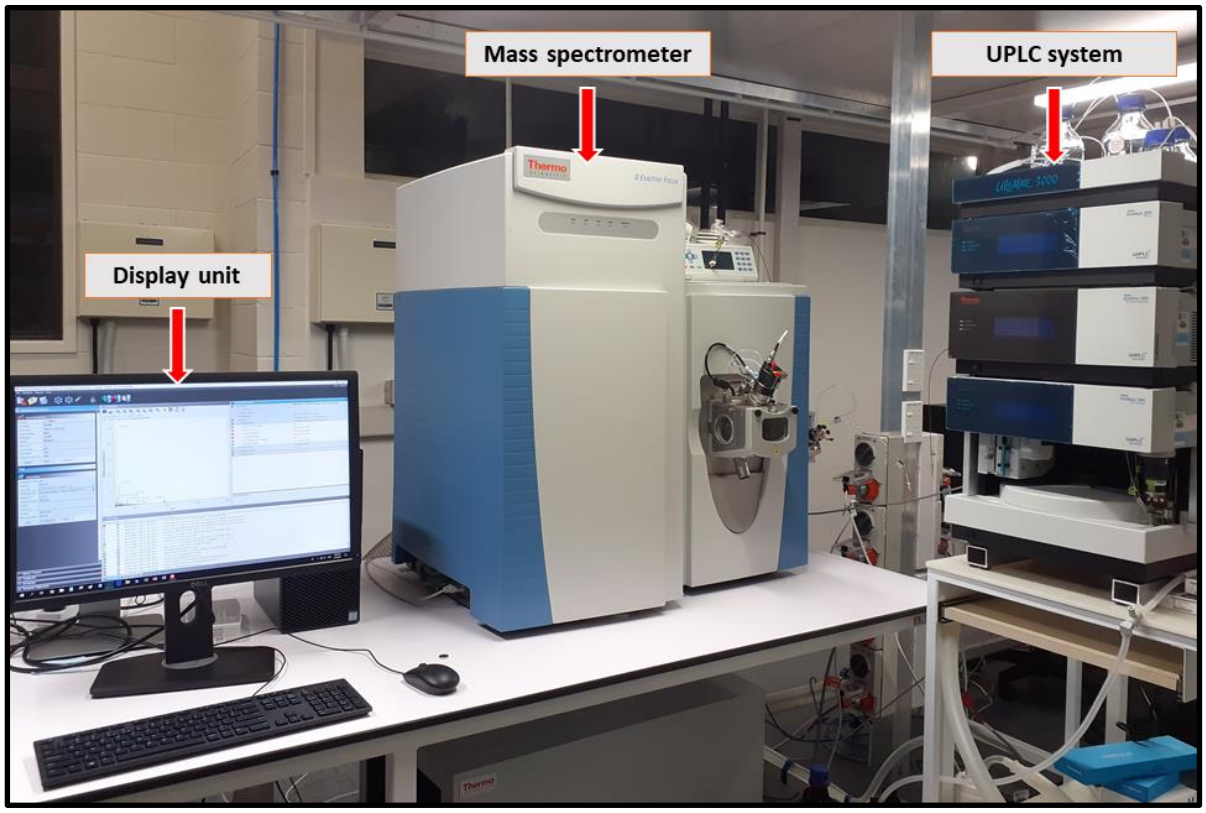


Figure 2.2. Picture of the LC-MS/MS setup in Massey University.

LC-MS/MS analysis was performed on a Thermofisher Dionex Ultimate 3000 UPLC system equipped with a Thermofisher-Q Enactive Focus-orbitrap mass spectrometer. The separation study of the plant extract was achieved using three columns. These columns were: 1) C18 column: Waters Symmetry Column 2.1 x 50 (mm) 3.5 µm (186000187); 2) C18 column: Thermofisher Hypersil Gold 100x2.1 (mm) 1.9 µm (THC25002-102130); 3) HILIC column: Thermofisher Hypersil GOLD 100x2.1 (mm) 1.9 µm HILIC (THC26502-102130).

Test samples were prepared in a 2 mL short thread vial (32x11.6 mm, clear glass, wide opening with thread cap). Concentration of each sample was 0.5 mg/mL in either DCM (fractions obtained from hexane or EtOAc extraction) or water (fractions obtained from water and MeOH extraction). The dried samples were first dissolved in their respective solvent at 1 mg/mL concentration in a 1.5 mL centrifuge tubes. The tubes were subjected to centrifugation (*Eppendorf Minispin, Eppendorf AG, UK)* for 12 min at 10000 rpm. For the fractions obtained

from hexane and EtOAc extraction, an aliquot (0.5 mL) from the tube was mixed with MeOH (0.6 mL) in a 2 mL short thread vial (32x11.6 mm, clear glass, wide opening with thread cap) and subjected to the LC-MS analysis. For the fractions obtained from water and MeOH extractions, an aliquot (1.2-1.5 mL) from the tube was directly transferred into a 2 mL short thread vial (32x11.6 mm, clear glass, wide opening with thread cap) and subjected to the LC-MS analysis.

Methodology of each column is described in the following sections.

2.3.11.1. Methodology of C18 Column

2.3.11.1.1. Using Waters Symmetry Column

The mobile phase for this C18 column was a gradient of water with 0.1% formic acid (buffer A) and acetonitrile (buffer B). The injection volume was 10 µL for each test sample at a flow rate of 0.300 mL/min. The running time was set to be 27 minute and the method is presented in Table 2.16.

RT (min)	***%B (Acetonitrile)***
0-10	25-50
10-15	50-80
15-16	80-100
16-21	100
21-22	100-25
22-26	25%
27	STOP RUN

Table 2.16. Method of LC run using Waters Symmetry C18 column with %B (Acetonitrile) applied at different RT (min).

2.3.11.1.2. Using Thermofisher Column

The mobile phase for this C18 column was a gradient of water with 0.1% formic acid (buffer A) and acetonitrile (buffer B). The injection volume was 5 µL for each test sample at a flow rate of 0.300 mL/min. The running time was set to be 23 minute and the method is presented in Table 2.17.

RT (min)	*%B (Acetonitrile)*
0-3	0
3-15	0-100
15-18	100-0
19-22	0
23	STOP RUN

Table 2.17. Method of LC run using Thermofisher C18 column with %B (Acetonitrile) applied at different RT (min).

The tune method for MS analysis for both positive (+) and negative (-) ionisations of C18 column both methods is presented below:

Scan range (+ or -): 120 to 1500 *m/z*

Spray voltage (mv): (+) 3300.0; (-) 3000.0

Capillary temp (+ or -): 320.0

Sheath gas (+ or -): 30.0

Max spray current (+ or -): 100.0

Probe heater temp (+ or -): 350.0

RF level: 50.0

ION: HESI

2.3.11.2. Methodology of HILIC column

The mobile phase for the HILIC column was a gradient of 10mM NH_4COOH (adjusted to pH 4 with HCOOH) (buffer A) and 97% CH_3CN, 3% H_20, 0.1% HCOOH (buffer B). The injection volume was 5 µL for each test sample at a flow rate of 0.200 mL/min. The running time was set to be 33 minute and the method is presented in Table 2.18.

RT (min)	*%B (Acetonitrile)*
0-1	95
1-6	95-55
6-20	55-10
20-23	10
23-24	10-95
24-32	95
33	STOP RUN

Table 2.18. Method of LC run using HILIC column with %B (Acetonitrile) applied at different RT (min).

The tune method for MS analysis for both positive (+) and negative (-) ionisations is presented below:

Scan range (+ or -): 120 to 1500 *m/z*

Spray voltage: (+) 4000.0; (-) 3300.0

Capillary temp (+ or -): 300.0

Sheath gas (+ or -): 20.0

Max spray current (+ or -): 100.0

Probe heater temp (+ or -): 350.0

RF level: 50.0

ION: HESI

2.3.11.3. Methodology of the Quebracho Extract Analysis

The LC-MS/MS analysis of the Quebracho samples were performed with an Thermofisher Synchronis aQ Column 50x2.1 (mm) 1.7 µm (THC97302-102130) which was designed for analysis of aqueous samples. The mobile phase for this C18 column was a

gradient of water with 0.1% formic acid (buffer A) and acetonitrile (buffer B). The injection volume was 10 µL for each test sample at a flow rate of 0.300 mL/min. The tune method for MS analysis for both positive (+) and negative (-) ionisations of C18 column both methods is presented below:

Scan range (+ or -): 200 to 2000 *m/z*

Spray voltage: (+) 4000.0; (-) 3300.0

Capillary temp (+ or -): 300.0

Sheath gas (+ or -): 20.0

Max spray current (+ or -): 100.0

Probe heater temp (+ or -): 350.0

RF level: 50.0

ION: HESI

Four different methodologies of LC run were used with both (+) and (-) ionisation mode for the Quebracho test samples.

1st Method: 0%-B

The method is presented in Table 2.19.

RT (min)	***%B (Acetonitrile)***
0-3	0
3-15	0-100
15-17	100
17-20	100-0
20-23	0
24	STOP RUN

Table 2.19. 1st method of LC run using Synchronis aQ column for the Quebracho samples with %B (Acetonitrile) applied at different RT (min).

2nd method: 25%-B

The method is presented in Table 2.20.

RT (min)	*%B (Acetonitrile)*
0-3	25
3-15	25-100
15-17	100
17-20	100-25
20-23	25
24	STOP RUN

Table 2.20. 2nd method of LC run using Synchronis aQ column for Quebracho samples with %B (Acetonitrile) applied at different RT (min).

3rd method: 40% B

The method is presented in Table 2.21.

RT (min)	*%B (Acetonitrile)*
0-3	40
3-15	40-100
15-17	100
17-20	100-40
20-23	40
24	STOP RUN

Table 2.21. 3rd method of LC run using Synchronis aQ column for Quebracho samples with %B (Acetonitrile) applied at different RT (min).

4th method: 60% B

The method is presented in Table 2.22.

RT (min)	*%B (Acetonitrile)*
0-3	60
3-15	60-100
15-17	100
17-20	100-60
20-23	60
24	STOP RUN

Table 2.22. 4th method of LC run using Synchronis aQ column for Quebracho samples with %B (Acetonitrile) applied at different RT (min).

2.4. In-vitro Nematocidal Analysis

2.4.1. Introduction

As discussed in Section 1.2.2, GIN have a six-phase life cycle and the 3rd larval stage (L3) of the GIN life cycle is the infective stage which is found in the environment and is infective by being ingested. For ruminants this is usually when it is present on the leaves on the pasture. A feature of L3 is that they maintain the old cuticle of the L2 stage around them and which protects them in the environment. These L3 are thus described as being ensheathed. The L1 and L2 stage occur in faeces and with adequate temperature they develop through to the longer lasting L3. Hence the L3 stage is commonly used as a readily available phase that can be utilised for exploratory drug studies. A feature of this stage is that when kept cool (<10°C) they will coil up and remain motionless, whereas at higher temperatures they will maintain an active sinusoidal swimming motion. Although active these larvae are unable to move unless they can flex against a solid object so in a body of water they remain on the bottom. When they are kept motionless at cool temperatures, they will typically remain alive for several weeks and for some species much longer. The process of culturing nematode eggs through to the L3 is readily achieved if the appropriate environmental conditions are used. For the collection of studies in this thesis, faeces from sheep infected with GIN were obtained through the use of opportunistic diagnostic faecal samples submitted to the Massey University Parasitology Laboratory. Routine diagnostic samples were obtained

from GIN infected goats that were held on a concrete floor whilst they defaecated or were donations of excess L3 cultured by *ARGL* for other studies (Waghorn peers' com). A key feature when collecting faeces is to avoid soil contamination and the resulting presence of free-living nematodes that results from that contamination. Faecal samples were collected from four dissimilar sources (*SA* farm*, AR* farm*, LATU* and *ARGL*) located in Manawatu-Wanganui region and some of these were known to be anthelmintic resistant isolates, although the actual level of anthelmintic resistance and to which anthelmintics was not ascertained. A useful feature of the L3 stage is that it is possible to morphologically identify each L3 as belonging to a particular genus of GIN.

2.4.2. Culture of GIN Infected Faeces and Recovery of L3 using the Baermann Technique

To obtain L3 larvae there were several steps involved. Infected faeces were firstly mixed with vermiculite and water. The vermiculite provides aeration to the resulting mix and sufficient water was added to ensure the mix remains moist but sufficient air remains in the pockets in the vermiculite to provide oxygen to the developing larvae (Muchiut et al., 2021). This was then held at an appropriate temperature for 10 days to allow the eggs to develop through to the L3 stage. The resulting L3 were then recovered from the faecal mix using the Baermann technique, or baermannisation, which involved holding the faecal mix over a fine sieve in water in room temperature. The L3 then swam through the sieve and accumulated in the bottom of the water body. Although there are many variations on the general approach, these particular principles have been described many times in the literature (Getachew et al., 2015, Ibrahim et al., 2012, Zharkikh et al., 2019). The culturing and baermannisation are summarised in Figure 2.3.

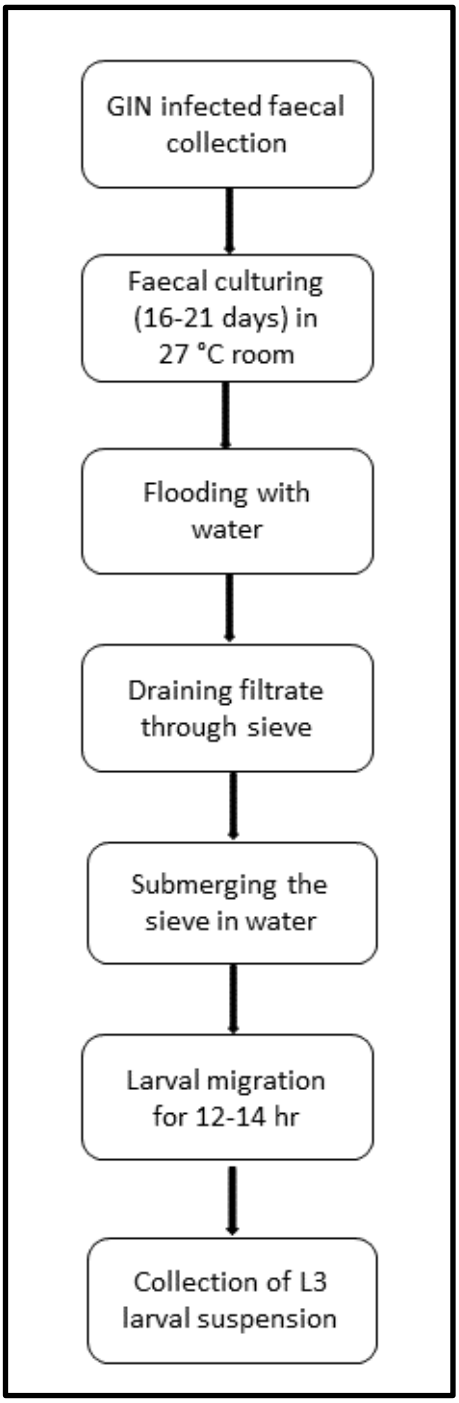


Figure 2.3. L3 larval suspension preparation through baermannisation.

After collection of nematode egg infected faeces, they were mixed with vermiculite (*Plantation Products' vermiculite*) and water. To ensure appropriate moisture the mix should appear relatively dry but when squeezed moisture beads around the material. This indicates that sufficient moisture is present in the spaces in the vermiculite but not overly moist to exclude air. The faeces were kept in a mason jar or similar container with a loosely fitted lid, in a 27 °C culture room (a room with adjustable temperature regulation facility and well-

spaced shelves) for 10 days. During this period, the faeces were watered periodically to keep them moist. After the incubation period, the faeces were taken out from the container, and put on a strainer. The base of the strainer was covered in a single layer of tissue paper which was held over a 500 mL cylindrical shaped bowl containing 200-250 mL water. The strainer was not fully submerged in the water and the base was slightly elevated so that the developed L3 larvae can swim through the tissue paper and into the water and accumulate on the bottom. The strainer was kept in this condition overnight (12-14 hours) at room temperature. After this period, the supernatant was filtered through three different sieves with different mesh sizes, 150 μm, 100 μm, and then collected on a 11 μm sieve. It was thoroughly washed with Milli-Q water in each step. Note, it is important not to use chlorinated water at this step as the chlorine has the effect of stimulating the L3 to lose the L2 sheath which is surrounding them. The L3 larvae readily pass through the 150 μm and 100 μm pore size but are too large to rapidly travel through an 11 μm pore. The larvae were then transferred to a 100 mL cell culture bottle and kept in water. Larval density was determined by counting the number of larvae in 100 μL of concentrated larval suspension under a microscope and their concentration was adjusted to approximately 100 per 100 μL. The bottle was stored in refrigerator at 6-10 °C until further use. The larvae were re-baermannised prior to usage for experiments.

2.4.2.1. L3 Larvae Selection and Microscopic Identification

A total of 13 different batches of larvae were procured throughout the duration of research from the four sources mentioned in Section 2.3.1. For larval identification and to determine the motility of the larval population, a sample of larval suspension (550 μL) was pipetted into a cavity slide (clear PVC tube of 17mm length and 5.8mm internal diameter superglued to microscope slide, Figure 2.7). For identification, Lugol's iodine solution (100 uL) was added to the solution which rapidly kills the larvae and lightly stains the larvae to allow for optimal identification. The mouth of the slide was covered by a 22 × 22 mm microscope coverslip. The cavity slide was inspected under x100 magnification. Larvae were identified using established morphometric and morphological methods (Knoll et al., 2021). The identified larvae were categorized into three groups based on their ensheathed tail length (Figure 2.3): a) short tail (25-50 μm) typical of *Trichostrongylus* spp. and *Teladorsagia circumcincta*; b) medium tail (51-89 μm) typical of *Cooperia* spp. and *Haemonchus contortus*;

and c) long tail (90-200 µm) typical of *Oesophagostomum* spp. and *Chabertia* spp. Several different morphological characteristics were then used for further identification: 1) full body length; 2) shape of head; and 3) shape of sheathed tail. A synopsis of the morphometric and morphological characteristics employed is presented in Table 2.19 and Figure 2.4. This is reasonably accurate for differentiating those with short and medium tails but those larvae with long tails present a challenge and are typically considered together as "Long Tails".

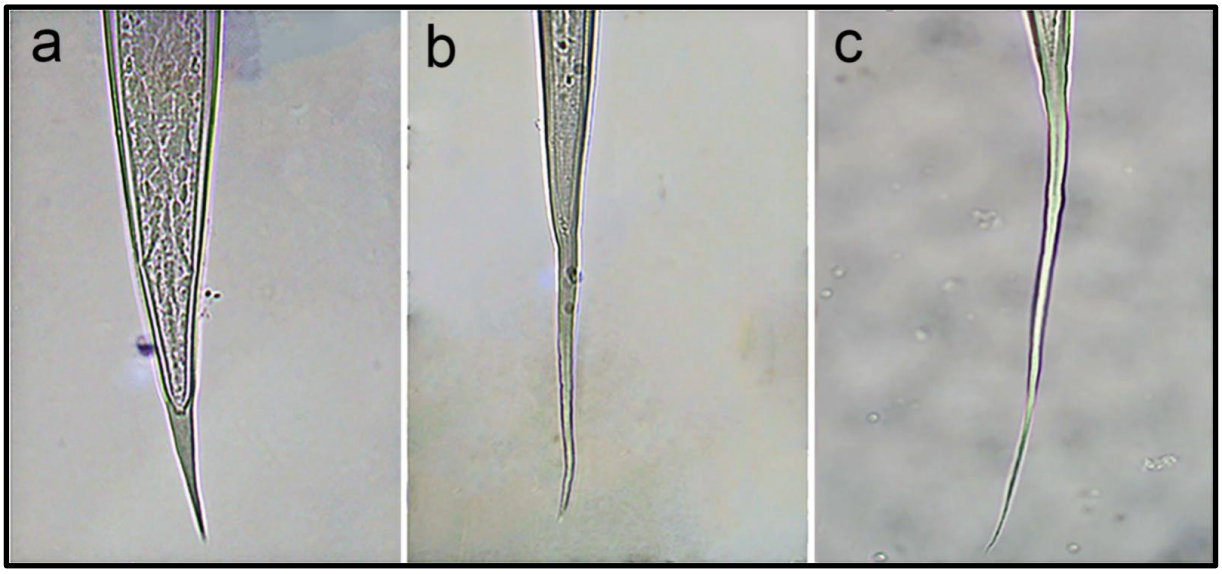


Figure 2.4. Preliminary classification method GIN based on sheathed tail length divided into three groups: a) short tail (25–50 µm), b) medium tail (51–89 µm) and c) long tail (90–200 µm) (Bowman, 2014). All images are shown at the same magnification.

Species/genus	***Full body length***	***Sheathed tail length***	***Group (ensheathed tail)***	***Shape of head***	***Shape of sheathed tail***
Teladorsagia circumcincta	≥720 µm	>35 µm	Short (a)	Flat/square, cranial inflexion	Conic
Trichostrongylus spp.	<720 µm	<35 µm	Short (a)	Rounded	Blunt
Haemonchus contortus	<790 µm	>65 µm	Medium (b)	Bullet-shaped	Curved
Cooperia spp.	≥790 µm	≤65 µm	Medium (b)	Square/rounded, 2	Pointed
Oesophagostomum spp.	>750 µm	>150 µm	Long (c)	Triangular	Bent
Chabertia spp.	≤750 µm	≤150 µm		Rectangular	

Table 2.19. Classification of different species of GIN L3 stage larvae based on different morphometric and morphological characteristics (Jackson and Coop, 2000, Knoll et al., 2021).

Different batches of larvae that were used throughout the research period, composition of the larval population and sources are presented below in Table 2.20. The identifications and the content determination were performed by Adlington and Gupta.

Larval Batch	***Larval Content***	***Source***	***Culture Date***
Batch 1	62% *Haemonchus contortus* 10% *Trichostrongylus* spp. 2% *Cooperia* spp. 26% LT	*SA* farm	7 January 2019
Batch 2	66% *Haemonchus contortus* 24% *Teladorsagia circumcincta* 6% *Trichostrongylus* spp. 1% *Cooperia* spp. 3% LT	*LATU*	18 February 2019
Batch 3	100% *Haemonchus contortus*	*ARGL*	24 March 2019
Batch 4	3% *Teladorsagia circumcincta* 73% *Trichostrongylus* spp. 8% *Cooperia* spp. 16% LT	*LATU*	10 August 2019
Batch 5	78% *Haemonchus contortus* 18% *Teladorsagia circumcincta* 1% *Trichostrongylus* spp. 3% LT	*SA* farm	15 August 2019
Batch 6	100% *Haemonchus contortus*	*ARGL*	07 November 2019
Batch 7	100% *Teladorsagia circumcincta*	*ARGL*	08 November 2019
Batch 8	33% *Haemonchus contortus* 18% *Teladorsagia circumcincta* 4% *Trichostrongylus spp.* 8% *Cooperia spp.* 32% LT	*LATU*	06 March 2020

(Contd. on the next page)

Larval Batch	***Larval Content***	***Source***	***Culture Date***
Batch 9	56% *Haemonchus contortus* *4% Teladorsagia circumcincta* 7% *Trichostrongylus* spp. 33% LT	*SA* farm	08 July 2020
Batch 10	44% *Haemonchus contortus* 2% *Teladorsagia circumcincta* 5% *Trichostrongylus* spp. 1% *Cooperia* spp. 48% LT	*SA* farm	04 August 2020
Batch 11	72% *Haemonchus contortus* 4% *Teladorsagia circumcincta* 4% *Trichostrongylus* spp. 20% LT	*SA* farm	12 October 2020
Batch 12	33% *Haemonchus contortus* 11% *Teladorsagia circumcincta* 11% *Trichostrongylus spp.* 44% *Cooperia spp.* 1% LT	*AR* farm	01 February 2021
Batch 13	44% *Haemonchus contortus* 1% *Teladorsagia circumcincta* 27% *Trichostrongylus spp.* 24% *Cooperia spp.* 2% LT 2% Nematodirus	*AR* farm	06 May 2021

Table 2.20. Different batches of larvae taken for the experiment conducted in this thesis, their larval content and source. LT= Long Tail: Oesophagostomum venulosum and Chabertia ovina.

2.4.3. Methodology of Nematocidal Larval Assay (NLA)

To estimate the nematocidal activity of the plant extracts against nematode L3 larvae under lab conditions, an assay, Nematocidal Larval Assay (NLA), was developed based on the Larval Motility Assay (Sujith et al., 2018, Barron-Bravo et al., 2020, Paras and Kaplan, 2020). The Larval Motility Assay typically involves treating the L3 with a test substance and then measuring how many migrate through a sieve. The technique used in this thesis involved incubating the L3 with the test substance then measuring how many remained motile after a set period of time. The schematic diagram of the NLA process is presented in Figure 2.6.

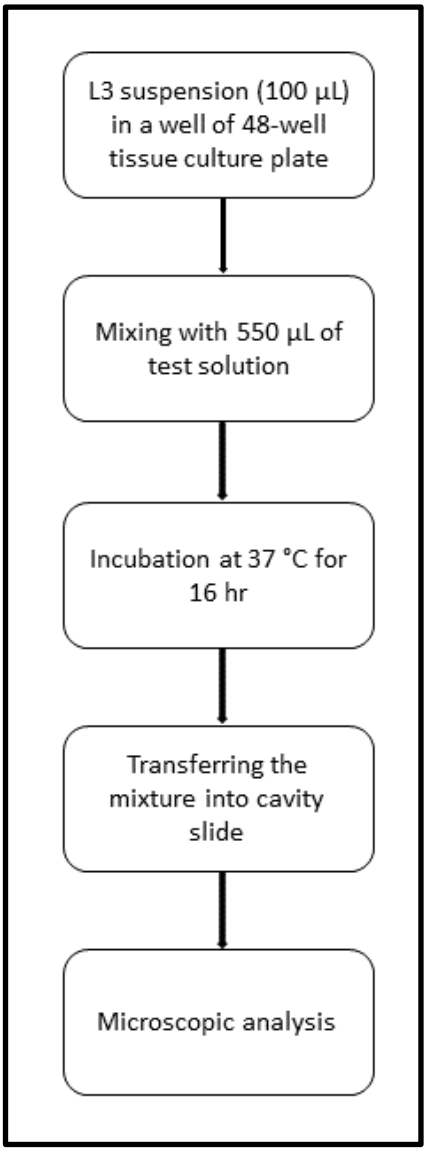
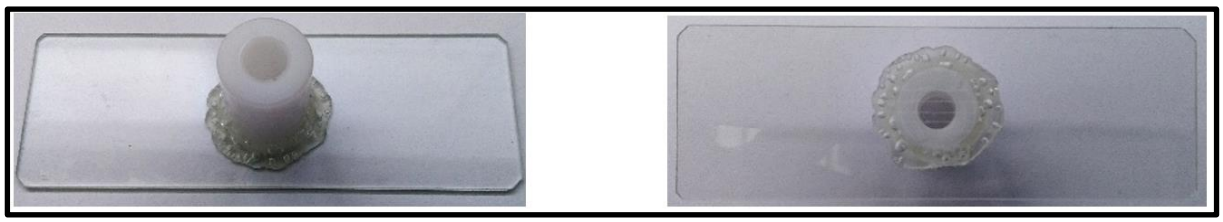


Figure 2.5. Schematic diagram of the NLA depicting various steps.

The method involved preparation of test solutions of plant extracts and L3 larval suspension. The mixture was combined and cultured in 48-well tissue culture plate. Each well of the tissue culture plate contained 100 µL of larvae solution (~100 L3 larvae) with 550 µL of test solution. The tissue culture plate was incubated at 37 °C for 16 h with the lid of the plate placed on top. The contents of each well was transferred into a cavity slide that was specially made for NLA experiments (clear PVC tube of 17mm length and 5.8mm internal diameter superglued to a glass microscope slide, Figure 2.7). The mouth of the cavity slide was covered with 4 x 4 mm microscopic coverslip and viewed under a microscope. The volume of the transferred fluid was sufficient to ensure the coverslip sat on the cavity with no air between the coverslip and the fluid to ensure good optical quality. Two microscopes were used throughout the research period. Larval identification of Batch 1-7 was performed using an OLYMPUS CX41 and NLA test analyses were performed using a Leica EZ4W microscope. The microscopic analysis was viewed on the Airlab app by Leica Corp. connected to an iPad. Both larval identification and NLA test analyses were performed using an OLYMPUS CX41 microscope for Batch 8-13 larvae. NLA test of each sample with a batch of larvae were performed in triplicate (n=3, T=1-3). A series of standard anthelmintics was taken as the positive control for the NLA experiments. These standards were: Benzimidazole (BZ), Abamectin (AB), and Ivermectin (IVM). Concentration of BZ was 3 mg/mL, AB and IVM 1 mg/mL, all in PBS.



(a) (b)

Figure 2.6. Picture of cavity slide designed for NLA clear PVC tube of 17mm length and 5.8mm internal diameter superglued to microscope slide; (a) front view; (b) back view.

Preparation of PBS:

In a beaker (2000 mL) with Milli-Q water (800 mL), NaCl (8 g), KCl (200 mg), Na_2HPO_4 (1.44 g), and KH_2PO_4 were added and thoroughly stirred in a magnetic stirrer (IKA basic) at RT

for ~10 min. The pH of the solution was adjusted to pH=7.4 on a pH instrument (*pH electrode InLab Routine, Mettler Toledo*) and it was transferred to a 1L volumetric flask. Water was added until the volume was to the mark.

2.4.3.1. Calculations and Statistics for NLA Test

The nematocidal activity of the plant fractions was expressed as % larval mortality which was calculated using the following equation:

$$\% \text{ Larval mortality} = \frac{N_T}{N_0 \times 100} \%$$

Where, N_0 = *Total count of motile larvae*

And

N_T = *Total count of immotile larvae*

The % larval mortality of each sample for individual NLA test performed in the 48 well tissue culture plate was calculated using the above equation. For those samples where several replicate cells were run, the data was extracted and analysed using GraphPad Prism 8 software to obtain the mean values of each sample. The mean % larval mortality is the average mortality of larvae obtained from three equivalent well readings in the 48 well tissue culture plate from a NLA test. Statistical analyses were carried out using GraphPad Prism 8 software. Analysis of variance (ANOVA) and Tukey's Multiple Comparison Test were used to compare the efficacies of the plant extracts, the reference anthelmintics and the non-treated (PBS) group. The results were summarized in tables as arithmetic means with SEM (standard error of mean) with Superscript (a, b, c)s next to the mean representing the statistical differences. Differences were considered significant at $P<0.05$.

2.4.4. Methodology of Dose Response Test

To find the optimal concentration of KK-leaf solvent and separated Fractions, a series of concentrations were chosen. These solvent fractions were selected: KK-May20-leaf-Water and KK-May20-leaf-MeOH. These following concentrations were chosen: 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, and 16 mg/mL in PBS. The experiments were done with Batch

9 larvae with both solvent fractions. The experiments were done in triplicate (n=3, T=1-3) following the standard NLA protocol described in Section 2.4.3.

For the separated fraction KK-May20-leaf-Water-Fraction-3, these following concentrations of the sample were made with in PBS: 1 mg/mL, 2 mg/mL, 4 mg/mL, 6 mg/mL, and 8 mg/mL. The experiments were done with Batch 9 larvae. The experiments were done in triplicate (n=3, T=1-3) following the standard NLA protocol.

2.4.4.1. Calculations and Statistics

Firstly, the nematocidal efficacy of the fraction of each concentration was defined as % larval mortality using the equation defined in Section 2.4.3.1. A dose response curve of % larval mortality v drug concentration was plotted in GraphPad Prism 8 to find the optimal concentration. This was the concentration with maximal efficacy of the sample studied and the LC_{50} (50% Lethal Concentration) concentration.

2.4.5. Methodology of Dead Larval Species Identifications

The dead larval species identifications were performed i.e., the investigation of the species of larvae that the samples were affecting. The identifications were performed with the batch of larvae having mixed larval population. The larvae that were killed by a sample were identified by investigating the morphology, and characteristics of the species as described in Section 2.4.2.1.

Note that the species identification was quite a challenging and cumbersome task. Therefore, even though the NLA tests with each sample were performed in triplicate (n=3, T=1-3), the identification was observed with only one test (T=1/2/3) using only one repetition. Total mortality of each larval species present in the larval population was counted and presented in Table as % (dead/total count out of 100).

Chapter 3. Nematocidal Study of the Quebracho Extract Through Bioassay-guided Fractionation and Chromatographic Separation

Nematocidal Study of the Quebracho Extract Through Bioassay-guided Fractionation and Chromatographic Separation

3.1. Introduction

In Chapter 1, the effect of condensed tannins (CT) containing plant extracts on GIN was discussed. The Quebracho extract is a commercially available source of CT which was reported to have both in-vitro and in-vivo usage for the treatment and control of GIN (Paolini et al., 2003, Athanasiadou et al., 2001, Athanasiadou et al., 2000a, Paolini et al., 2005, Whitney et al., 2011, Martínez-Ortiz-De-Montellano et al., 2019, Lucianer et al., 2019). The extract originates from the bark of the tree *Schinopsis balansae*. It is to be noted that this tree is native to South America and the presence of the tree in New Zealand has not yet been reported. The commercially available extract is obtained from the bark of the tree which is a fine powder with a chocolaty-brown colour and a coffee like aroma. It is readily soluble in water and forms an intense brown coloured solution when dissolved (Casanova et al., 2021). The Quebracho crude extract with concentrations from 0 to 1.2% (w/v) quadratically reduced ($P< 0.001$) larval viability by 89.4, 65.5, 22.8, and 9.2% in rumen fluid infected with *H. contortus* collected from three goats (Whitney et al., 2011). The Quebracho crude extract with concentrations of 12, 6 and 3% (w/v) was found to decrease the viability of L3 species of *H. contortus*, *T circumcincta* and *Trichostrongylus* spp. In an in-vitro study (Athanasiadou et al., 2001). The Quebracho extract, was found to cause a reduction in worm fecundity and egg output when added to the diet of two groups of cull goats infected *with H. contortus* and fed for 8 days (Paolini et al., 2003). It was found to decrease the worm count compared to the control groups by 33% and 38% in two groups of goats infected with *H. contortus* when fed for 3 days (although the difference was not found to be significant) (Paolini et al., 2005). However, all these reported studies in the literature were with the application of the crude Quebracho extract. The purification of the extract and study of the nematocidal efficacy of the separated and purified samples obtained from the crude extract was not previously attempted.

In this chapter, the bioassay-guided fractionation and chromatographic separation of the commercially available Quebracho extract are presented. In the bioassay-guided fractionation, a crude plant extract is fractionated either by solvent partition based on polarity, or chromatographically with respect to difference in molecular size or polarity (Dauda and Mudi, 2013). Chromatography is an established and effective method to separate, isolate, purify and analyse natural products (Gogoi et al., 2016). Chromatographic techniques

are based on distinctive separation procedures and the nature of the stationary and mobile phases employed. Sephadex LH-20 column chromatography (SLCC) is a widely established chromatography technique which has been reported to have been used for the fractionation of CT from plants yielding low molecular weight phenolics and CT-containing fractions (Tibe et al., 2013). The reverse phase flash chromatography (RP-FC) techniques have been used extensively to separate, and isolate PSM from different plant extracts (Ramaswamy et al., 2021, Chanda et al., 2021). However, the usage of RP-FC for the separation of condensed tannins was not reported in the literature. For the studies undertaken in this chapter, the overall aim was to obtain discrete fractions from the Quebracho extract. To achieve this aim, the initial step was the liquid-liquid solvent partition of the Quebracho extract to obtain different solvent fractions. The nematocidal efficacy of the resulting solvent fractions were then compared. The TLC study of the Quebracho extract was performed with a wide selection of mobiles phase to find the most suitable one. The mobile phases designed for the TLC analysis had a wide range of polarity with mixture of different solvents. The Quebracho powder was suspended in each of these solvent systems to obtain a series of soluble and insoluble samples and nematocidal experiments were conducted with each. It was hypothesised (**Hypothesis 3.1**) that the nematocidal efficacy of some of these samples might be higher than the samples obtained from the liquid-liquid solvent partition of the Quebracho crude extract. The most effective samples from this experiment were then subjected to nematocidal studies with two batches of larvae having different larval populations. It was hypothesised (**Hypothesis 3.2**) that the efficacy of the samples when examined against two batches of larvae having different larval populations will not vary. The effective solvent fractions (obtained from the liquid-liquid solvent partition work) were subjected to further chromatographic separation and nematocidal studies were evaluated against two batches of L3 larvae. This was performed to examine if further separation resulted in a fraction with higher nematocidal efficacy than the parent sample. The dead larval identification tests were conducted with an effective Quebracho sample to observe its impact on the different L3 species in the larval population. Therefore, this research with the Quebracho extract was a good starting point towards finding effective isolates which might provide an answer to the anthelmintic issues discussed throughout Chapter 1.

3.2. Materials and methods

3.2.1. Collection of the Quebracho Crude Powder

The Quebracho crude powder (QCP) was collected from the source mentioned in Section 2.3.2.

3.2.2. Solvent Partition of the Quebracho Extract

The QCP was dissolved in 400 mL water at a ratio of 1:8 (w/v). It was then extracted with equal volume of hexane, followed by dichloromethane (DCM) and n-butanol following the method described in Section 2.3.6. The extracts were concentrated to dryness by rotary evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland*), and freeze-dried (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)* following the usual method described in Section 2.3.6. The dry matter yield of the four solvent fractions was recorded. These solvent fractions were: QHF (Hexane Fraction), QDF (DCM Fraction), QBF (Butanol Fraction), and QWF (Water Fraction).

The QCP was also suspended in MeOH at the same ratio of 1:8 (w/v). The insoluble sample from the suspension was filtered out and freeze-dried to remove any residual solvent. The MeOH extract was concentrated to dryness by rotary-evaporation and freeze-dried to remove any residual solvent. The two samples from this extraction were: QMe (MeOH Extract), QMe-I (Insoluble sample from MeOH extraction).

3.2.3. Experiment 3.1: Nematocidal Analysis of The Quebracho Samples

The QCP, QBF, QWF, QMe, QMe-I samples were subjected to NLA tests with Batch 1 larvae (Source: *SA* farm) following the protocol in Section 2.4.3. The concentration of each sample was 8 mg/mL in PBS. PBS was used as a negative control group. The experiments were done in duplicate (n=2). These studies are detailed as Experiment 3.1.

3.2.4. TLC Study of The Quebracho Extract

The TLC study of the QCP was performed using the method described in Section 2.3.7. Vanillin-HCl was used as the staining reagent following the method described in Section 2.3.7.2. The solvents were arranged based on their polarity in ascending order (low to high). In Table 3.1, the mobile phase selected for the TLC analysis is presented.

# Mobile phase	*Mobile phase solvent ID*
TLC-1	EtOAc:AcOH:H_2O = 18:1:1
TLC-2	BuOH:AcOH:H_2O = 5:1:4
TLC-3	BuOH:AcOH:Acetone:H_2O = 3:1:2:4
TLC-4	BuOH:AcOH:Acetone:H_2O = 2:1:3:4
TLC-5	BuOH:AcOH:Acetone:H_2O = 1:2:3:4
TLC-6	Acetone:MeOH=1:1
TLC-7	PrOH:AcOH:H_2O = 6:1:3
TLC-8	PrOH:AcOH:Acetone = 6:1:3
TLC-9	PrOH:AcOH:MeOH = 6:1:3
TLC-10	IPA:AcOH:H_2O = 6:1:3
TLC-11	IPA:AcOH:Acetone = 6:1:3
TLC-12	IPA:MeOH = 1:1
TLC-13	IPA:AcOH:H_2O = 7:1:2
TLC-14	IPA:H_2O = 8:2
TLC-15	IPA:AcOH:H_2O = 6:2:2
TLC-16	IPA:AcOH:H_2O = 8:1:1

Table 3.1. Mobile phase for the TLC analysis of the QCP and solvent ID for each.

3.2.5. Suspension of the Quebracho Crude Powder into the Solvent Systems of The TLC Mobile Phases

The QCP (100 mg) was suspended in the respective solvent systems (100 mL) of each mobile phase taken in respective 250 mL conical flasks. It was then subjected to stirring (*IKA RCT Basic; IKA Works (Asia) Sdn Bhd, Malaysia*) at RT for 60 minutes. After that, the insoluble sample (if any) was collected by filtration and freeze-dried to remove any trace of solvent. The dry matter yields of the insoluble samples were recorded. The soluble sample present in the respective solvent systems was concentrated to dryness by rotary evaporation and freeze-dried to remove any residual solvents.

3.2.6. Experiment 3.2: Nematocidal Analysis of the Samples Obtained from the TLC Mobile Phases

The soluble and insoluble samples obtained from the TLC solvent systems were subjected to NLA tests with Batch 1 larvae. Similar protocols and concentrations mentioned in Section 3.2.3 was followed. The experiments were done in duplicate (n=2, T=1-2). These studies were detailed as Experiment 3.2.

3.2.7. Experiment 3.3-3.4: Nematocidal Analysis of the Effective Samples from Experiment 3.2 with Two Batches of Larvae

The most effective samples from the Experiment 8.2 were studied against two batches of larvae with different population, Batch 2 (Source: *LATU*) and 3 (Source: *ARGL*) larvae. Tests with the QCP, QWF, and QBF samples were also carried out with these two batches of larvae. The protocols and concentrations of samples mentioned in Section 3.2.3 were followed. These experiments were done in triplicate (n=3, T=1-3). The studies with Batch 2 larvae are detailed as Experiment 3.3 and with Batch 3 larvae are detailed as Experiment 3.4.

3.2.8. Separation of Quebracho Water Fraction (QWF) using Reverse Phase Flash Chromatography (RP-FC)

The RP-FC separation of QWF was performed using the method described in Section 2.2.9.4. The fractions obtained from the RP-FC run were combined using the method described in Section 2.3.9.3. The mixtures were concentrated by rotary evaporation and freeze-dried to remove any trace of solvent residue. The dry matter yield of each sample was recorded.

3.2.9. Separation of Quebracho Butanol Fraction (QBF) Using Reverse Phase Flash Chromatography (RP-FC)

The RP-FC separation of QBF was performed using the method described in Section 2.2.9.4. The fractions obtained from the RP-FC run were combined using the method described in Section 2.3.9.3. The mixtures were concentrated by rotary evaporation and freeze-dried to remove any trace of solvent residue. The dry matter yield of each sample was recorded.

3.2.10. Experiment 3.5-3.6: Nematocidal Analysis of the Separated Fractions Obtained from the RP-FC of QWF and QBF with Two Batches of Larvae

The separated fractions obtained from the RP-FC separations of QWF and QBF were subjected to NLA tests with Batch 4 (Source: *LATU*), and Batch 5 (Source: *SA* farm). Tests with QCP, QWF, and QBF were also carried out with these two batches of larvae. Similar protocols and concentrations mentioned in Section 3.2.3 were followed. The experiments were done in triplicate (n=3, T=1-3). These studies with Batch 4 larvae are detailed as Experiment 3.5, and with Batch 5 are detailed as Experiment 3.6.

3.2.10.1. Dead Larval Species Identification

For the NLA experiments conducted with QWF (T1) in Experiment 3.6, the identification of the dead larvae was made using the method described in Section 2.4.5.

3.2.11. Separation of QWF Using Sephadex LH-20 Column Chromatography

The separation of QWF was performed using Sephadex LH-20 Column Chromatography with the method described in Section 2.3.10. The fractions obtained from this run was combined based on the qualitative colour detection. The mixtures were concentrated by rotary evaporation and freeze-dried.

3.2.12. Experiment 3.7: Nematocidal Analysis of the Separated Fractions Obtained from the Sephadex LH-20 Column Chromatography

The fractions obtained from the Sephadex LH-20 separation of QWF were subjected to NLA tests with Batch 5 (Source: *SA* farm). A similar protocol and concentration mentioned in Section 3.2.3 were followed. The experiments were done in duplicate (n=2, T=1-2). These studies with Batch 5 are detailed as Experiment 3.7.

3.2.13. Calculation and Statistics for Nematocidal Analysis

The nematocidal efficacy of each Quebracho test sample was specified as % larval mortality using the equation described in Section 2.4.3.1. The efficacy of each sample was compared with one another using an ANOVA and post-hoc Tukey's test.

3.2.14. LC-MS Analysis of Quebracho Samples

The LC-MS analysis of the various Quebracho samples was performed using the methodology described in Section 2.3.11.3`. All four different LC methods were tested to find out the most suitable.

3.3. Results

3.3.1. Dry Matter Yield of Quebracho Solvent Extracts and Fractions

The Quebracho Fractions, obtained from the liquid-liquid solvent partition and dry matter yield of each fraction are presented in Figure 3.1.

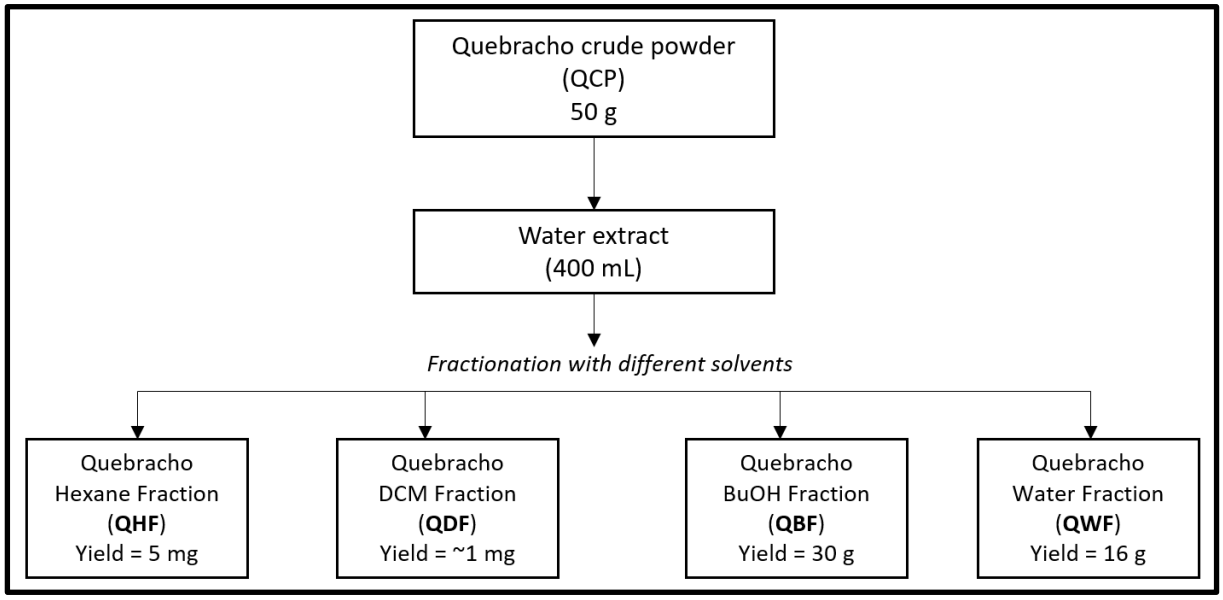


Figure 3.1. Schematic representation of the liquid-liquid solvent partition with the Quebracho extract and dry matter yield of different fractions.

The Quebracho powder was found to be fully soluble in water at 125 mg/mL concentration which indicated the high-water solubility of the crude powder. The water extract was fractionated with three different solvents, and it was found that the Hexane and DCM Fractions had little to no yield compared to the BuOH and the remaining Water Fractions. The schematic representation of the Quebracho MeOH extraction and the dry matter yield of the insoluble sample is presented in Figure 3.2.

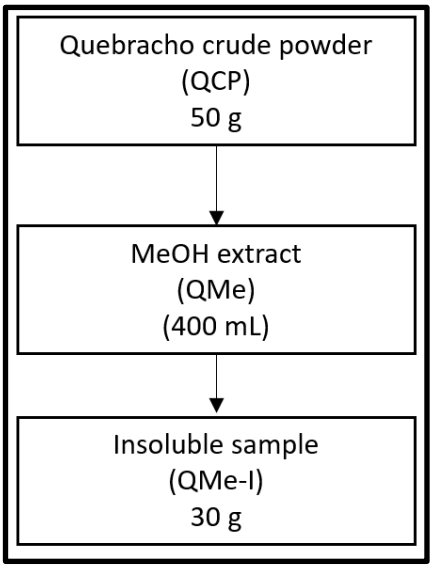


Figure 3.2. Schematic representation of the MeOH extraction with the Quebracho extract and dry matter yield of the insoluble sample.

The Quebracho powder was found to have around 40% solubility in MeOH at 125 mg/mL concentration from the dry matter yield of the insoluble sample.

3.3.2 Result of Experiment 3.1: Nematocidal Analysis of The Quebracho Samples

NLA tests of the QCP, QBF, QWF, QMe, and QMe-I samples were performed with Batch 1 larvae. The result of Experiment 3.1 is presented in Table 3.2.

Sample	***% Larval mortality***		***Mean of % mortality ± SEM***
	T1	***T2***	
QMe-I	25	25	25.0 ± 0^{b}
QMe	25	24	24.5 ± 0.5^{b}
QWF	30	31	30.5 ± 0.5^{a}
QBF	30	30	30.0 ± 0^{a}
QCP	10	11	10.5 ± 0.5^{c}
PBS	0	0	0

Table 3.2. Result of Experiment 3.1: Mean of % mortality of the different Quebracho samples (n=2; T1-2) against Batch 12 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

It was found that the QWF and QBF had comparable efficacy with one another ($P>0.05$, NS), and both had higher efficacy than the parent sample QCP ($P<0.05$). The efficacy of QMe and QMe-I were similar ($P>0.05$, NS) and higher than the parent QCP ($P<0.05$). However, they had lower efficacy than both the QBF and QWF ($P<0.05$).

3.3.3. Result of the TLC study of the Quebracho Sample

The pictures of the TLC plate obtained from the respective mobile phases (described in Table 3.1) are presented in **Appendix 3.1**.

3.3.4. Result of The Suspension of the Quebracho Crude Powder into the Solvent Systems of The TLC Mobile Phases

The QCP (100 mg) was dissolved in each of the solvent systems (100 mL) taken during the TLC analysis. The solvent systems based on their polarity (low to high) and the insoluble sample obtained in each are presented in Table 3.3.

Polarity	***# Mobile phase***	***Mobile phase solvent ID***	***Insoluble sample (mg)***
Low ↓ High	TLC-1	EtOAc:AcOH:H_2O = 18:1:1	49.1
	TLC-11	IPA:AcOH:Acetone = 6:1:3	49.8
	TLC-8	Propanol:AcOH:Acetone = 6:1:3	51.7
	TLC-6	Acetone:MeOH=1:1	31.6
	TLC-5	BuOH:AcOH:Acetone:H_2O = 1:2:3:4	-
	TLC-16	IPA:AcOH:H_2O = 8:1:1	-
	TLC-14	IPA:H_2O = 8:2	21.5
	TLC-13	IPA:AcOH:H_2O = 7:1:2	22.8
	TLC-12	IPA:MeOH = 1:1	22.9
	TLC-15	IPA:AcOH:H_2O = 6:2:2	26.1
	TLC-9	Propanol:AcOH:MeOH = 6:1:3	44.7
	TLC-4	BuOH:AcOH:Acetone:H_2O = 2:1:3:4	-
	TLC-10	IPA:AcOH:H_2O = 6:1:3	-
	TLC-3	BuOH:AcOH:Acetone:H_2O = 3:1:2:4	-
	TLC-7	Propanol:AcOH:H_2O = 6:1:3	14.1
	TLC-2	BuOH:AcOH:H_2O = 5:1:4	-

Table 3.3. The dry matter yield of the insoluble sample when suspended in the mobile phases of the TLC analysis.

It was found that the QCP was fully soluble in all the BuOH based systems. The quantities of insoluble sample in the low polar solvents were found to be slightly higher than the high polar solvent systems except the TLC-9 solvent system.

3.3.5. Result of Experiment 3.2: Nematocidal Analysis of the Samples Obtained from the TLC Mobile Phases

NLA tests of the soluble and insoluble samples obtained from the previous chemical analysis were performed with Batch 1 larvae. The result of Experiment 3.2 is presented in Table 3.4.

Sample	***% Larval mortality***		***Mean of % mortality ± SEM***
	T1	***T2***	
QCP-TLC-16	10	10	10.0 ± 0^{d}
QCP-TLC-15	23	23	23.0 ± 0^{b}
QCP-TLC-15-Ins	25	25	25.0 ± 0^{b}
QCP-TLC-14	29	28	28.5 ± 0.5^{a}
QCP-TLC-14-Ins	31	31	31.0 ± 0^{a}
QCP-TLC-13	24	24	24.0 ± 0^{b}
QCP-TLC-13-Ins	30	30	30.0 ± 0^{a}
QCP-TLC-12	19	20	19.5 ± 0.5^{c}
QCP-TLC-12-Ins	19	18	18.5 ± 0^{c}
QCP-TLC-11	30	28	29.0 ± 1.0^{a}
QCP-TLC-11-Ins	32	31	31.5 ± 0^{a}
QCP-TLC-10	10	9	9.5 ± 0.5^{c}
QCP-TLC-9	20	20	20.0 ± 0^{c}
QCP-TLC-9-Ins	31	31	31.0 ± 0^{a}
QCP-TLC-8	30	31	30.5 ± 0.5^{a}
QCP-TLC-8-Ins	29	28	28.5 ± 0.5^{a}
QCP-TLC-7	28	29	28.5 ± 0.5^{a}
QCP-TLC-7-Ins	20	19	19.5 ± 0.5^{c}
QCP-TLC-6	28	27	27.5 ± 0.5^{a}
QCP-TLC-6-Ins	27	27	27.0 ± 0^{a}
QCP-TLC-5	9	10	9.5 ± 0.5^{d}
QCP-TLC-4	10	11	10.5 ± 0.5^{d}
QCP-TLC-3	10	10	10.0 ± 0^{d}
QCP-TLC-2	11	11	11.0 ± 0^{d}
QCP-TLC-1	27	27	27.0 ± 0^{a}
QCP-TLC-1-Ins	31	30	30.5 ± 0.5^{a}
PBS	0	0	0

Table 3.4. Result of Experiment 3.2: Mean of % mortality of the soluble and insoluble Quebracho samples from TLC analysis (n=2; T1-2) against Batch 1 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05). Ins = insoluble. Samples highlighted in yellow represent the highest efficacy.

It was found that the difference between the efficacy of the soluble and insoluble sample of a TLC solvent system was not significant (P>0.05, NS) for most systems except TLC-13, TLC-9, and TLC-7 where the difference was found to be significant (P<0.05). The efficacies of the soluble samples from the solvent systems with non-occurrence of insoluble sample, was found to be comparable with one another (P>0.05) and identical with the parent QCP sample from Experiment 3.1. The highest efficacies obtained with the samples (highlighted in yellow in the Table 3.4) was found to be comparable with the efficacy of QWF and QBF on a separate Tukey's test (P<0.05, NS).

3.3.6. Results of Experiment 3.3-3.4: Nematocidal Analysis of the Effective Samples from Experiment 3.2 With Two Batches of Larvae

NLA tests of the most effective samples from the previous experiment and QWF, QBF and QCP were performed with Batch 2 and 3 larvae. The result of Experiment 3.3 with Batch 2 is presented in Table 3.5 and Experiment 3.4 with Batch 3 is presented in Table 3.6.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
QCP-TLC-14	20	22	24	$22.0 \pm 1.1^{c,d}$
QCP-TLC-14-Ins	17	18	17	$17.3 \pm 0.3^{e,f}$
QCP-TLC-13-Ins	25	24	25	$24.6 \pm 0.3^{b,c}$
QCP-TLC-11	19	17	17	$17.6 \pm 0.6^{e,f}$
QCP-TLC-11-Ins	12	8	11	10.3 ± 1.2^{g}
QCP-TLC-9-Ins	25	24	24	$24.3 \pm 0.3^{b,c}$
QCP-TLC-8	32	31	30	$31.0 \pm 0.5^{a,b}$
QCP-TLC-8-Ins	27	28	29	28.0 ± 0.5^{b}
QCP-TLC-7	21	20	19	$20.0 \pm 0.5^{d,e}$
QCP-TLC-6	30	31	29	30.0 ± 0.5^{a}
QCP-TLC-6-Ins	28	29	28	28.3 ± 0.3^{a}
QCP-TLC-1-Ins	12	15	16	14.3 ± 1.2^{f}
QBF	21	23	24	$22.6 \pm 0.8^{c,d}$
QWF	28	29	26	27.6 ± 0.8^{b}
QCP	17	17	16	$16.6 \pm 0.3^{e,f}$
PBS	0	0	0	0

Table 3.5. Result of Experiment 3.3: Mean of % mortality of the Quebracho samples (n=3; T1-3) against Batch 2 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05). Ins = insoluble.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
QCP-TLC-14	20	21	20	20.3 ± 0.3^{e}
QCP-TLC-14-Ins	18	19	18	18.3 ± 0.3^{e}
QCP-TLC-13-Ins	27	25	24	$25.3 \pm 0.8^{c,d}$
QCP-TLC-11	18	17	15	16.6 ± 0.8^{e}
QCP-TLC-11-Ins	16	17	15	16.0 ± 0.5^{e}
QCP-TLC-9-Ins	23	24	23	$23.3 \pm 0.3^{d,e}$
QCP-TLC-8	20	21	20	20.3 ± 0.3^{e}
QCP-TLC-8-Ins	20	23	20	21.0 ± 1.0^{e}
QCP-TLC-7	26	25	24	$25.0 \pm 0.5^{c,d}$
QCP-TLC-6	19	16	18	17.6 ± 0.8^{e}
QCP-TLC-6-Ins	29	30	32	30.3 ± 0.8^{b}
QCP-TLC-1-Ins	30	31	28	29.6 ± 0.8^{b}
QBF	27	28	27	$27.3 \pm 0.3^{b,c}$
QWF	35	36	35	35.3 ± 0.3^{a}
QCP	20	19	20	19.6 ± 0.3^{e}
PBS	0	0	0	0

Table 3.6. Result of Experiment 3.4: Mean of % mortality of the Quebracho samples (n=3; T1-3) against Batch 3 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05). Ins = insoluble.

It was observed that the efficacy of some of the samples varied depending on the batch of larvae. For example, efficacy of the QWF was higher in Batch 3 compared to Batch 2 (P<0.05, performed with a separate Tukey's test). Similar observations were found with the QCP-TLC-11-Ins (higher in Batch 3 than Batch 2), QCP-TLC-8 (higher in Batch 2 than Batch 3), QCP-TLC-8-Ins (higher in Batch 2 than Batch 3), QCP-TLC-7 (higher in Batch 3 than Batch 2), QCP-TLC-6 (higher in Batch 2 than Batch 3), QCP-TLC-1-Ins (higher in Batch 3 than Batch 2), QBF (higher in Batch 3 than Batch 2). The efficacy of the QCP was higher than the QCP-TLC-11-Ins sample when studied with Batch 2 larvae.

3.3.7. Result of the RP-FC Separation of the QWF

The flash chromatogram obtained from the RP-FC separation of the QWF is presented in **Appendix 3.2.1**. Initially, 32 fractions were obtained from the run. They were combined with one another following the hypotheses presented in Section 2.3.9.3. A total of four fractions were obtained from the combination work. In Table 3.7, the fractions obtained from the run and the yield of each are presented.

Combination of the fractions obtained from RP-FC run	***Hypothesis followed (Section 2.2.9.3)***	***Final fraction***	***Dry matter yield (in mg)***
2-3	1st hypothesis	QWF-RP-Fr-1	125.5
7-12	3rd and 4th hypothesis	QWF-RP-Fr-2	60.2
13-28	2nd hypothesis	QWF-RP-Fr-3	10.5
29-32	3rd and 4th hypothesis	QWF-RP-Fr-4	100.2

Table 3.7. Combination of the fractions obtained from the RP-FC run of the QWF Sample and the dry matter yield of each.

3.3.8. Result of the RP-FC Separation of the QBF

The flash chromatogram obtained from the RP-FC separation of the QBF is presented in **Appendix 3.2.2**. Initially, 32 fractions were obtained from the run. They were combined with one another following the hypotheses presented in Section 2.3.9.3. A total of three fractions were obtained from the combination work. In Table 3.8, the fractions obtained from the run and the yield of each are presented.

Combination of the fractions obtained from RP-FC run	***Hypothesis followed (Section 2.2.9.3)***	***Final fraction***	***Dry matter yield (in mg)***
2-3	1st hypothesis	QBF-RP-Fr-1	204.5
7-27	2nd hypothesis	QBF-RP-Fr-2	40.6
28-32	3rd and 4th hypothesis	QBF-RP-Fr-3	188.5

Table 3.8. Combination of the fractions obtained from the RP-FC run of the QBF Sample and the dry matter yield of each.

3.3.9. Results of Experiments 3.5-3.6: Nematocidal Analysis of the Separated Fractions Obtained from the RP-FC of QWF and QBF With Two Batches of Larvae

NLA tests with the separated fractions and the parent QWF and QBF samples were carried out with Batch 4 and 5 larvae. The result of Experiment 3.5 with Batch 4 is presented in Table 3.9 and Experiment 3.6 with Batch 5 is presented in Table 3.10.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
QBF-RP-Fr-3	15	15	14	14.6 ± 0.3^{c}
QBF-RP-Fr-2	20	20	18	19.3 ± 0.6^{b}
QBF-RP-Fr-1	24	22	25	23.6 ± 0.8^{a}
QBF	24	25	24	25.0 ± 0.5^{a}
QWF-RP-Fr-4	8	8	7	$7.6 \pm 0.3^{d,e}$
QWF-RP-Fr-3	7	7	6	6.6 ± 0.3^{e}
QWF-RP-Fr-2	10	10	8	9.3 ± 0.6^{d}
QWF-RP-Fr-1	8	9	8	8.3 ± 0.3^{d}
QWF	14	15	14	14.3 ± 0.3^{c}
QCP	4	3	2	3.0 ± 0.5^{f}
PBS	0	0	0	0

Table 3.9. Result of Experiment 3.5: Mean of % mortality of the Quebracho samples (n=3; T1-3) against Batch 4 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
QBF-RP-Fr-3	53	52	53	52.6 ± 0.3^{a}
QBF-RP-Fr-2	52	51	51	51.3 ± 0.3^{a}
QBF-RP-Fr-1	52	51	51	51.3 ± 0.3^{a}
QBF	48	50	52	50.0 ± 1.1^{a}
QWF-RP-Fr-4	39	34	38	37.0 ± 1.5^{d}
QWF-RP-Fr-3	41	41	38	$40.0 \pm 1.0^{c,d}$
QWF-RP-Fr-2	47	47	44	46.0 ± 1.0^{b}
QWF-RP-Fr-1	42	43	41	42.0 ± 0.5^{c}
QWF	52	50	53	51.6 ± 0.8^{a}
QCP	23	24	23	23.3 ± 0.3^{e}
PBS	0	0	0	0

Table 3.10. Result of Experiment 3.6: Mean of % mortality of the Quebracho samples (n=3; T1-3) against Batch 5 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

It was observed that the efficacy of each sample was higher in Batch 5 than Batch 4 larvae. With Batch 4, the QBF was found to have higher efficacy than the QWF sample (P<0.05). The efficacy of the parent QWF sample was higher than its separated fractions (P<0.05) with Batch 4 larvae. The efficacy of the QBF and its fraction QBF-RP-F-1 was similar (P>0.05, NS) but the efficacy of the other separated fractions was found to be lower (P<0.05) with this batch of larvae. With Batch 5, the QBF and QWF had similar efficacy (P>0.05, NS). The efficacy of the separated fractions of QBF was similar with the parent sample (P>0.05, NS). The efficacy of the QWF sample was higher than all its separated fractions (P<0.05). With both batch of larvae, all the Quebracho samples had higher efficacy than the parent crude powder (QCP) (P<0.05).

3.3.9.1. Result of Dead Larval Identifications

The result of the identification of the larval species that were killed by the sample QWF (T2) is presented in Table 3.11.

Larval species	***Mortality count of QWF (%)*** (*immotile/motile for that species*)
H. contortus (78%)	51% (40/78)
T. circumcincta (18%)	66% (12/18)
Trichostrongylus spp*. (1%)*	0% (1/1)
LT *(3%)*	0% (0/3)
Total dead count (%) (dead/total)	52% (52/100)

Table 3.11. Dead larval identification of the species of Batch 5 larvae against the QWF.

It was found that the sample QWF was 51% effective against the *H. contortus* and 66% effective against the *T. circumcincta* species present in the larval population of Batch 5. The QWF did not affect the other species that were present in the population.

3.3.10. Result of Separation of QWF Using Sephadex LH-20 Column Chromatography (SLCC)

From the SLCC initially 36 fractions were obtained. These fractions were combined with one another based on the qualitative colour detection to get four final fractions: QWF-SLCC-Fr-1 to QWF-SLCC-Fr-4. The fractions that were combined are presented in Table 3.12.

Combination of the fractions obtained from SLCC run	***Final fraction***	***Dry matter yield (in mg)***
1-2	QWF-SLCC-Fr-1	220.5
3-7	QWF-SLCC-Fr-2	266.2
8-20	QWF-SLCC-Fr-3	253.5
21-36	QWF-SLCC-Fr-4	190.2

Table 3.12. Combination of the fractions obtained from the SLCC run of the QWF.

3.3.11. Result of Experiment 3.7: Nematocidal Analysis of the Separated Fractions Obtained from the Sephadex LH-20 Column Chromatography

NLA tests of the separated fractions obtained from the SLCC separation of QWF with Batch 5 larvae. The result of Experiment 3.7 is presented in Table 3.13. The efficacies of QCP and QWF from Experiment 3.6 were analysed together to compare the values with one another.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
QWF-SLCC-Fr-4	51	51	-	51.0 ± 0^{a}
QWF-SLCC-Fr-3	50	51	-	50.5 ± 0.5^{a}
QWF-SLCC-Fr-2	34	34	-	34.0 ± 0^{b}
QWF-SLCC-Fr-1	27	28	-	27.0 ± 0.5^{c}
QWF	52	50	53	51.6 ± 0.8^{a}
QCP	23	24	23	23.3 ± 0.3^{d}
PBS	0	0	0	0

Table 3.13. Result of Experiment 3.7: Mean of % mortality of the separated Quebracho fraction from SLCC (n=2; T1-2) against Batch 5 larvae with SEM=Standard Error of Mean. Values of the QCP and QWF are from Experiment 3.6. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

It was found that the efficacy of the SLCC-Fraction-1 and SLCC-Fraction-2 were lower than the efficacy of their parent sample the QWF ($P<0.05$). The efficacy of SLCC-Fraction-3 and SLCC-Fraction-4 were comparable with the QWF ($P>0.05$, NS). All the separated SLCC QWF fractions had higher efficacy than the QCP ($P<0.05$).

3.3.12. Result of the LC-MS analysis of the Quebracho Samples

From the comparison of LC-MS analysis of the QWF sample using different methods (from Section 2.3.11.3) presented in **Appendix 3.3.1**, it was found that the 3rd and 4th methods were the most ideal. The analysis of the Quebracho samples was performed using the 4th method. From the comparison of the LC-MS chromatograms (result presented in **Appendix**

3.3.3) of the QCP, QWF, QBF, QMe and QMe-Ins, all the samples had identical chromatograms i.e., there was no difference in their LC and MS traces. From the comparison of LC-MS analysis QWF and its separated fractions from the RP-FC separation, all the samples had identical traces i.e., there was no difference of LC and MS trace with the parent and its separated fractions (result presented in **Appendix 3.3.4**). Similar observations were noted with QBF and its separated fractions from RP-FC (result presented in **Appendix 3.3.5**), and QWF and its separated fractions from SLCC (result presented in **Appendix 3.3.6**).

3.4. Discussion

In this chapter, the nematocidal efficacy of the commercially available Quebracho extract though bioassay-guided fractionation and chromatographic separation is presented. The overall aim was to observe if further chemical separation improved the efficacy of the QCP. The crude powder was first suspended into water. It was soluble in water at 125 mg/mL concentration which indicated the highly polar nature of the commercial powder. Upon liquid-liquid solvent partitions, it was found that the Quebracho extract had little to no partition to the hexane and DCM solvents, which indicated that there was negligible presence of low-medium polar components in the crude powder. Two fractions were obtained from the partition work, the QBF (BuOH Fraction) and QWF (Water Fraction). It was observed that the QCP was 40% soluble in MeOH. Whereas, in water it had 100% solubility which further attested the highly polar nature of the powder. The two samples obtained from the MeOH extraction, QMe and QMe-Ins, and the QWF and QBF were subjected for nematocidal analysis with Batch 1 larvae. It was found that all of these samples had higher efficacy than the QCP (Experiment 3.1). This result indicated that the bioassay-guided fractionation improved the efficacy of the crude powder. This finding was novel as all the previous work in the literature with the Quebracho extract was with its crude aqueous extract. However, further in-vivo studies are required with these separated fractions as the previously reported studies of Quebracho were performed in-vivo (Athanasiadou et al., 2000a, Paolini et al., 2005).

The TLC study of the QCP was conducted with a series of mobile phases. Vanillin-HCl forms complexes with CT which can be identified from the reddish/pinkish spots they make on a silica plate (Tibe, 2012, Tibe et al., 2013). From the TLC study, it was found that these mobile phases were not able to provide a good separation, as a long tail was obtained with

each. The spots were indistinct, and no separation was observed on the plate. This indicated that the TLC analysis of the QCP was not an ideal way to get an understanding of the components present in the crude extract. However, the series of solvents that were prepared for the TLC mobile phase had a wide range of polarity. The QCP was suspended in them to gather soluble and insoluble samples from each. From the nematocidal study of these samples conducted against Batch 1 larvae (Experiment 3.2), it was found that the soluble samples collected from the solvent systems in which the QCP was completely soluble and had identical efficacy with the QCP sample ($P>0.05$). This was expected as the parent sample was totally soluble in these solvent systems, and upon removing the solvent through rotary-evaporation and subsequent freeze-drying, the accumulated sample was the parent QCP. In the solvent systems with which there were occurrences of both soluble and insoluble samples, it was observed that the soluble and insoluble samples had either different or similar efficacy with one another. However, the highest efficacies obtained with the samples (highlighted in yellow in the Table 3.4) were found to be comparable with the efficacy of QWF and QBF. Therefore, the **Hypothesis 3.1** that the nematocidal efficacy of some of these samples might be higher than the samples obtained from the liquid-liquid solvent partition of the Quebracho crude extract was found to be **not correct**. The most effective samples from Experiment 3.1 and the QCP, QWF, and QBF were subjected to nematocidal analysis with two different batches of larvae (Experiment 3.3-3.4). The nematocidal efficacies of many samples were found to be different in these two batches of larvae. The efficacy of a Quebracho sample was found to be dependent on the larval population of the batch of larvae. From the dead larval identifications of the QWF performed during Experiment 3.6, it was found that the sample was more effective against the *T. circumcincta* species than the *H. contortus* species present in the larval population of Batch 5. Therefore, the **Hypothesis 3.2** that the efficacy of the samples when examined against two batches of larvae having different larval populations will not vary was found to be **not correct**.

The separation of the QWF and QBF samples was performed using RP-FC. From the nematocidal experiment conducted with the separated fractions of QWF and QBF against Batch 4 and 5 larvae, none of the separated fractions had higher efficacy than the parent QWF and QBF samples. This result indicated that the further chromatographic separation of these samples did not result towards a fraction with a higher nematocidal efficacy. Nonetheless,

the efficacy of almost every sample was found to be different in both batches of larvae, further indicating the selective nature of the Quebracho sample against the L3 species present in a larval population. The larval populations of Batch 4 and 5 were different from each other. The QWF sample was also subjected to SLCC. The SLCC has been previously reported to separate and purify water-soluble highly polar plant fractions (Gu et al., 2002, Spencer et al., 2007). The elution started with the gradient E1 which had 0% MeOH. It was able to move the analyte through the column but reached a saturation point after the 5th fraction when E2 was used. Obtaining the saturation point, E3 was applied, and this procedure was repeated until the use of E4 and the total transfer of the QWF analyte through the Sephadex LH-20 column was achieved. A total of 36 fractions were obtained from this run. The combination of these fractions was performed using qualitative colour detection to obtain four final fractions. The qualitative colour detection was performed by observing the amount of tannin coloration in the fractions obtained from the column and quantitively combining the similar group of fractions having an identical colour. For example, the Fractions 1-2 had similar colouration, which was different from the colour of Fractions 3-7 (as presented in Table 3.12). From the nematocidal analysis of these four fractions (Experiment 3.7), it was found that none of the separated fractions had higher efficacy than the parent QWF sample. The efficacies of the QWF-SLCC-Fr-1 and QWF-SLCC-Fr-2 were lower than the QWF ($P<0.05$). Therefore, similar to the RP-FC observation, further separation of the QWF using SLCC did not result in a fraction with higher efficacy.

The most challenging aspect of the Quebracho research was encountered with its characterisation through LC-MS analysis. The analysis was performed using a Synchronis aQ Column with low particle size, a column designed to provide optimal retention of polar analytes (Thermofisher-Scientific, 2018, Jia et al., 2014). The LC-MS analysis of the QWF using the 1st method described in Section 2.3.11.3, resulted in poor resolution of the analytes present and occurrence of distinct peaks at RT = 21 min which was during the washing phase of the column (chromatograms presented in **Appendix 3.3.1**). This method was used with the KK samples which provided good resolution and retention of analytes in their LC-MS chromatograms (see **Chapter 7**). Hence, it was modified with a higher percentage of acetonitrile (buffer B) from 0% (1st method) to 25% in the LC mobile phase (2nd method). It resulted in better resolution of the analytes but, there was no occurrence of peaks until the

washing phase of the column (RT = 20 min). The method was then further modified (3rd method) with a higher percentage of buffer B (40%). More distinct peaks were observed which indicated the better separation of analytes present in the QWF using this method. An additional method was used (4th method) with a higher percentage of buffer B (60%). The chromatogram obtained with this method, the peaks had sharper edges and the resolution was better than the 3rd method (**Appendix 3.3.2**). Hence, the 4th method was selected to conduct the LC-MS analysis studies of the various Quebracho samples. From the LC-MS chromatograms comparison (**Appendix 3.3.3**) of the QCP and its resulting fractions from the liquid-liquid solvent extraction (QWF, QBF, QMe and QMe-Ins), there was no difference of LC-MS trace in the chromatogram of each. This result indicated that all these samples were identical to one another having similar components and no apparent deviation from the parent QCP sample. However, the nematocidal efficacy of the fractionated samples were different from the QCP sample, which indicated that these samples were distinctive from one another. If they were chemically similar, they would have identical nematocidal efficacy. Similar LC-MS results were observed with the separated fractions of the QWF and QBF, obtained from both RP-FC (**Appendix 3.3.4** and **Appendix 3.3.5**) and SLCC (**Appendix 3.3.6**), with no distinct variation of peaks between the parent sample and its separated fractions. However, the nematocidal efficacies of the parent sample and its separated fractions were different which indicated that they possessed different components which were not detectable by the LC-MS analysis. Therefore, the characterisation of the Quebracho extract using LC-MS analysis was not found to be effective.

Thus, the bioassay-guided fractionation and chromatographic separation of the Quebracho extract were encountered with many challenges. The bioassay-guided fractionation resulted in solvent fractions having higher nematocidal efficacy than the crude commercially available powder. However, further chromatographic separation did not improve towards the efficacy. Therefore, it was necessary to investigate other naturally derived sources to further the nematocidal research of this PhD and finding a potentially stronger candidate with greater anthelmintic efficacy as compared to the Quebracho extract. Hence, a series of NZ endemically found plants with known medicinal properties was accumulated and nematocidal efficacy was studied with each to find the most effective. This is presented in the next chapter, **Chapter 4**.

Chapter 4. Medicinal Plants of New Zealand and Their Nematocidal Efficacies

Medicinal Plants of New Zealand and Their Nematocidal Efficacies

4.1. Introduction

As discussed in Chapter 1, one of the alternative methods of worm control in GIN infested ruminants is the use of medicinal plants (Hrckova and Velebny, 2013, Jan et al., 2021, Ogedengbe-Olowofoyeku et al., 2021, El Shanawany et al., 2019, Pandey et al., 2018). In many parts of the developing world, the usage of medicinal plants has long been relied on as a traditional method of deworming (Mirazei and Mirazei, 2013). Several plants have been found to contain valuable secondary metabolites which possess nematocidal properties (Ndlela et al., 2021, Torres-Fajardo and Higuera-Piedrahita, 2021). They have the potential to act as alternatives or supplements to the synthetic anthelmintics and provide an answer to the anthelmintic resistance issue (Bizhani, 2015, Zaman et al., 2017). There are many reported studies in recent years of bioactivities of phytoconstituents of plant origin against parasites, and other pathogens (Molan and Faraj, 2010, Mansi et al., 2021, Salehi et al., 2020, Sakti et al., 2020, Tasdemir et al., 2020, French et al., 2018). As discussed in the Section 1.5, there are several medicinal plants found in NZ which have been used as traditional medicines by Māori as well as some other introduced species which may also have medicinal activity. These plants are *P. Excelsum*, S. *aviculare*, C. *laevigatus, P. tenax*, and *G. officinalis.* The objective of the study reported in this chapter was to screen these five medicinal NZ plants for their anthelmintic properties using L3 larvae procured from GIN infected ruminant using the In-vitro Nematocidal Analysis as described in Chapter 2. To date studies of these plants for their nematocidal properties have yet to be reported in the literature. It was hypothesised that these plants could be high in secondary metabolites possessing anthelmintic properties. This chapter details the first of a series of studies conducted to evaluate the nematocidal activity of these medicinal plants. The initial study examined the nematocidal activity of crude extract of these plants to find the most effective one. This led to subsequent studies detailed in the following chapters which involved the extensive separation, and purification of various components of the most effective plant found from this series of plants.

4.2. Materials and Methods

4.2.1. Collection and Drying of Plants

A series of medicinal plants were collected from Bledisloe Park, Palmerston North and Massey University's outer campus as discussed in Section 2.3.2. The plants were identified,

and the components that were collected and extracted for nematocidal analysis as given below in Table 4.1.

Plant	***Component collected***
Kawakawa (*Piper Excelsum*)	Fruit
Poroporo (*Solanum aviculare*)	Leaf
Karaka (*Corynocarpus laevigatus*)	Leaf
Harakeke (*Phormium tenax*)	Leaf
Goat's Rue (*Galega officinalis*)	Leaf

Table 4.1. List of plants collected, and components selected to be evaluated for nematocidal activity.

These plants were collected during October-November 2018. Drying and grinding of plant components were performed using the method described in Section 2.3.3.

4.2.2. Preparation of Crude Plant Extract for Nematocidal Study

Preparation of crude plant extracts of the five plant components was achieved following the method described in Section 2.3.4. The dry matter yield of each plant extract was recoded.

4.2.3. Experiment 4.1: Nematocidal Analysis of Different Plant Fractions

The dried plant fractions (20g) were suspended in PBS-T in DMSO (3.5% Tween-80 PBS in 1.5% DMSO) at 6 mg/mL concentration in a centrifuge tube. It was then subjected to centrifugation followed with ultrasound sonication for 2-3 hr. The nematocidal experiments were carried out using the NLA method described in Section 2.4.3 with Batch 2 larvae (66% *H. contortus*, 24% *T. circumcincta*, 6% *Trichostrongylus* spp., 1% *Cooperia* spp., 3% *LT,* Source: LATU, 18 Feb 2019). PBS was used as a negative control. The experiments were undertaken for each plant component in triplicate (n=3, T=1-3). These studies are detailed as Experiment 4.1.

4.2.3.1. Calculations and Statistics

Nematocidal efficacy of each was described as % larval mortality using the equation defined in Section 2.4.3.1. The mean values of efficacy of each plant extract were compared

with one another using an ANOVA with a post hoc Tukey's test to compare between the different plant components and control.

4.3. Results

4.3.1. Dry Matter Yield of Plant Component Extracts

The plant components, as given in Table 4.1, were subjected to extract preparation, and dried to prepare fractions to be tested for nematocidal efficacy. The dry matter yield of each plant component is given in Table 4.2.

Plant component extract	***Dry matter yield***	***% Of usable component***
Kawakawa fruit	3.62 g	18.1
Poroporo leaf	2.78 g	13.9
Karaka leaf	3.87 g	19.3
Harakeke leaf	2.12 g	10.6
Goat's Rue leaf	1.87 g	9.3

Table 4.2. *Dry matter yield of plant component extracts subjected to nematocidal analysis.*

The dry matter yield indicated the useable quantity of component per 20 g of the dried sample. It was found that the dry matter of the KK fruit and Karaka leaf extract had comparable yield of >3.5g. This indicated that both had ~20% of usable component for phytochemical analysis. Hence, both had significant usable phytochemical components present in them as extracted with absolute MeOH compared to other plant components.

4.3.2. Results of Experiment 4.1: NLA Tests of Different Plant Extracts

The result of Experiment 4.1 with mean values of % of larval mortality with SEM are given below in Table 4.3.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
Kawakawa fruit	28	30	32	30.0 ± 1.1^{a}
Poroporo leaf	15	14	16	15.0 ± 0.5^{c}
Karaka leaf	10	11	10	10.3 ± 0.3^{c}
Harakeke leaf	15	14	15	14.6 ± 0.3^{c}
Goat's Rue leaf	23	24	21	22.6 ± 0.8^{b}
PBS-T in DMSO	0	0	0	0

Table 4.3. Result of Experiment 4.1: Mean of % mortality of different plant extracts against Batch 2 larvae with SEM=Standard Error of Mean. T=Test number. Superscript (a, b, c) represents statistical significance difference obtained from Tukey's test (P<0.05).

The results of Experiment 4.1 showed the KK fruit extract demonstrated the highest nematocidal efficacy with a mean mortality of 30.0±1.1% at the concentration of 6 mg/mL, which was higher than any other plant components (P>0.05). The second highest was Goat's Rue leaf fraction with a mean mortality of 22.7±0.8%. Thus, the KK fruit fraction showed the highest nematocidal efficacy among the endemically found NZ plants which were compared.

4.4. Discussion

In this chapter, a screening study of five medicinal plants found in NZ for their nematocidal properties has been presented. Firstly, a crude plant extract of each plant component was prepared in absolute MeOH. The extract yield is dependent on factors such as methods of extraction, temperature, extraction time, and pH (Dent et al., 2013). All these factors were controlled apart from pH. The yields were dependent on polarity of solvent employed in the extraction; in this case this was absolute MeOH. From the result of dry matter yield of plant component extracts in absolute MeOH, it indicated that the Karaka leaf and KK fruit had more phytoconstituents present that were soluble in MeOH than the other plant components. A question might arise that why MeOH was the solvent of choice and no other solvents of different polarities such as hexane and ethyl acetate were taken for this screening study. MeOH has been widely used as the solvent of choice for initial screening study of plant extract and evaluating nematocidal properties (Ndlela et al., 2021). Methanol can extract

both hydrophilic and lipophilic molecules from plant parts. After extraction it can be removed low temperature by distillation as methanol is highly volatile.

The plant extracts were suspended in PBS-T in DMSO (3.5% Tween-80 PBS in 1.5% DMSO) at 6 mg/mL concentration for NLA test. The NLA test is based on a larval motility assay which is a widely used bioassay to assess anthelmintic properties. It has been used to measure the extent of L3 paralysation when incubated with various plant extracts (da Silva et al., 2021, Jacob et al., 2021). In Experiment 4.1, the KK fruit extract was found to be the most effective of the five plant extracts studied ($P<0.05$). This could be because of the presence of secondary metabolites in the extract which either paralysed or killed the larvae (Olmedo-Juárez et al., 2020). The second most effective was the Goat's Rue leaf extract with 22.7±0.8% efficacy. Thus, the KK fruit extract showed the highest nematocidal efficacy among the endemically found NZ plants which were compared. It is acknowledged that Goat's Rue is not found natively in NZ. This plant is also a recognised toxic plant for ruminants (Fraiture, 2014). Although this does not necessarily exclude it from having useful non-toxic components, but this was a further reason for not pursuing this plant for future studies. This is in contrast to KK fruit extract which is from a plant with a long history of being used safely for various medicinal purposes (Briggs, 1941, Butts et al., 2019a).

The batch of larvae which was used for this study, Batch 2, had a mixed larval population with *H. contortus* being the most dominant species present in the population. The finding of Experiment 4.1 was in tandem with the other reported studies of plant extracts in the literature. This finding was also consistent with the hypothesis that extracts prepared from selected plant materials based on traditional medicinal uses are likely to contain biologically active components of medicinal interest. A number of studies using a similar approach have examined the anthelmintic properties of various plants. For example, the water and MeOH extracts of *Iris kashmiriana* rhizome resulted in 85% and 100% inhibition of motility respectively, as measured with an LMI against *H. contortus* L3 (Khan et al., 2018). Similarly, ethanolic leaf extract of *Moringa oleifera* at 5 mg/ml showed and 98% and 100% mortality of *H. contortus* (Tayo et al., 2014) and William et al. (2016) have reported the MeOH extract of *Cichorium intybus* possessed anthelmintic activity against larval stages of the parasites *Ascaris suum* and *Oesophagostomum dentatum*. However, anthelmintic properties

of the methanolic extract of five plants studied in this report have not yet been reported in the literature. Thus, the finding in this chapter is novel.

In conclusion, it was found that the KK fruit was the most effective nematocidal component in the series of plant components studied. The NLA finding supported the view that this plant has nematocidal properties. Based on the NLA experiment, KK fruit was deemed to be the most qualifying candidate for further separation study and nematocidal analysis. In the following chapter, the bioassay-guided fractionation study of KK fruit and study of its nematocidal efficacy is presented.

Chapter 5. Nematocidal Study of Kawakawa Fruit Through Bioassay-guided Fractionation and Chromatographic Separation

Nematocidal Study of Kawakawa Fruit Through Bioassay-guided Fractionation and Chromatographic Separation

5.1. Introduction

As documented in the previous chapter, from the comparative nematocidal analysis of crude extracts of different components of five medicinal plants found in NZ, KK fruit was found to be the most effective. In this chapter, bioassay-guided separation and nematocidal studies of the separated fractions of the KK fruit is presented. There is a mixture of secondary metabolites present in any medicinal plant which requires a series of different separation techniques to separate and isolate individual active compounds from the primary crude extract of the plant component (Chaturvedi and Gupta, 2021). The crude extract is separated into discrete fractions and then bioassayed to identify fractions containing the most active phytoconstituents which possess the highest or strongest anthelmintic property (Valente et al., 2021, Gado et al., 2021). As previously introduced in Chapter 3, the bioassay-guided fractionation is used to fractionate a crude plant extract either by solvent partition or chromatographically until a pure biologically active compound is isolated (Dauda and Mudi, 2013). Chromatography is an established and effective method to separate, isolate, purify and analyse natural products (Gogoi et al., 2016). Chromatographic techniques are based on distinctive separation procedures and the nature of the stationary and mobile phases employed. The silica-gel based chromatography coupled with TLC is a widely used technique to effectively separate, isolate and determine the number and nature of the constituents in a crude plant extract (de Paula Carlis et al., 2019). For the studies undertaken in this chapter, the aim was to perform bioassay-guided fractionations of the KK fruit to obtain discrete fractions. Studies were then carried out to determine if some of these fractions had higher nematocidal efficacy than the crude extract of the KK fruit. To achieve this overall aim, the initial step was Soxhlet extraction of KK fruit and then the nematocidal efficacy of the resulting crude solvent extracts were compared. The most effective solvent extract was subsequently subjected to further separation and nematocidal studies were evaluated against two batches of larvae to examine if further separation resulted in a fraction with higher nematocidal efficacy than the parent fraction.

5.2. Materials and Methods

5.2.1. Collection of the Kawakawa Fruit

Fresh unripe KK fruits were collected following the procedure described in Section 2.3.2. The fruits were collected in early December 2018 from two native Kawakawa trees grown in Bledisloe Park, Palmerston North area which were the same trees used for all experiments. After collection, the fruits were immediately processed for drying.

5.2.2. Drying and Grinding of the Kawakawa Fruit

Drying of the KK fruit and grinding of dried material to obtain fine, even sized particles was achieved following the method detailed in Section 2.3.3.

5.2.3. Soxhlet Extraction of the Kawakawa Fruit

The crushed fine powder was subjected to Soxhlet extraction following the method protocol described in Section 2.3.5. Briefly, KK fruit (250 g) was added to methanol (1500 mL) for Soxhlet extraction which was run for 9 hrs. After extraction, the non-soluble portion of the extracted KK fruit sample remained in the thimble and the solvent in the flask contained all the soluble components of the sample.

5.2.4. Solvent Partitions of KK Fruit Using Liquid-Liquid Extraction

The parent Water-Methanol Fraction was obtained by following the procedure presented in Section 2.3.6. This diluted solution was extracted first with 500 mL of hexane and followed with the same volume of EtOAc following the usual procedure. These solvents were evaporated to dryness by rotary evaporation followed by freeze drying to remove any residual solvent. Hexane, EtOAc and Water-Methanol Fractions were obtained. The dry matter yield of each solvent fraction was recorded.

5.2.5. TLC Study of the Fractions of the Kawakawa Fruit

TLC study of the three crude solvent fractions was performed using the protocol detailed in Section 2.3.7. The mobile phase of the TLC study for the Hexane and Ethyl Acetate Extracts was hexane:acetone = 70:30. For the Water-Methanol Extract it was dichloromethane:methanol = 20:80. Diethylamine (1%) was added in the mobile phase to

prevent any tailing and good separation of alkaloids was achieved. Vanillin-H_2SO_4 was used for spotting.

5.2.6. Experiment 5.1 and 5.2: Nematocidal Analysis of Kawakawa Fruit Crude Solvent Extracts Using Two Different Batches of Larvae

NLA tests were carried out with Batch 2 and Batch 3 larvae for the crude solvent extracts as obtained from Soxhlet extractions following the protocol described in Section 2.4.3. At the time of conducting this experiment, Batch 2 and 3 were available for the studies. Batch 2 had a mixed larval composition (66% *H. contortus*, 24% *T. circumcincta*, 6% *Trichostrongylus* spp., 1% *Cooperia* spp., 3% LT, Source: LATU, 18 Feb 2019) and the composition of Batch 3 was 100% *H. contortus* (Source: ARGL, 24 March 2019). The experiments of each solvent fraction with both batches of larvae were performed in triplicate (n=3, T=1-3). Concentration of the dry KK fruit crude solvent extracts were 6 mg in 1 mL in PBS-T in DMSO (3.5% Tween-80 PBS in 1.5% DMSO). The studies with Batch 2 are detailed as Experiment 5.1, and with Batch 3 as Experiment 5.2.

5.2.7. Separation of the KK-fruit-Hexane-Fraction by Silica Gel Gravity Chromatography

The dried KK-fruit-Hexane Extract (4 g) was subjected to gravity chromatography on a silica gel (230-400 mesh) column following the protocol described in Section 2.3.8. Seven different solvent gradients were used as presented in Table 5.1. The quantity of each gradient was 500 mL.

Elution number	***Elution ID (Solvent gradient)***	***Mobile phase for TLC***
1	Hexane 100%	Hexane 100%
2	Hexane 98%, DCM 2%	10% acetone in hexane
3	Hexane 90%, DCM 10%	
4	Hexane 75%, DCM 25%	
5	Hexane 70%, Acetone 30%	30% acetone in DCM
6	DCM 70%, Acetone 30%	
7	Methanol 100%	

Table 5.1. Solvent gradients used in the KK-fruit Hexane-Fraction separation.

TLC of each initial fraction obtained from the above run was carried out following the method described in Section 2.3.7. Vanillin-H_2SO_4 was used as the staining reagent, following the method described in Section 2.3.7.2. Then, the initial fractions were combined with one another on the basis of the TLC result. Mixtures were then concentrated by rotary evaporation and freeze-dried to remove any residual solvent to obtain final fractions.

5.2.8. Experiment 5.3 and 5.4: Nematocidal Analysis of the Separated Fractions of the KK-fruit-Hexane-Fraction Using Two Different Batches of Larvae

The NLA tests of the seven fractions obtained from the separation of the parent Hexane-Fraction were conducted with Batch 2 and 3 larvae following the same NLA protocol. Concentration of the fractions were 6 mg in 1 mL in PBS-T in DMSO (3.5% Tween-80 PBS in 1.5% DMSO). The studies with Batch 2 larvae are detailed as Experiment 5.3 and with Batch 3 as Experiment 5.4.

5.2.9. Normal Phase Flash Chromatography of the KK-fruit-Hexane-Fraction-5

The KK-fruit-Hexane-Fraction-5 was subjected to further separation using normal phase flash chromatography (NP-FC) with the protocol described in Section 2.3.9.1. Briefly, the dried Fraction 5 (1.5 g) was dry-loaded into an empty cartridge (PP 40/150) containing silica gel (230-400 mesh). A gradient of hexane (solvent A), and acetone (solvent B) was used set to a flow rate of 100 mL/min. Collection volume was 45 mL. Firstly, a series of initial fractions was obtained from this run. TLC of each initial fraction was carried out following the method described in Section 2.2.7 with Vanillin-H_2SO_4 as a staining reagent (Section 2.3.7.2). The mobile phase used for each initial fraction obtained from this NP-FC run of KK-fruit-Fraction-5 is presented in Table 2.3 of Section 2.3.9.1. Then, the initial fractions were combined on basis of the TLC results. Thus, a series of final sub-fractions from this separation study of Hexane-Fraction-5 were obtained. The sub-fractions were then concentrated by rotary evaporation and freeze-dried to remove any residual solvent. The dry matter yield of each sub-fraction was recorded.

Fraction	***Mobile phase for TLC study***
3-6	100% Hexane
7-25	10% DCM in hexane
26-50	20% DCM in hexane
51-70	30% DCM in hexane

Table 5.2. Mobile phase for TLC study for different initial fractions obtained from NP-FC run.

5.2.10. Experiment 5.5 and 5.6: Nematocidal Analysis of Fractions of the KK-fruit-Hexane-Fraction-5 Using Two Different Batches of Larvae

The NLA tests of the eight sub-fractions obtained from the separation of the KK-fruit-Fraction-5 were performed with Batch 2 and 3 larvae following the usual protocol. Concentration of the fractions were 6 mg in 1 mL in PBS-T in DMSO (3.5% Tween-80 PBS in 1.5% DMSO). These studies with Batch 2 larvae are detailed as Experiment 5.5 and with Batch 3 larvae as Experiment 5.6.

5.2.11. LC-MS Analysis of the Separated Kawakawa Fruit Fractions

LC-MS analysis of the separated KK fruit fractions was performed with a Waters Symmetry column. The method described in Section 2.3.11.1 was followed.

5.2.12. Calculation and Statistics for Nematocidal Experiments

The nematocidal efficacy of each fraction was specified as % larval mortality using the equation described in Section 2.4.3.1. The efficacy of each fraction was compared with one another using an ANOVA and post-hoc Tukey's test.

5.3. Results

5.3.1. Dry Matter Yield of Kawakawa Fruit Crude Solvent Fractions

The dry matter yields of the extracts obtained from the Soxhlet extraction of the KK fruit were recorded. The schematic diagram of the KK fruit extraction with dry matter yield of each fraction is given in Figure 5.1.

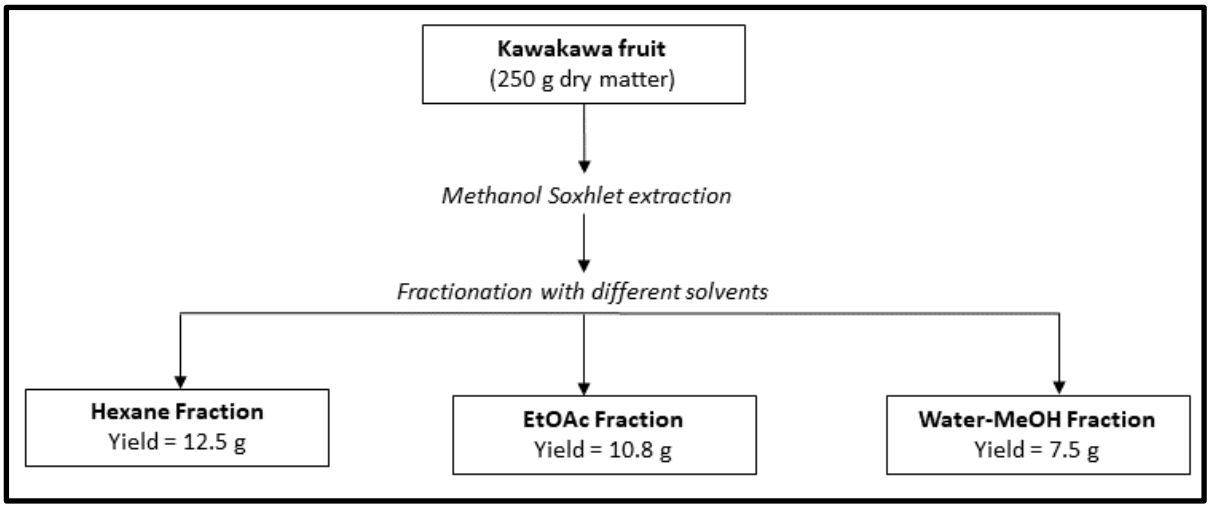


Figure 5.1. Schematic diagram of the Kawakawa fruit extraction and dry matter yield of each fraction.

It was observed that the Hexane-Fraction had the highest dry matter yield out of 3 crude solvent fractions with 12.5 g followed by the EtOAc Extract with 10.8 g and the Water-MeOH Fraction with 7.5 g yield. This yield indicated that there were more components with low polarity present in the KK fruit component. The Hexane-Fraction dissolved the low polar components from fruit extract which had the highest dry matter yield, followed with low to medium polar components dissolved in the EtOAc and high polar components in the Water-MeOH Fraction.

5.3.2. Results of Experiment 5.1 and 5.2: Nematocidal Analysis of Kawakawa Fruit Crude Solvent Extracts

The mean values of % of larval mortality with SEM values are given in Table 5.2 and in Table 5.3 for Experiment 5.1 and 5.2 respectively.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-fruit-Water	34	36	35	35.0 ± 0.5^{b}
KK-fruit-EtOAc	28	32	30	30.0 ± 1.1^{c}
KK-fruit-Hexane	41	42	40	41.0 ± 0.5^{a}
PBS-T in DMSO	0	0	0	0

Table 5.3. Result of Experiment 5.1: Mean of % mortality of Kawakawa fruit crude solvent extracts (n=3; T1-3) against Batch 2 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-fruit-Water	34	32	34	33.3 ± 0.6^{b}
KK-fruit-EtOAc	25	24	26	25.0 ± 0.5^{c}
KK-fruit-Hexane	41	45	44	43.3 ± 1.2^{a}
PBS-T in DMSO	0	0	0	0

Table 5.4. Result of Experiment 5.2: Mean of % mortality of Kawakawa fruit crude solvent extracts (n=3; T1-3) against Batch 3 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

From the results of Experiment 5.1 and 5.2 presented in Table 5.2 and 5.3, it was found that the KK-fruit-Hexane-Fraction had the highest nematocidal efficacy out of the three crude fractions ($P<0.05$) with both Batch 2 and 3 larvae. Additionally, it was observed that the KK-fruit-Hexane-Fraction had similar efficacy against a mixed larval composition and a single composition of *H. contortus* larvae. The efficacy of the Hexane Fraction with Batch 2 larvae was 41.0 ± 0.5% and with Batch 3 was 43.3 ± 1.2% which were very comparable ($P>0.05$, NS). Hence, further chromatographic separation of the Hexane-Fraction was carried out as it was found to be the most effective fraction.

5.3.2. Result of Normal Phase Silica Gel Gravity Chromatography and TLC Analysis of the KK-fruit-Hexane -Fraction

The dried sample of the Hexane-Fraction (4 g) was subjected to further separation using silica gel gravity chromatography. The overall runtime to complete the separation was 4 hrs. The polarity of solvent gradients used in the separation for mobile phase was gradually increased as per the progression of separation as viewed on the column and according to the TLC profiling of each fraction. Primarily, a total of 30 initial fractions were obtained. The number of initial fractions obtained from each elution is summarised in Table 5.4. The pictures of TLC plates of initial fractions 1-30 is presented in Figure 5.2.

Elution number	***Elution ID (Solvent gradient)***	***Mobile phase used for TLC study***	***Number of initial fractions obtained***
1	Hexane 100%	Hexane 100%	2
2	Hexane 98%, DCM 2%	10% acetone in hexane	4
3	Hexane 90%, DCM 10%		4
4	Hexane 75%, DCM 25%		5
5	Hexane 70%, Acetone 30%	30% acetone in DCM	5
6	DCM 70%, Acetone 30%		5
7	Methanol 100%		5

Table 5.5. *Solvent gradients used in the KK-fruit-Hexane-Fraction separation and number of initial fractions obtained from each elution.*

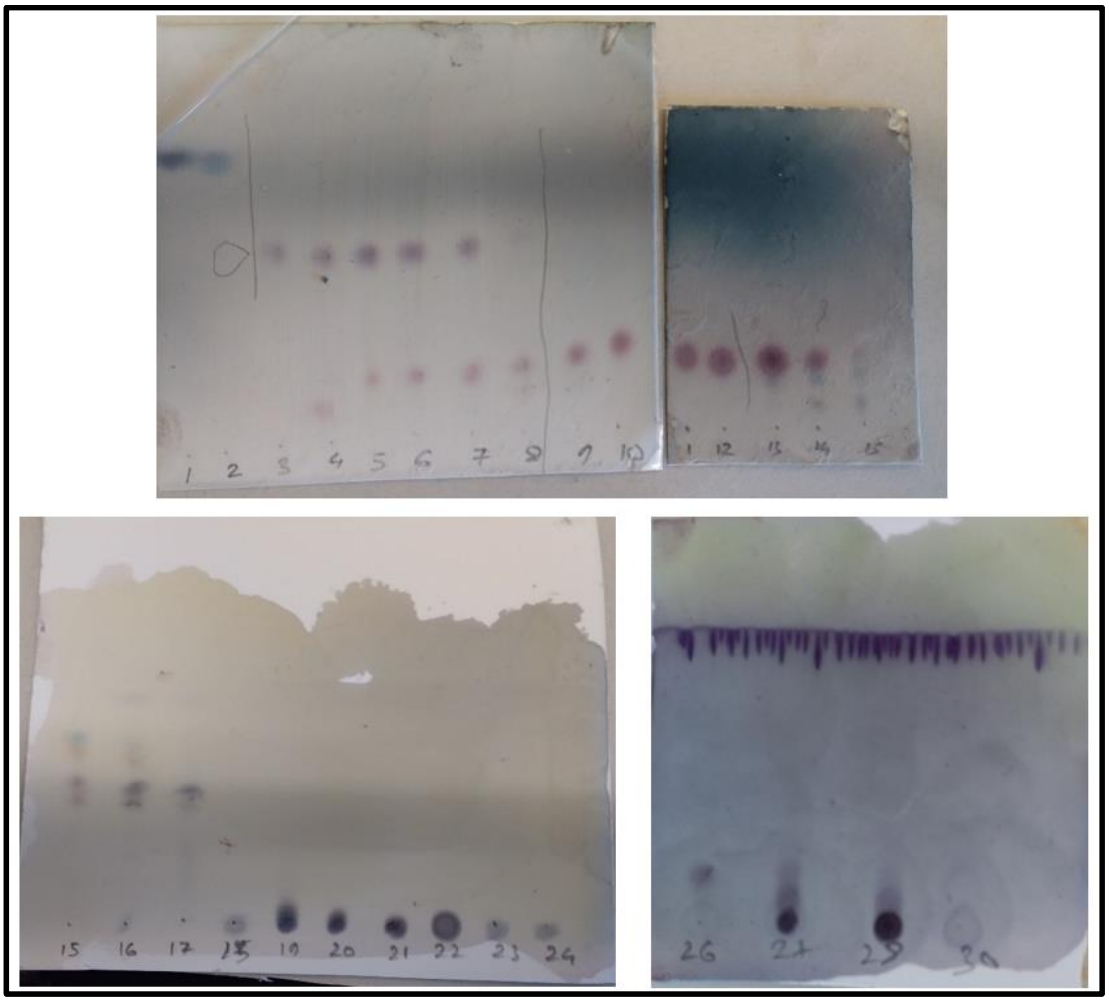


Figure 5.2. Picture of the TLC plates of the initial fraction 1-30 from the KK-fruit-Hexane-Fraction separation.

Based on the TLC result, the initial fractions were combined to get 7 final fractions and yield of each fraction was recoded. The initial fractions that were combined to get the final fractions and yield of each fraction are presented below in Table 5.6.

Combination of initial fractions	***Final fraction***	***Dry matter yield of final fraction (mg)***
1-2	KK fruit Hexane Fr 1	9
3-8	KK fruit Hexane Fr 2	300
9-14	KK fruit Hexane Fr 3	440
15-17	KK fruit Hexane Fr 4	1050
18	KK fruit Hexane Fr 5	1710
19-22	KK fruit Hexane Fr 6	310
23-30	KK fruit Hexane Fr 7	100

Table 5.6. Combination of initial fractions obtained from the separation of the KK-fruit-Hexane-Fraction and dry matter yield of final fractions.

5.3.3. Result of Normal Phase Flash Chromatography of the KK-fruit-Hexane-Fraction-5

The KK-fruit-Hexane-Fraction-5 was subjected to further separation using NP-FC separation. A total of 70 initial fractions were obtained. The flash chromatogram is presented in Figure 5.3. TLC of each fraction was carried out and fractions were combined to get 8 final composite fractions (Table 5.7). Note that the TLC plates were not photographed. Results of the TLC profiling were observed and documented by Gupta. In Table 5.8, a proposed combination of initial fractions based on chromatogram is presented.

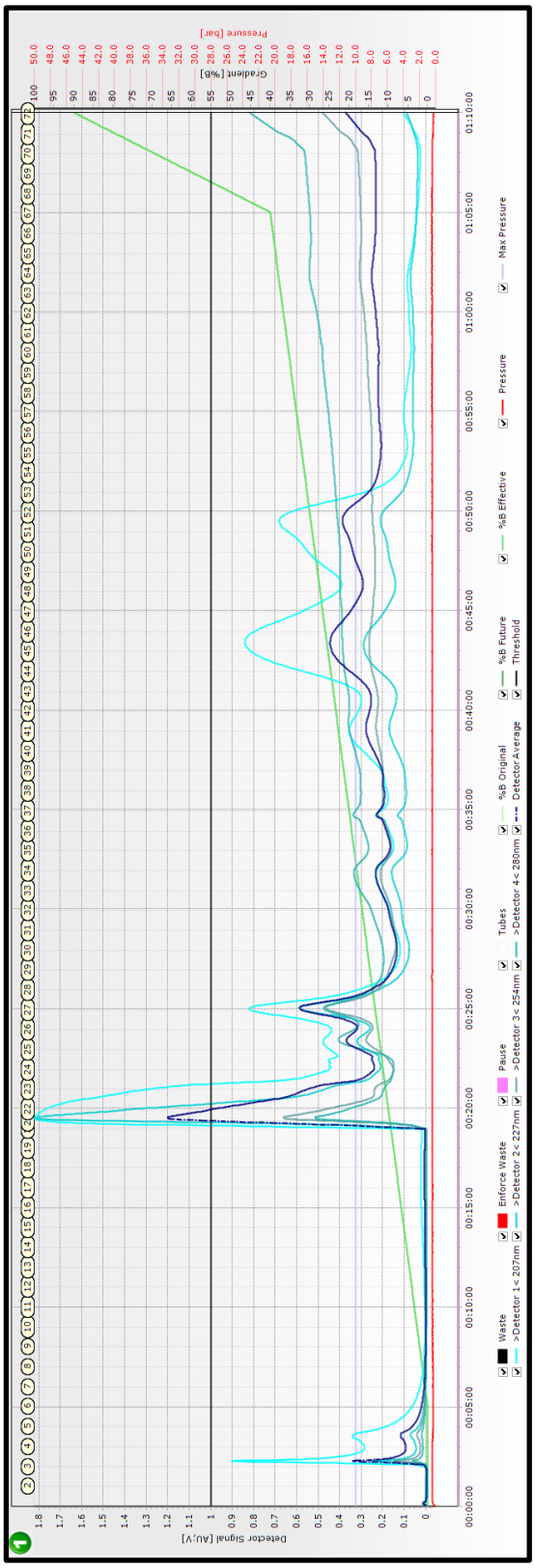


Figure 5.3. Flash chromatogram of the normal phase flash chromatography run of the KK-fruit-Hexane-Fraction-5.

Combination of initial fractions	***Final fraction***	***Dry matter yield of final fraction (mg)***
3-20	KK fruit Hexane Fr 5-1	3
21-22	KK fruit Hexane Fr 5-2	20
23-25	KK fruit Hexane Fr 5-3	110
26-39	KK fruit Hexane Fr 5-4	9
32-39	KK fruit Hexane Fr 5-5	290
40-50	KK fruit Hexane Fr 5-6	120
51-62	KK fruit Hexane Fr 5-7	250
63-72	KK fruit Hexane Fr 5-8	300

Table 5.7. Combination of initial fractions of the KK-fruit-Hexane-Fraction-5 to get final 8 sub-fractions and dry matter yield of each fraction.

Combination of initial fractions based on the peak areas in chromatogram	3-4
	21-22
	23-24
	25
	26
	27
	33-34
	35-37
	39-41
	42
	43-47
	48-52
	53-72

Table 5.8. Proposed combination of initial fractions based on the peak areas in flash chromatogram.

The combination of initial fractions through TLC study and flash chromatogram were not always complimentary of one another. The result of the combination of initial fractions

from TLC and chromatogram analysis is presented below. For the proposed combination of initial fractions based on the peak areas in the flash chromatogram, similar hypotheses were taken to obtain final fractions as described in Chapter 3, the combination of Quebracho Water and BuOH initial fractions from flash chromatogram, where TLC analysis was not possible. From the chromatogram presented in Figure 5.3, the Fractions 3 and 4 were found to be UV-absorbent. TLC results of both had similar traces. Hence, they were combined. Fractions 5-20 did not show any UV absorbance. Fractions 21-22 showed a strong peak on the chromatogram. TLC of these fractions had similar traces. Hence, they were combined with one another to get the final sub-fraction KK fruit Hexane Fr 5-2. Similarly, initial fractions 23-25 were combined to obtain the final sub-fraction KK fruit Hexane Fr 5-3 based on the TLC trace. From Fraction 26 onward, there was a huge disparity with the TLC result and peak areas in the chromatogram. This has been thoroughly discussed in the discussion section of this chapter. All the other fractions were combined based on the TLC result. KK fruit Hexane Fr 5-5 had the highest dry matter yield with 290 mg.

5.3.4. Results of Experiment 5.3-5.6: Nematocidal Analysis of Fractions of the KK-fruit-Hexane-Fraction and the Hexane-Fraction-5

The NLA tests results of Experiment 5.3 and 5.5 with Batch 2 larvae are presented together. The mean values of % larval mortality with SEM for these two experiments are presented in Table 5.9.

Sample	***% Larval Mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-fruit-Hexane-Fraction 5	52	55	52	53.0 ± 1.0^{b}
KK-fruit-Hexane-Fraction 6	47	47	44	46.0 ± 1.0^{c}
KK-fruit-Hexane-Fr 5-3	49	50	47	48.6 ± 0.8^{a}
KK-fruit-Hexane-Fr 5-4	43	44	43	43.3 ± 0.3^{c}
KK-fruit-Hexane-Fr 5-5	52	50	49	$50.3 \pm 0.8^{a,b}$
KK-fruit-Hexane-Fr 5-6	46	48	42	$45.3 \pm 1.7^{c,a}$
KK-fruit-Hexane-Fr 5-7	38	38	39	38.3 ± 0.3^{d}
KK-fruit-Hexane-Fr 5-8	37	38	37	37.0 ± 0.5^{d}
PBS-T in DMSO	0	0	-	0

Table 5.9. Results of Experiment 5.3 and 5.5: Mean of % mortality of the separated KK fruit Hexane fractions (n=3; T1-3) against Batch 2 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

The NLA tests results of Experiment 5.4 and 5.6 with Batch 3 larvae are presented together. The mean values of % larval mortality with SEM for these two experiments are presented in Table 5.10.

Sample	***% Larval Mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-fruit-Hexane-Fraction 5	50	51	48	49.6 ± 0.8^{a}
KK-fruit-Hexane-Fraction 6	47	45	42	44.6 ± 1.4^{b}
KK-fruit-Hexane-Fr 5-3	49	48	47	$48.0 \pm 0.5^{b,a}$
KK-fruit-Hexane-Fr 5-4	40	40	39	39.6 ± 0.3^{c}
KK-fruit-Hexane-Fr 5-5	46	47	43	45.3 ± 1.2^{b}
KK-fruit-Hexane-Fr 5-6	48	48	49	$48.3 \pm 0.3^{b,a}$
KK-fruit-Hexane-Fr 5-7	34	32	31	32.3 ± 0.8^{d}
KK-fruit-Hexane-Fr 5-8	32	31	30	31.0 ± 0.5^{d}
PBS-T in DMSO	0	0	-	0

Table 5.10. Results of Experiment 5.4 and 5.6: Mean of % mortality of the separated Kawakawa fruit Hexane fractions (n=3; T1-3) against Batch 3 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

The results of Experiment 5.3-5.6 (presented in Table 5.6 and 5.7) indicated that the KK-fruit-Hexane-Fraction-5 was more effective than Fraction-6 ($P<0.05$) with both Batch 2 and 3 larvae. The Hexane-Fractions 1, 2, 3, 4 and 7 were oil-like and suspending them in PBS-T in DMSO, resulted in emulsion formation and the NLA assay could not be performed as the worms were not visible under the microscope (due to the turbidity of the emulsion). LC_{50} was achieved only with Fraction-5 at the concentration 6 mg/mL. Hence, further chromatographic separation of the Fraction-5 was carried out. However, as found with Experiment 5.3-5.6, the efficacies in the NLA tests of the separated sub-fractions obtained from Fraction 5 did show statistically different efficacies although the actual values did not change dramatically compared to the efficacy of the parent Fraction-5 with both Batch 2 and 3 larvae. The efficacies were very comparable, but the parent Fraction-5 was found to have a slightly higher efficacy than the separated Fraction 5-3 ($P<0.05$), Fraction 5-4 ($P<0.05$), Fraction 5-6 ($P<0.05$), Fraction 5-7 ($P<0.05$) and Fraction 5-8 ($P<0.05$) with Batch 2 larvae. When compared with the efficacy of the separated Fraction 5-5, the difference in efficacy was not significant ($P>0.05$). An LC_{50} was achieved with Fraction 5-5 at the same concentration of 6 mg/mL. With Batch 3

larvae, the parent Fraction-5 was found to have slightly higher efficacy than the Fraction 5-4 ($P<0.05$) and the Fraction 5-5 ($P<0.05$), Fraction 5-7 ($P<0.05$) and Fraction 5-8 ($P<0.05$). However, the difference in efficacy was not significant with the separated Fraction 5-3 ($P>0.05$) and Fraction 5-6 ($P>0.05$). Therefore, it was found that the further separation of the KK fruit Fraction 5 did not reveal any sub-fractions with substantially increased efficacy.

5.3.5. LC-MS Analysis of Separated Fractions of Kawakawa Fruit Hexane Extract

5.3.5.1. LC-MS Analysis of the Fractions Obtained from Kawakawa Fruit Hexane Extract

The seven fractions obtained from silica gel chromatography were subjected to LC-MS analysis following the method described in Section 5.2.11. Both positive (+) and negative (-) ionisation modes were run. However, the (-) mode did not result in any peaks in any of the fractions. This indicated that the separated fractions did not possess any components with fragments that could be negatively ionised. These are the ions that originate from the analyte molecule present in the sample by abstraction of a proton [M-H]-, such as phenols and acids (Hua et al., 2001). The chromatograms of the LC-MS analysis with (+) ionisation of the separated fractions of the KK-fruit-Hexane is presented in Figure 5.4.

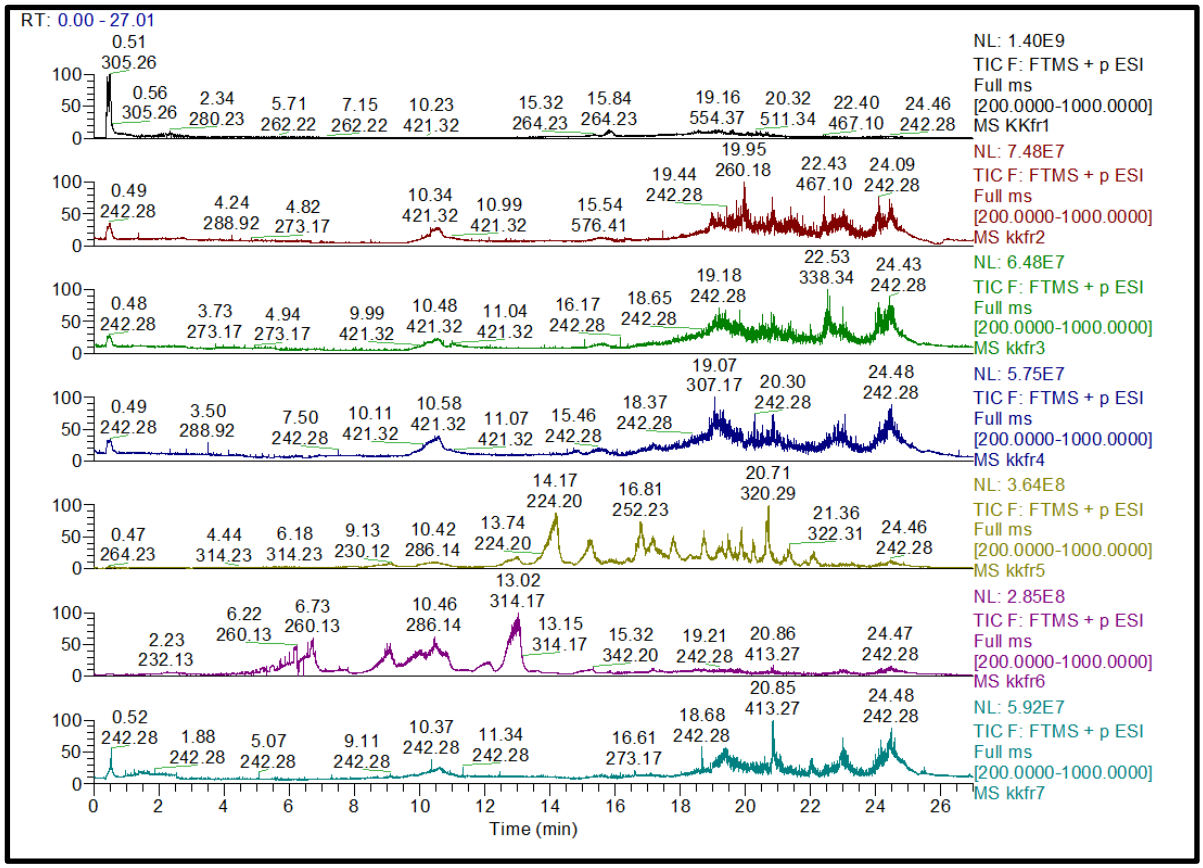


Figure 5.4. LC-MS chromatograms of the separated fractions 1-7 of the KK-fruit-Hexane-Fraction with (+) ionisation mode. Labels above each peak represent RT and m/z of base peak.

Interestingly, Fractions 2, 3 and 4 had similar LC-MS chromatograms. From the TLC result presented in Figure 5.2 and combination of initial fractions to obtain the seven fractions presented in Table 5.4, the Fraction-2 consisted of initial fractions 3-8, Fraction 3 consisted of initial fractions 9-14, and the Fraction-4 consisted of initial fractions 15-17. TLC results were different for these initial fractions as presented in Figure 5.2. However, from the LC-MS analysis presented above in Figure 5.4, the Fractions 2, 3 and 4 were found to have a very similar trace with each other. This finding is discussed thoroughly in the discussion section of this chapter (Section 5.4).

5.3.5.2. LC-MS Analysis of the Separated Sub-Fractions of the KK-fruit-Hexane-Fraction-5

LC-MS analyses of the eight separated sub-fractions of the KK-fruit-Hexane-Fraction-5 obtained using NP-FC were conducted. Both positive (+) and negative (-) iodination mode were run. However, (-) mode did not result in any peak in any of the fractions. This indicated that the separated fractions do not possess any components with fragments that could be

negatively charged. These are the ion originating from the analyte molecule by abstraction of a proton [M-H]-, such as phenols and acids (Hua et al., 2001). The chromatograms of LC-MS analysis with (+) ionisation of sub-fraction 1-8 of KK fruit Hexane Fraction 5 are presented below in Figure 5.5.

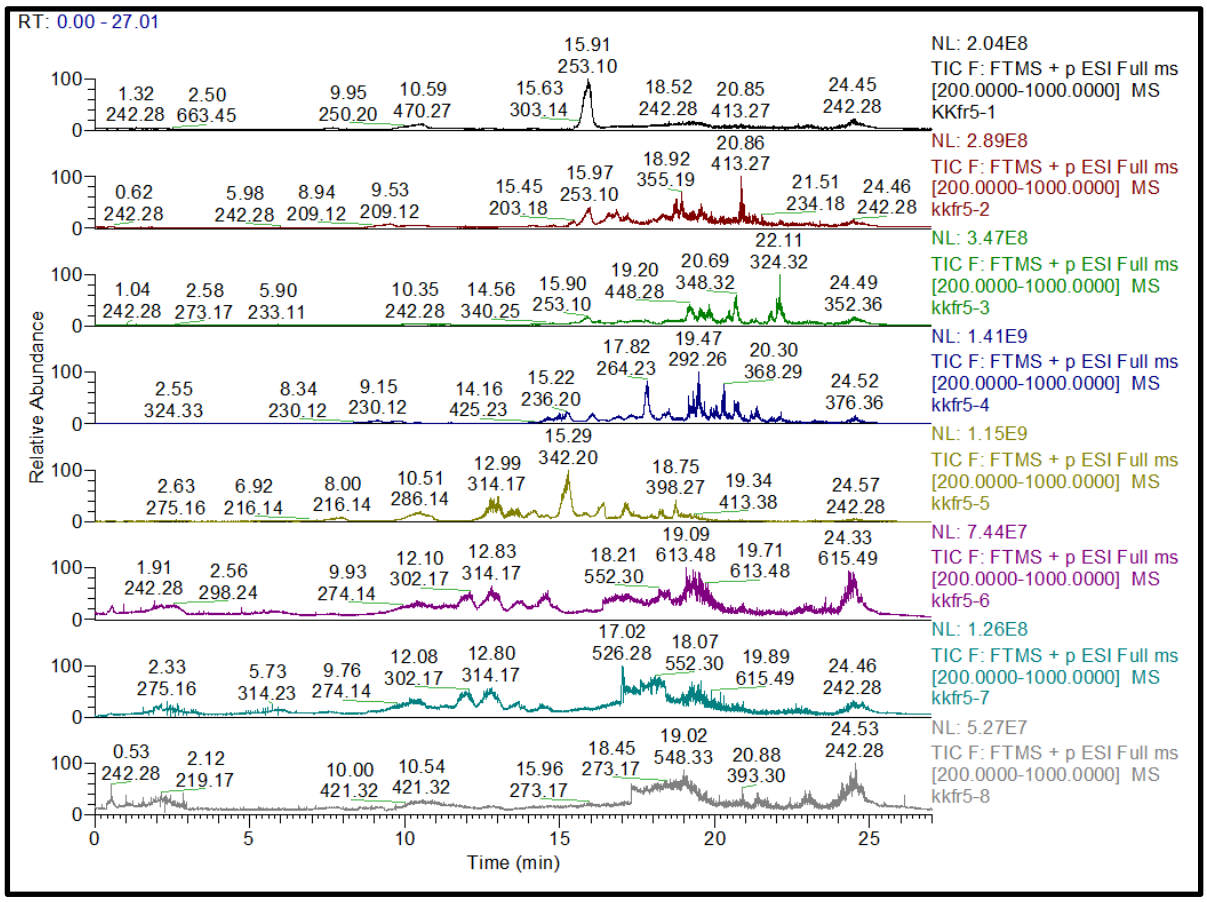


Figure 5.5. LC-MS chromatograms of the separated sub-fractions 1-8 of the KK-fruit-Hexane-Fraction-5 with (+) ionisation mode. Labels above each peak represent RT and m/z of base peak.

From the chromatograms of LC-MS analysis as presented above; all fractions were distinctive of one another. This finding is discussed thoroughly in the discussion Section 5.4.

5.4. Discussion

In this chapter, bioassay-guided fractionation and chromatographic separation of the KK fruit has been presented. The KK fruit was found to be the most effective plant component from the comparative study presented in the previous chapter, Chapter 4. The bioassay-

guided separation techniques have been widely used to initially separate and then purify fractions from a crude plant extract. These pure fractions are then assessed to identify those with higher anthelmintic efficacy than the parent crude extract (Ogedengbe-Olowofoyeku et al., 2021, Valente et al., 2021, Pournaghi et al., 2021, Wang et al., 2011, Samoylenko et al., 2008). In the present series of studies, three solvent fractions were prepared through Soxhlet extraction of the KK fruit and liquid-liquid solvent partitioning. NLA tests of these fractions were then carried out with two batches of larvae having different larval population. At the time of conducting these experiments, Batch 2 and 3 were available for studies. The reason for choosing two different batches of larvae was based on the hypothesis that the efficacy of each fraction will not vary with the species of larvae present in the larval population. It was found that the Hexane-Fraction had the highest nematocidal efficacy out of the three solvent fractions studied ($P<0.05$). The Hexane-Fraction showed an efficacy of 41.0 ± 0.5% against Batch 2 larvae which was higher than the efficacy of crude KK fruit extract described in Chapter 4 with an efficacy of 30.0 ± 1.1% ($P<0.05$). Therefore, separation of the KK fruit extract into different solvent fractions resulted in increased efficacy. Efficacy of the KK-fruit-Hexane-Fraction against Batch 2 and 3 was found to be comparable indicating that efficacy did not vary with the species of larvae present in the larval population. For each experiment the comparisons were made within that experiment and not between experiments which may have been compromised by the different genera of larvae present.

The KK-fruit-Hexane-Fraction was separated using silica gel gravity chromatography. It was observed that there was a long-time involved (4 hrs) for the separation to complete and manually transferring each solvent gradient into the column was cumbersome. A total of thirty fractions was initially obtained from the separation. Through TLC work, the initial fractions with similar traces on the TLC were combined, and seven final fractions were obtained. These were subjected to NLA assay to identify the most effective fraction. These fractions were subjected to LC-MS analysis which revealed an interesting result. It was found that the Fractions 2, 3 and 4 had identical LC-MS chromatograms. However, from the TLC profiling, the initial fractions that constituted these three fractions had different traces present on the TLC plate. This signified two different possibilities: 1) the components present in these fractions were not separated on the column used for LC-MS analysis; 2) combination of fractions through TLC profiling might not be accurate. From the dry matter yield of Fraction

2, 3 and 4 presented in Table 5.6, all of them had different yields. This observation of disparity between the LC-MS analysis and TLC profiling was taken into consideration during future studies. The Fractions 1, 2, 3 and 4 were not important in the wider scope of the research, as the NLA test could not be performed with them. This pointed out a limitation of the technique used in this assay as preparation of the test solution was found to be dependent on the nature of the test sample and the medium used. In this case this was PBS-T in DMSO. This will be addressed in the next chapter (**Chapter 6**) with a selection of different PBS and organic solvent-based media employed to perform the NLA tests with.

The KK-fruit-Hexane-Fraction-5 was found to be the most effective one when studied with two different batches of nematode larvae. LC_{50} of this fraction with both batches of larvae was estimated to be 6 mg/mL. Hence, the **hypothesis** that the efficacy of each fraction will not vary with the species of larvae present in larval population was found to be **correct**. Additionally, the KK-Fruit-Hexane-Fraction-5 had slightly higher efficacy than the parent Hexane Extract with both batches of larvae ($P<0.05$). Therefore, further chromatographic separation was found to produce a fraction having slightly higher nematocidal efficacy than the parent fraction.

Further separation of the KK-fruit-Hexane-Fraction-5 was carried out using NP-FC. The NP-FC was found to be a very quick procedure with 70 minutes of total runtime. Additionally, it was found to be very convenient being a fully automated process. A total of 70 initial fractions were obtained. The initial fractions were combined based on TLC profiling of each. Combination of initial fractions through peak areas in the chromatogram was proposed, taking similar approaches discussed previously in Chapter 3. It was found that from the initial fraction 26 onward, the combination of fractions with TLC profiling and through similar peak areas in chromatogram were not complimentary of one another. For example, Fractions 26-39 were combined because they had similar TLC traces. However, from the flash chromatogram, there were many distinctive peak areas present between the region of these fractions. This signified two possibilities: 1) the fractions were distinctive of one another as indicated by the flash chromatogram; 2) there was a common component present in this series of fractions that was responsible for the UV-absorbance in the chromatogram. Since, TLC profile of Fractions 26-39 were similar, the 2nd possibility was thought to be correct. The combination of Fractions 26-39 resulted in final sub-fraction 5-4. From the LC-MS result of

sub-fraction 1-8 in Figure 5.5, Fraction 5-4 was distinctive of all the other fractions. This indicated that the combination of initial fractions resulted in a distinctive fraction. This finding was taken into consideration for future studies with chromatographic separation using FC. However, based on the results of NLA tests presented in Section 5.3.4, further separation of the Fraction-5 did not result in a fraction with increased efficacy but resulted in a slightly decreased efficacy with some separated fractions with both batches of larvae. Based on this observation, further chromatographic separation was found to not result in fractions having higher nematocidal efficacy than the parent fraction. This finding contradicted with the previous two findings.

In conclusion, the KK fruit had significant nematocidal efficacy, and bioassay-guided purification resulted in better nematocidal efficacy with some of its separated fractions. However, further chromatographic separation did not result in identifying a sub-fraction with very high nematocidal efficacy. Moreover, as observed with Experiment 5.3-5.6, NLA studies could not be performed with a lot of fractions obtained from the separation study of the KK fruit because of the nature of the fractions as they were not found to be soluble in the medium. Therefore, the studies reported here did not establish that KK fruit was an ideal candidate for research aimed towards finding a solution to the anthelmintic resistance issue. In the next stage of research with KK, the collection of its different components (leaf, stem, and root) was carried out and their nematocidal efficacies were investigated to determine which component of this plant had the highest nematocidal efficacy and thus could be the most effective.

Chapter 6. Collection and Extraction of Different Components of Kawakawa and their Nematocidal Efficacies

Collection and Extraction of Different Components of Kawakawa and their Nematocidal Efficacies

6.1. Introduction

The previous chapter concentrated on the extraction, bioassay-guided fractionation and nematocidal study of the KK fruit component. It was found that the preliminary separation of KK fruit generated fractions with higher nematocidal efficacy than the parent fruit extract, but the efficacy did not dramatically improve any further with its separated sub-fractions. There was a need to conduct a screening study of the whole plant to find if there is any other plant component possessing plant secondary metabolites (PSM) that have significant anthelmintic properties, potentially higher than the KK fruit component. Screening studies of medicinal plants through collection of their different components and evaluating their nematocidal efficacy by making extracts of each has been widely reported in the literature (Chhabra et al., 2014, Buza et al., 2020, Ahmed et al., 2020). Some medicinal plants are reported to possess the most effective PSM in their root extracts (Baby and Regi Raphael, 2014, Bodke et al., 2013), stembark extracts (Wahab Obeng et al., 2021, De Amorim et al., 2021), leaf extracts (Jan et al., 2021, Hadi et al., 2021), fruit extracts (Wahab Obeng et al., 2021, Chennuru et al., 2021), and flower extracts (Turan and Mammadov, 2020, Ahmed et al., 2020). The practice of using KK as a traditional Māori medicinal plant includes the usage of its fruit, leaf and root (Butts et al., 2019b). However, the evaluation of nematocidal efficacy of its different components is yet to be reported in the literature. It was hypothesised that the four components of KK (leaf, stem, fruit, and root) would likely contain different PSM which may have distinctive nematocidal efficacy. In this chapter, the studies of the collection of each plant component followed by the preparation of extracts of each are presented. A wide range of media was selected. Quantification of the insoluble matter of the plant component in each media was also investigated. NLA tests were conducted with these media alone to determine if they had any negative effect on nematode larvae by either paralysing or killing them. This elucidated which of these media were not suitable as a media for the NLA test and which were found to be suitable. NLA tests of each plant component were then performed by dissolving the dry component fraction in the right media. The NLA tests were carried out with two different batches of larvae. From this study, the KK plant component with the highest nematocidal efficacy was identified. This led to the further bioassay-guided fractionation and purification study of that plant component which is presented in the next chapter.

6.2. Materials and Methods

6.2.1. Collection and Extraction of Different Components of Kawakawa

KK plant components (leaf, fruit, steam and root) were collected from the same KK plants which had been used for collections previously and were found in Bledisloe Park, the area neighbouring Massey University. A picture of the KK plant of Bledisloe Park and the components that were selected are illustrated in Figure 6.1. Extracts of each component were prepared using the protocols described in the Section 2.3.4 after drying and grinding of each plant component following the protocol of Section 2.3.3.

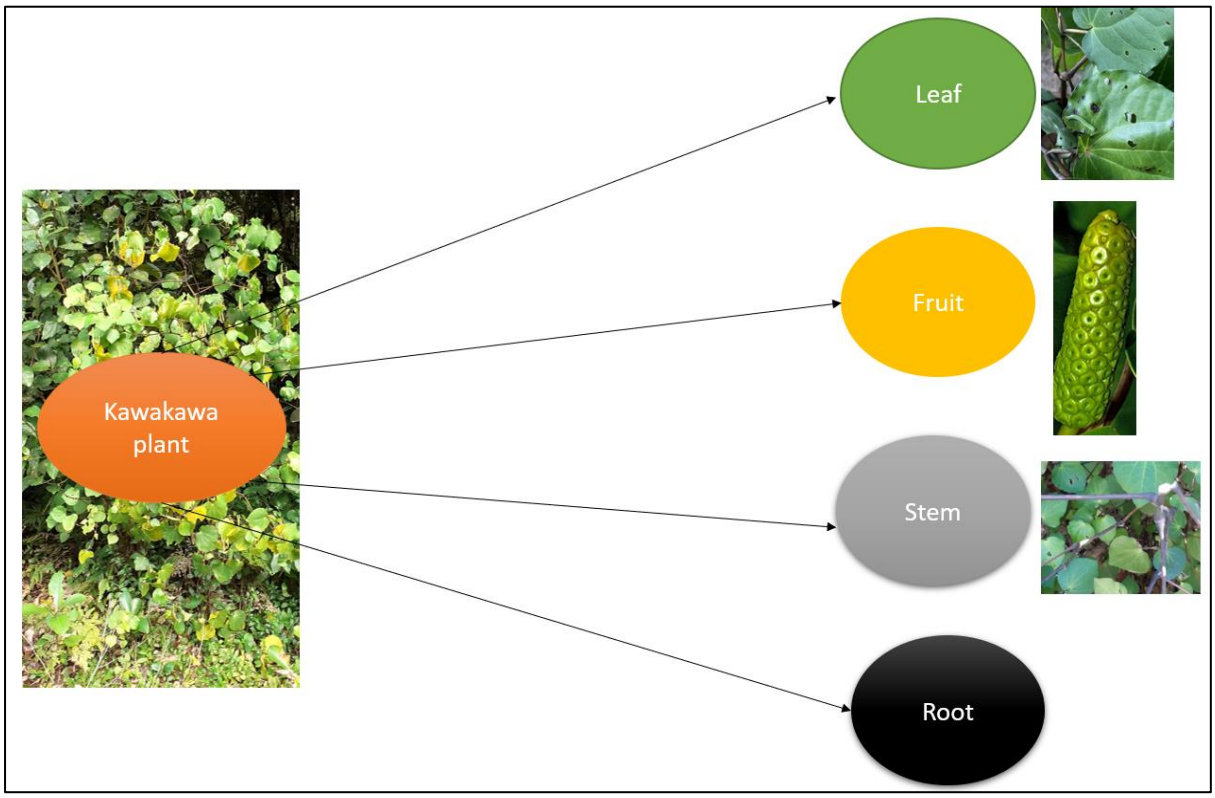


Figure 6.1. Picture of the Kawakawa plant and its different components.

6.2.2. Solubility of Dried Extracts in Different Medias

The dried component extracts of each plant component were suspended in four PBS media that were later to be used as medias for an NLA test. This was performed to quantify the soluble and insoluble matter present in a respective media. The 4 PBS compositions that were chosen and the respective media number, are presented in Table 6.1. Note that the insoluble matter in Media 4 was assessed qualitatively.

Media number	***ID of Composition***	***Abbreviation of composition***
Medium 1	PBS	PBS
Medium 2	PBS in 3% Tween-80	PBS-T
Medium 3	PBS-T in 1.5% DMSO	PBS-T in DMSO
Medium 4	PBS-T in 3% DMSO	PBS-T in 3% DMSO

Table 6.1. List of PBS medium taken to quantify the soluble and insoluble matter of dried component fraction.

A series of organic solvents was also selected to quantify the solubility of the sample. The series of organic solvent that was selected is presented in Table 6.2.

Media Number	***ID of Composition***
Medium 5	100% EtOH
Medium 6	100% MeOH
Medium 7	25% EtOAc+75% EtOH
Medium 8	25% EtOAc+75% MeOH
Medium 9	50% (25% EtOAc+75% EtOH) +50% PBS

Table 6.2. List of organic solvents taken to quantify the soluble and insoluble matter of dried decoction fraction.

The dried extract of respective components was dissolved in the respective medium (1.5 mL of the medium in a centrifuge tube, weighed at empty) at 8 mg/mL. The suspension was then subjected to ultra-sonication followed by centrifugation. The supernatant was decanted carefully using a micropipette and transferred into a clear vial. The remaining contents in the centrifuge tube was freeze-dried and weighed, and the quantity of the insoluble matter (if any) was recorded.

6.2.3. Experiment 6.1: Observation of the Effect of Medium Alone on Nematode Larvae

To evaluate whether any of the medium presented in Section 6.2.2 have any effect on nematode larvae, NLA tests were carried out with Batch 3 (100% *H. contortus*, source: ARGL, 24 March 2019) larvae following the standard NLA method described in Section 2.3.3. These studies are detailed as Experiment 6.1.

6.2.3.1. Calculation and Statistics

The effect of media alone on nematode larvae present in Batch 3 was described as survival rate of each medium. The number of larvae killed/paralysed by a medium per 100 larvae was expressed as % survival rate. A graph of % survival rate of each medium was plotted using GraphPad Prism 8.

6.2.4. Experiment 6.2 and 6.3: Nematocidal Analysis of Different Kawakawa Plant Components Using Two Different Batches of Larvae

The NLA tests of the four dried KK plant component (leaf, stem, root, and fruit) extracts were carried out with Batch 3 and 4 larvae. Batch 3 was 100% *H. contortus* (Source: ARGL, 24 March 2019) and Batch 4 was of a mixed larval composition (3% *T. circumcincta*, 73% *Trichostrongylus* spp., 8% *Cooperia* spp., 16% LT, Source: LATU, 10 August 2019). The studies of NLA tests with KK plant components with Batch 3 larvae are detailed as Experiment 6.2 and with Batch 4 are detailed as Experiment 6.3. Standard NLA protocol was followed, and experiments were done in triplicate (n=3). Concentrations of each sample was 8 mg/mL in Medium 3.

6.3.4.1. Calculations and Statistics

The nematocidal efficacy of each fraction was specified as % larval mortality using the equation described in Section 2.4.3.1. The efficacy of each plant extract was compared with one another using an ANOVA and post-hoc Tukey's test.

6.3. Results

6.3.1. Result of Solubility of Decoctions in Different Medium

The dry matter yields of insoluble matter present in each medium are presented in Table 6.3.

Sample	***Medium***	***Insoluble matter (in mg)***
KK leaf dried extract	Medium 1	-
	Medium 2	-
	Medium 3	-
	Medium 5	-
	Medium 6	-
	Medium 7	-
	Medium 8	-
	Medium 9	-
KK root dried extract	Medium 1	3.1 mg
	Medium 2	2.7 mg
	Medium 3	1.5 mg
	Medium 5	0.9 mg
	Medium 6	0.7 mg
	Medium 7	-
	Medium 8	-
	Medium 9	-
KK stem dried extract	Medium 1	5.8 mg
	Medium 2	4.6 mg
	Medium 3	3.9 mg
	Medium 5	1.2 mg
	Medium 6	0.9 mg
	Medium 7	-
	Medium 8	-
	Medium 9	-

(Contd. on the next page)

Sample	***Medium***	***Insoluble matter (in mg)***
KK fruit dried extract	Medium 1	5.9 mg
	Medium 2	4.9 mg
	Medium 3	3.1 mg
	Medium 5	2.4 mg
	Medium 6	1.3 mg
	Medium 7	0.9 mg
	Medium 8	-
	Medium 9	0.3 mg

Table 6.3. The series of medium taken and insoluble matter present in each for the respective plant component.

It was found that the dried extract sample of KK leaf component was totally soluble in all the medium. Solubility of the rest of the KK plant components varied depending on the medium. Solubility of KK root, stem, fruit varied from one medium to the another. For example, for the dried sample of KK fruit extract, there was a presence of insoluble matter in all PBS based media. However, PBS-T in DMSO (Medium 3) had the least amount of insoluble matter present. Among the organic based media, all were demonstrated to have better solubility for each KK sample (except KK leaf which was fully soluble in PBS) than the PBS based media.

6.3.2. Results of Experiment 6.1: Observation of the Effect of Medium Alone on Nematode Larvae

The graph of the % of survival rate of larvae of Batch 3 with each medium alone is presented in Figure 6.2.

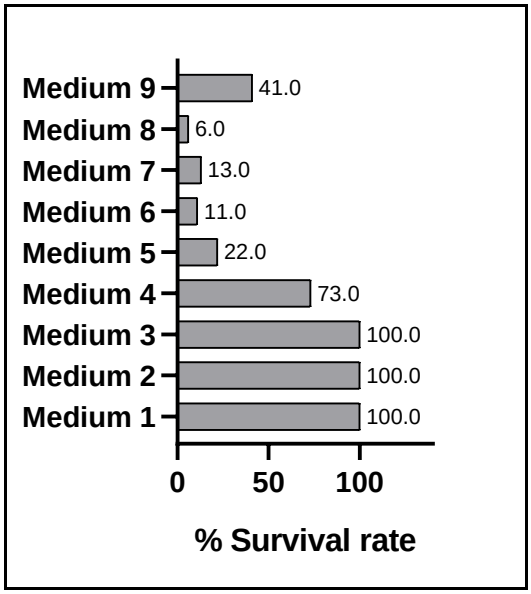


Figure 6.2. Graph of % of survival rate of the larvae of Batch 3 larvae (100% H. contortus) in different medium.

It was found with Experiment 6.1 that all the three PBS based media did not have any adverse effect on larvae present in Batch 3 (100% survival rate of larvae). Upon increasing the concentration of DMSO from 1.5% to 3% (PBS-T in 3% DMSO, designated as Medium 4), it increased the solubility for the KK fruit sample (not quantified and observed qualitatively). However, it had an adverse effect on the larval population with survival rate of 73%. On the other hand, Medium 5-9, the organic solvent-based media, were better solvents for each plant component. Nevertheless, all of them had an adverse effect on larvae with very low % of survival rate, which indicated that most of the larvae present in the population did not survive these media. Therefore, they were not fit to be used as a media for NLA test.

6.3.3. Results of Experiment 6.2 and 6.3: Nematocidal Analysis of Different Kawakawa Plant Components

The mean values of % larval mortality with SEM for the Experiment 6.2 are presented in Table 6.4. The mean values of % larval mortality with SEM for the Experiment 6.3 are presented in Table 6.5.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK leaf	66	68	66	66.6 ± 0.6^{a}
KK root	25	26	25	25.3 ± 0.3^{c}
KK stem	9	9	10	9.3 ± 0.3^{d}
KK fruit	34	36	33	34.3 ± 0.8^{b}
PBS-T in DMSO	0	0	0	0

Table 6.4. Result of Experiment 6.2: Mean of % mortality of different Kawakawa components (n=3, T=1-3) against Batch 3 larvae with SEM=Standard Error of Mean. T=Test number. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK leaf	60	62	60	60.7 ± 0.7^{a}
KK root	25	28	27	26.7 ± 0.8^{b}
KK stem	8	10	7	8.3 ± 0.8^{d}
KK fruit	12	18	14	14.7 ± 1.7^{c}
PBS-T in DMSO	0	0	0	0

Table 6.5. Result of Experiment 6.3: Mean of % mortality of different Kawakawa components (n=3, T=1-3) against Batch 4 larvae with SEM=Standard Error of Mean. T=Test number. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

From Experiments 6.2 and 6.3, it was found that KK leaf component had the highest nematocidal efficacy with both batches of larvae when compared to the other three KK plant components (P<0.05). Interestingly, Batch 4 had a mixed larval population which did not have any *H. contortus* present (predominantly *Trichostrongylus* spp.). However, the KK leaf fraction had similar efficacy with both batches of larvae. With Batch 3, the KK leaf extract had 66.6 ± 0.6% efficacy and with Batch 4, it was 60.7 ± 0.7%. The activity of the KK fruit varied greatly on the other hand with Batch 3 and 4. With Batch 3, KK fruit had 34.3 ± 0.8% efficacy but with

Batch 4, the efficacy dropped to 14.7 ± 1.7%. The efficacy of KK stem and KK root fractions did not vary considerably from one batch to another, but they were much lower than that of KK leaf fraction. Therefore, the KK leaf was found to be the most effective KK plant component.

6.4. Discussion

In this chapter, the collection, extraction and nematocidal study of four different components of KK have been presented. It was hypothesised that these four components will have different nematocidal efficacies due to being distinctive components and possessing different PSM. This hypothesis was found to be correct. KK leaf component was found to be more effective than the other three components against two batches of nematode larvae with different larval populations. A similar hypothesis to the one described in Chapter 5 was made: the efficacy of a fraction will not dramatically vary with the species of larvae present in the larval population. This was found to be correct with KK leaf, root, and stem components as their efficacies with both Batch 3 and 4 were found to be similar. However, efficacy of KK fruit was found to be higher with Batch 3 than Batch 4. Thus, the hypothesis was found to be not correct with KK fruit. Nevertheless, KK leaf was found to be the most effective fraction. Therefore, it was found that there is a series of worm paralysing compounds present in KK whole plant which are effective against nematode larvae. The most effective ones are present in the leaf component of the plant which had comparable nematocidal efficacy against two batches of larvae with different larval populations.

An array of different media was taken to perform NLA tests with. Firstly, the quantification analysis of the insoluble matter of different KK plant components present in these media was performed. This was carried out to find the most suitable media which had the least amount of insoluble matter present. It was found that KK leaf did not have any insoluble matter present in all the PBS and organic solvent-based media. The other components had insoluble matter present in the PBS based media. It was found that PBS-T in DMSO had the least amount of insoluble matter present for each component. There was a wide selection of organic solvent-based media. It was found that all these media had better solubility result for each component (except leaf which was fully soluble in all PBS based media). However, upon conducting the NLA test with these media to determine if they would qualify as a medium for the NLA test, all of them had an adverse effect on the larvae present

in Batch 3 larval population. All the organic solvent-based media demonstrated a very low survival rate of larvae in them. This result indicated that they will not qualify as media to conduct NLA test with as they cannot be validated for their usage as negative control. Therefore, the NLA test with four different KK components were performed using Media 3, which had the lowest insoluble matter present for each component (except KK leaf) and the survival rate of larvae was 100% in this media. Nevertheless, upon conducting this NLA test, KK leaf was found to be the most effective which was fully soluble in PBS.

In conclusion, KK leaf was found to be most effective of all the components found in the KK plant. The dried KK leaf extract sample was found to be completely soluble in PBS which did not have any effect on larval population of Batch 3. Therefore, the research to find the answer to the anthelmintic resistance issue was advanced with KK leaf. Further bioassay-guided purification study of the leaf component was needed to be conducted to evaluate the efficacy of its purified fractions, and to separate and identify the key PSM present. In the next chapter, these aspects of research are presented with the bioassay-guided fractionation and nematocidal study of KK leaf component.

Chapter 7. Bioassay-guided Fractionation and Purification Study of Kawakawa Leaf and Its Nematocidal Efficacy

Bioassay-guided Fractionation and Purification Study of Kawakawa Leaf and Its Nematocidal Efficacy

7.1. Introduction

From the previous study presented in Chapter 6, the KK leaf was found to be the most effective plant component having the highest nematocidal efficacy out of the four components studied (leaf, stem, root, and fruit). This is consistent with other reports in the literature where the leaf fractions from many different medicinal plants have demonstrated anthelmintic properties. Some recently reported in-vitro studies include: the methanolic leaf fractions of *Cyperus compressus,* a native tree from Africa, which caused parasite mortality at 30 mg/mL (Soren and Yadav, 2021); the hydroalcoholic extract of *Moringa oliifera* leaves, a plant native to the Indian subcontinent which exhibited significant anthelmintic activities against *Trichuris* spp. and *Teladorsagia* spp. (Pedraza-Hernández et al., 2021); the aqueous leaf fraction of *Indigofera tinctoria L,* a plant native to the Malay Archipelago which killed *H. contortus* at 220 mg/mL with 93.3% mortality (Muda et al., 2021). Nevertheless, the efficacies of the KK leaf extract, or any plant fractions native to NZ, have yet to be reported in the literature. Additionally, most of the research that has been reported in the literature regarding the usage of a medical plant leaf fraction, has focused on application of its crude solvent fractions. Very few researchers have isolated semi-pure or pure compounds from leaf fractions to evaluate their efficacy against GIN as previously discussed in Chapter 1 (Gogoi et al., 2016, Ogedengbe-Olowofoyeku et al., 2021, Ramadwa et al., 2021).

In this chapter, the bioassay-guided fractionation and chromatographic separation of KK leaf sample is presented to evaluate its anthelmintic potential. Firstly, the crude MeOH extract was made with the dried KK leaf sample. The MeOH extract was then further fractionated with a range of solvents to obtain different crude solvent fractions. The nematocidal efficacy of these solvent fractions was then evaluated to find the most effective one. To further separate the solvent fractions, several different separation chromatographic techniques were used. These included normal phase silica gel chromatography (NP-FC) which is the most widely reported chromatography technique to separate crude plant fractions into more discrete fractions (Chimi Fotso et al., 2021, Yaermaimaiti et al., 2021, Lee and Cho, 2021, Liu et al., 2021a). The second technique, as previously introduced in Chapter 3, was Sephadex LH-20 column chromatography which is a commonly used method to separate highly polar methanolic or aqueous plant fractions (Mbopi et al., 2021, Liao et al., 2021). The third technique was reverse phase flash chromatography (RP-FC) which has also been used

extensively to separate, and isolate PSM from different plant fractions (Ramaswamy et al., 2021, Chanda et al., 2021). LC-MS/MS analysis was then used to investigate and determine the constituents of the separated fractions of plant fractions obtained from the aforementioned techniques (Chanda et al., 2021, Wan et al., 2021, Liu et al., 2021b). The separation study of different solvent fractions from KK leaf fraction was attempted using all these methods.

The bioassay-guided fractionation and chromatographic separation studies of KK leaf was conducted with leaf samples collected from 3 different seasonal periods to observe the seasonal effects (if any) on its PSM and subsequent nematocidal efficacies. The nematocidal efficacies of its various crude solvent fractions and their separated fractions were evaluated with batches of nematode larvae having different larval populations. It was hypothesised that the efficacy of KK leaf samples would not be affected by variations of the larval population (**Hypothesis 7.1**). There was a wide selection of different batches of larvae from four different sources, all of which were opportunistic samples as mentioned previously in Section 2.4.1. The L3 larvae stored inside a lab environment will usually lose their viability after 2-3 months from their culture date (Zajac and Garza, 2020, Choudhury and Cole, 2019). Hence, it was not possible to conduct all the nematocidal analyses of the KK samples and fractions with a single batch of larvae as this study extended over a period of 2 years. However, a nematocidal experiment with the most effective solvent fractions of two KK leaf samples from two different seasonal periods were conducted with a single batch of larvae to compare the efficacy with each other. It was hypothesised that the nematocidal efficacy would be irrespective of the seasonal variation and the effective fractions from two different seasons will have similar efficacy (**Hypothesis 7.2**).

The chromatographic separation of the KK-leaf solvent fractions was performed using the NP-FC, Sephadex LH-20 and RP-FC separation techniques. This was performed with the 3 KK leaf samples that were collected over three different seasonal periods to find out if there was a consistency to the separation pattern. For the separation using RP-FC, the flow rate of the eluent (solvent gradient of water and MeOH) was set to variable for a Water Sub-fraction separation. It was hypothesised that if the flow rate was variable and if it was gradually increased, a better separation of the high-polar water-soluble components present in the fraction would likely to take place (**Hypothesis 7.3**). The chromatographic separation of a

parent solvent fraction will produce a group of separated sub-fractions. It was hypothesised that the nematocidal efficacy of a few of these sub-fractions would be higher than the parent fraction (**Hypothesis 7.4**). This hypothesis was similar to the previous hypotheses described in Chapter 3 and 5.

The Nematocidal Larval Assays (NLA**)** tests were performed with several batches of larvae which had mixed species in their larval population. This was carried out to determine the susceptible species of larvae that were affected by the KK-leaf fractions and the standard anthelmintics. Additionally, some dose response tests of the crude solvent fraction and their separated sub-fractions were conducted to determine both the dose required to kill 50% of the larvae and their optimal nematocidal concentration i.e., the concentration at which a test sample had the highest efficacy against a larval population. Dose response tests have been frequently reported in the literature for the assessment of anthelmintic efficacy of plant fractions (Sarkar et al., 2019, Davuluri et al., 2020, Acevedo-Ramírez et al., 2019). It was hypothesised that a separated fraction from a crude solvent fraction will reach its maximum efficacy at a lower concentration than the optimal concentration of its parent crude fraction (**Hypothesis 7.5**).

Moreover, a series of novel formulations were created comprising a combination of the effective KK-leaf fractions and standard reference anthelmintics (BZ, AB, and IVM). Many of these larval batches were obtained from nematodes that were resistant to some of the standard anthelmintics and hence were not being killed at usual dose concentrations. A series of NLA tests were performed with these combination formulations. It was hypothesised that the combination of effective KK-leaf fractions with the standard anthelmintic will increase the efficacy of the latter (**Hypothesis 7.6**), especially where anthelmintic resistance was present in this population to one or more of the standard anthelmintics. This approach of using formulations made with a plant fraction and the standard anthelmintic has not yet been reported in the literature. This particular approach could provide an answer to the anthelmintic resistance issue if the formulations were found to have better efficacy than the reference anthelmintic, as previously discussed in Chapter 1. Thus, this chapter extensively presents the bioassay-guided separation study of the KK leaf fraction and evaluates its potential for usage as an anthelmintic.

7.2. Materials and Methods

7.2.1. Sampling, Drying and Grinding of the Kawakawa Leaf

Fresh KK leaves were plucked from the shrubs growing in Bledisloe Park which are the same source plants as for the previous collection of KK fruit. The leaf samples were collected over four periods, covering three seasons. This facilitated a comparison of phytochemical changes (if any) and their nematocidal activity. The leaves were gently plucked without doing any damage to the tree, thus allowing new leaves to flourish. After plucking, the stems were discarded, and the leaves were subjected to drying and grinding to obtain fine KK leaf powder following the method described in Section 2.3.3. The collection date of the leaf sample over the four different periods is presented in Table 7.1.

Kawakawa Leaf Sample	***Collection date***	***Season***
KK-Dec19	02 December 2019	Summer
KK-May20	31 May 2020	Winter
KK-Oct20	05 October 2020	Spring
KK-Dec20	18 December 2020	Summer

Table 7.1. Kawakawa leaf samples from different seasons and their collection dates.

7.2.2. Soxhlet Extraction of the Dried Kawakawa Leaf Components

The crushed fine powder of the KK leaf sample was subjected to Soxhlet extraction with the technique described in Section 2.3.5. The method was the same for all four KK leaf samples studied.

7.2.3. Solvent Partitions Using Liquid-Liquid Partition

The parent Water-MeOH, Hexane, and EtOAc Fractions were obtained with the four different KK leaf samples following the method presented in Section 2.3.6. The dry matter yield of each solvent fraction obtained from the KK-Oct20 and KK-Dec20 leaf samples were recorded quantitatively. The dry matter yield of solvent fractions obtained from the KK-May20 and KK-Dec19 samples were assessed qualitatively. The dried solvent fractions were stored in a freeze-drier until required following the method described in Section 2.3.4.

7.2.4. Analysis of Solvent Fractions Obtained from Kawakawa Leaf Samples

The solvent fractions obtained from the four samples of KK leaf were subjected to a series of chemical and nematocidal analyses presented below in Table 7.2.

Solvent fractions of sample	***Analyses performed***
KK-Dec19-leaf	Nematocidal analysis of the solvent fractions.
	Nematocidal analysis of combination formulations made with the effective solvent fractions and the standard anthelmintics.
KK-May20-leaf	Nematocidal analysis of the solvent fractions.
	Dose response NLA test of the most effective solvent fractions.
	Chromatographic separation of the solvent fractions.
	Nematocidal analysis of the separated fractions.
	Dose response NLA test of the most effective fractions.
KK-Oct20-leaf	Nematocidal analysis of the solvent fraction.
	Chromatographic separation of the effective crude solvent fractions.
	Nematocidal analysis of the separated fractions.
	Further separation of a separated fraction obtained from the crude solvent fraction.
KK-Dec20-leaf	Nematocidal analysis of the solvent fractions.
	Chromatographic separation of the effective crude solvent fractions.
	Nematocidal analysis of the separated fractions.
	Further separation of a separated fraction.
	Further separation of separated sub-fraction.

Table 7.2. The series of analyses performed with solvent fractions from each Kawakawa leaf sample.

In the next two sections, the methodology of the chromatographic separation and nematocidal analysis are presented.

7.2.4.1. Chromatographic Separation of Different Kawakawa Leaf Samples

In the following table, a summary of the methodology of the separation study for the different KK samples are presented. Note that in the results section of this chapter, a summary of the chromatographic separation will be provided. For a detailed version of the results for the separation of a specific fraction, refer to the individual Appendix as presented below in Table 7.3.

Kawakawa sample	***Chromatographic separation performed***	***Methodology described in***	***Result presented in***
KK-May20-leaf-Hexane	NP-FC	2.3.9.1	Appendix 7.1.1
KK-May20-leaf-EtOAc			Appendix 7.1.2
KK-May20-leaf-Water	Sephadex LH-20	2.3.10	-
	RP-FC	2.3.9.2	Appendix 7.1.3
KK-May20-leaf-MeOH	RP-FC		Appendix 7.1.4
KK-Oct20-leaf-Water			Appendix 7.1.5
KK-Oct20-leaf-MeOH			Appendix 7.1.6
KK-Oct20-leaf-MeOH-Fr-1			Appendix 7.1.7
KK-Oct20-leaf-MeOH-Fr-1-2			Appendix 7.1.8
KK-Dec20-leaf-MeOH (RUN 1-3)			Appendix 7.1.9
KK-Dec20-leaf-Water (RUN 1-2)			Appendix 7.1.10
KK-Dec20-leaf-Water-Fr-1			Appendix 7.1.11
KK-Dec20-leaf-Water-Fr-1-1			Appendix 7.1.12

Table 7.3. Depiction of the presentation of the methodology and separation study of different Kawakawa samples.

7.2.4.2. Nematocidal analysis of the Different Kawakawa Leaf Solvent Fractions

The different KK solvent fractions and their separated fractions were subjected to nematocidal analysis following the NLA test method presented in Section 2.4.3. The nematocidal experiments were done in triplicate (n=3, T=1-3) for each test sample. The standard anthelmintics: BZ, AB and IVM were used (Source: *Sigma-Aldrich New Zealand Company, Auckland, NZ)* as the positive controls at their respective concentration (3 mg/mL, 1 mg/mL and 1 mg/mL respectively). Medium for all the KK sample and standards was PBS. In some experiments the larvae which died were individually identified following the method described in Section 2.4.5 but only with one repetition of the sample group. This provides some indication as to whether there is some differential activity against different genera of nematodes. In Table 2.4, a summary of the nematocidal analyses is presented with the respective experiment number for each analysis and the dead larval species identification experiments performed with the group of samples for each experiment.

Leaf sample	*Nematocidal analysis*	*Experiment number*	*Batch of larvae taken*	*Dead larval identification performed with*
KK-Dec19	Nematocidal analysis of the crude solvent Fractions	Experiment 7.1	Batch 6	-
		Experiment 7.2	Batch 7	-
		Experiment 7.3	Batch 8	-
	Nematocidal analysis of the combination formulations made with the effective solvent Fractions and the standard anthelmintics.	Experiment 7.4	Batch 6	-
		Experiment 7.5	Batch 7	-
		Experiment 7.6	Batch 8	-
	Nematocidal analysis of the combination formulations made with the effective solvent Fractions and the Quebracho Water Fraction.	Experiment 7.7	Batch 6	-
		Experiment 7.8	Batch 7	-
		Experiment 7.9	Batch 8	-
KK-May20	Nematocidal analysis of the crude solvent Fractions.	Experiment 7.10	Batch 9	KK-May20-leaf-Water-MeOH (T1)
				KK-May20-leaf-Water (T1)
				KK-May20-leaf-MeOH (T1)
				AB (T1)
	Dose response test of the Water Fraction	Experiment	Batch 9	-
	Dose response test of the MeOH Fraction	Experiment 7.12	Batch 9	-
	Nematocidal analysis of the separated fractions of the Hexane Fraction	Experiment 7.13	Batch 9	-
	Nematocidal analysis of the separated fractions of the EtOAc Fraction	Experiment 7.14	Batch 9	-

(Contd. on next page)

Sample	*Nematocidal analysis*	*Experiment number*	*Batch of larvae taken*	*Dead larval identification performed with*
KK-May20	Nematocidal analysis of the separated fractions of the Water Fraction.	Experiment 7.15	Batch 9	-
	Dose Response NLA Test of KK-May20-leaf-Water Fraction 3	Experiment 7.16	Batch 9	KK-May20-leaf-Water-Fr 3 (T1)
	Nematocidal analysis of the separated fractions of the MeOH Fraction	Experiment 7.17	Batch 10	KK-May20-leaf-MeOH-Fr 2 (T2)
KK-Oct20	Nematocidal analysis of the crude solvent Fractions	Experiment 7.18	Batch 11	KK-May20-leaf-Water-MeOH (T1)
				KK-May20-leaf-Water (T1)
				KK-May20-leaf-MeOH (T1)
				IVM (T3)
	Nematocidal analysis of the separated fractions of the MeOH Fraction	Experiment 7.19	Batch 11	KK-Oct20-leaf-MeOH-Fr 4 (T2)
	Nematocidal analysis of the separated fractions of the Water Fraction	Experiment 7.20	Batch 11	KK-Oct20-leaf-Water-Fr 1 (T1-3)
KK-Dec20	Nematocidal analysis of the crude solvent Fractions	Experiment 7.21	Batch 12	KK-Dec20-leaf-Water-MeOH (T1)
				KK-Dec20-leaf-Water (T3)
				IVM (T3)
	Nematocidal analysis of the separated fractions of the MeOH Fraction	Experiment 7.22	Batch 13	-
	Nematocidal analysis of the separated fractions of the Water Fraction	Experiment 7.23	Batch 13	-

Table 7.4. Nematocidal analysis performed with the different Kawakawa samples and their respective experiment numbers.

7.2.4.2.1. Comparative Nematocidal Analysis of Various MeOH and Water Fractions Obtained from Different Seasonal Periods

The nematocidal analyses of the Water and MeOH Fractions of KK-Oct20 and KK-Dec20 were performed with Batch 12 larvae. Concentration of each sample was 8 mg/mL in PBS. BZ, AB and IVM were used as positive control standards at their usual concentrations. PBS was used as the negative control group. The experiments were performed in triplicate (n=3, T=1-3) following the usual NLA method. These studies are detailed as Experiment 7.24.

7.2.4.2.2. Combination Strategy: Combining KK-Dec19 Leaf Solvent Fractions with Reference Anthelmintics

The reference anthelmintic and the effective KK solvent fractions were mixed in the cell of 48-well plate at 1:1 at their respective and NLA tests were performed with 3 batches of larvae, Batch 6-8. The experiments were performed in triplicate (n=3, T=1-3). PBS was used as the negative control group. In Table 7.5, the series of combination formulations made with their abbreviations, and the batches of larvae that were selected for the respective NLA tests are presented. This series of experiments are detailed as Experiment 7.4 for Batch 6, Experiment 7.5 for Batch 7, and Experiment 7.6 for Batch 8 larvae.

Abbreviation	***ID of combination formulation***	***NLA tests performed with***
BZ+AB	Benzimidazole + Abamectin	Batch 6,7,8
KK-W+BZ	KK-Dec19-leaf-Water Fraction + BZ	Batch 6,7,8
KK-W+AB	KK-Dec19-leaf-Water Fraction + AB	Batch 6,7
KK-W+IVM	KK-Dec19-leaf-Water Fraction + IVM	Batch 6,7,8
KK-Me+BZ	KK-Dec19-leaf-MeOH Fraction + BZ	Batch 6,7,8
KK-Me+AB	KK-Dec19-leaf-MeOH Fraction + AB	Batch 6,7
KK-Me+IVM	KK-Dec19-leaf-MeOH Fraction + IVM	Batch 6,7,8

Table 7.5. Abbreviations of the combination formulations and NLA performed with the Batches of larvae.

The KK-W and KK-Me Fractions were also mixed with QWF in 1:1 at 8 mg/mL concentration. NLA tests were carried out with Batch 6 and 7 larvae and performed in triplicate (n=3, T=1-3) for each combination formulations. This series of experiments are

detailed as Experiment 7.7 for Batch 6, Experiment 7.8 for Batch 7 larvae, and Experiment 7.9 for Batch 8 larvae.

7.2.4.2.3. Calculations and Statistics of NLA Tests

The nematocidal efficacy of each test sample was specified as % larval mortality using the equation described in Section 2.4.3.1. The efficacy of each sample was compared with one another using an ANOVA and post-hoc Tukey's test. For the dose response rests, methodology described in Section 2.4.4.1 was followed.

7.2.5. LC-MS Analysis of KK Leaf Samples

The LC-MS analysis of the MeOH and Water Fractions of KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 samples were performed to compare the phytochemical tracing of each sample with one another. The LC-MS analysis of the most effective separated fractions of MeOH and Water Fractions from KK-May20, KK-Oct20, and KK-Dec20 were performed using the C18 column (Thermofisher Hypersil Gold 100x2.1 (mm) 1.9 µm (THC25002-102130)) and the HILIC column (Thermofisher Hypersil GOLD 100x2.1 (mm) 1.9 µm HILIC (THC26502-102130). The methodology described in Section 2.3.11 was followed. The concentration of each test sample was 1 mg/mL in 20% MeOH in water. Note that in this chapter, the identification of the effective compounds and structure prediction are not presented, refer to Chapter 10 for an in-depth LC-MS/MS analysis towards the identification of a few active compounds.

7.3. Results

7.3.1. Dry Matter Yield of Kawakawa Leaf Crude Solvent Fractions

The leaf samples of KK-Dec19, KK-May20, KK-Oct20, and KK-Dec20 were subjected to Soxhlet extraction and liquid-liquid solvent partitions. Initially 3 crude solvent-fractions were obtained. The Water-MeOH Fraction was further separated into Water and MeOH Fractions. The schematic diagram of the KK-Oct20 leaf extraction with dry matter yields of each fraction is presented in Figure 7.1 and of the KK-Dec20 is presented in Figure 7.2. The dry matter yields of solvent fractions obtained from KK-May20 and KK-Dec19 were assessed qualitatively. The dry matter yields of each fraction from these two leaf samples were qualitatively found to be comparable with KK-Dec20 and KK-Oct20 samples.

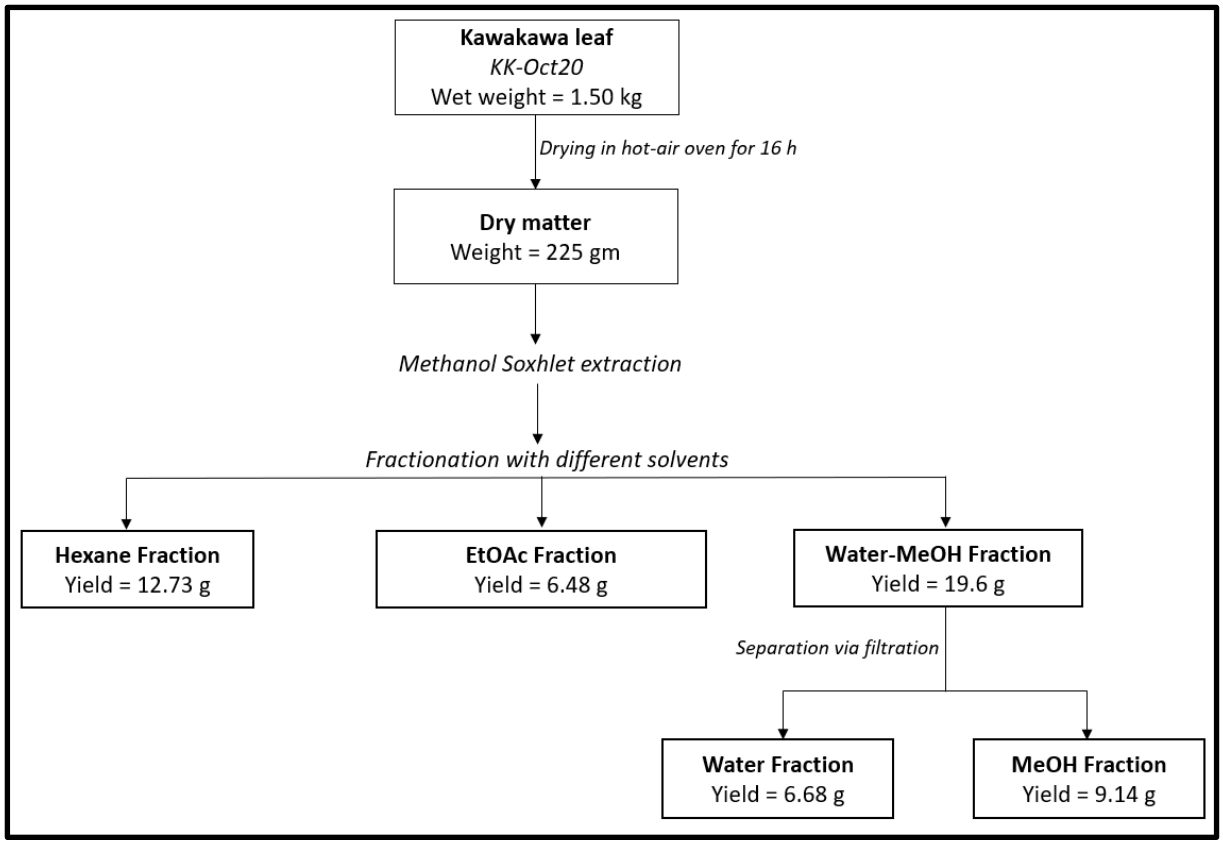


Figure 7.1. Schematic diagram of the KK-Oct20 leaf extraction and step fractionation with different solvents and the dry matter yield of each fraction.

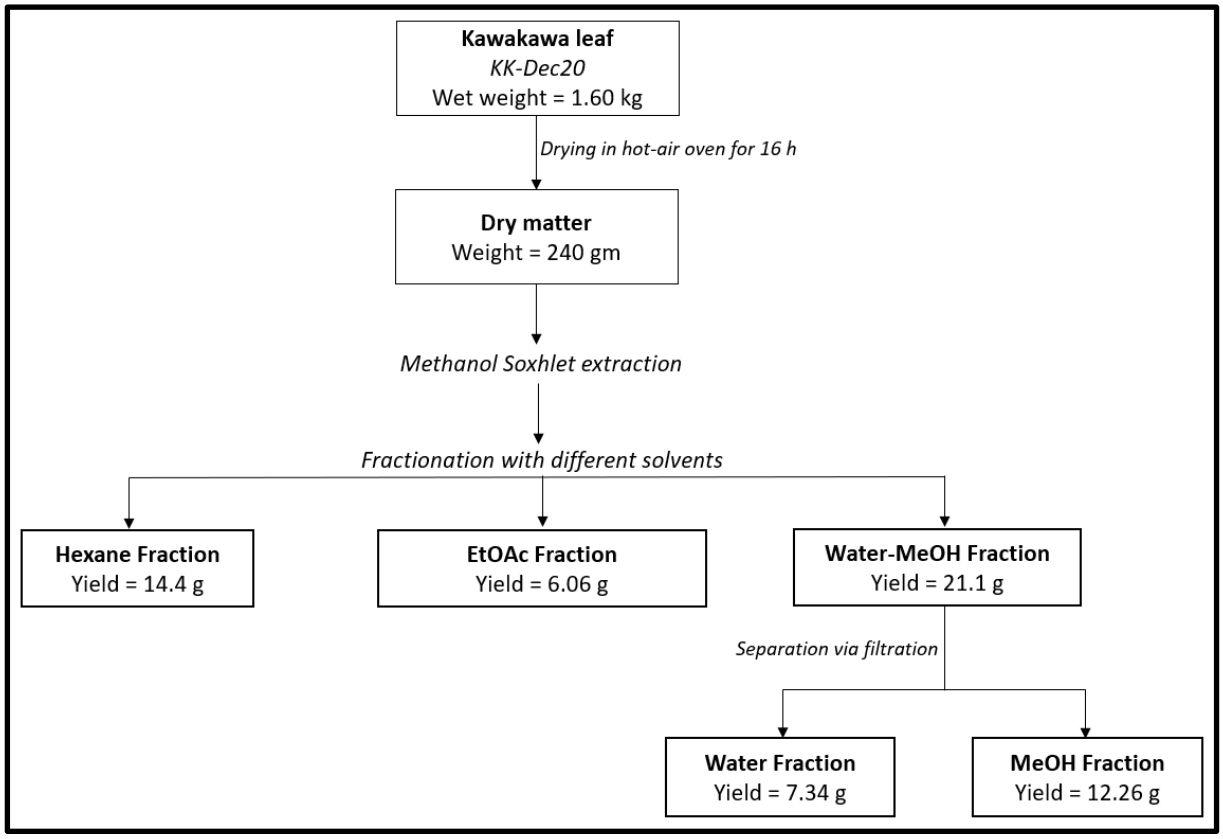


Figure 7.2. Schematic diagram of the KK-Dec20 leaf extraction and step fractionation with different solvents and the dry matter yield of each fraction.

It was observed that unlike the solvent fractions obtained from the KK fruit component (results presented in Section 5.3.1), which had a higher quantity of low-polar components than high polar components, the KK leaf consisted of more high-polar components than low polar components. The dry matter yield of the Water-MeOH Fraction of KK-Oct20 leaf sample was 19.6 g, whereas yield of the Hexane Fraction was found to be 12.73 g and the EtOAc Fraction was 6.48 g. Upon further separation of the Water-MeOH Fraction, the dry matter yield of the Water Fraction was 6.68 g, and the MeOH Fraction was 9.14 g. This indicated that the highly polar components that were present in Water-MeOH Fraction, were more soluble in MeOH than Water. A similar pattern of yields was recorded with the KK-Dec20 leaf sample.

Therefore, it was found that the KK leaf component had a considerable amount of highly polar components that were more soluble in MeOH than water.

7.3.2. Results of the Nematocidal Analysis of the Kawakawa Leaf Crude Solvent Fractions

A summary of the results of nematocidal experiments of crude solvent Fractions of KK-leaf sample from four different periods and the positive control standards are presented in Table 7.6. In the following sections, the results of each leaf sample are presented. Note that only a summary of the nematocidal analysis is provided. For the detailed version of the results for a specific nematocidal experiment, refer to **Appendix 7.2**.

7.3.2.1. Nematocidal Efficacy of the Crude Solvent Fractions of KK-Dec19-leaf Sample against Three Batches of larvae and Comparison with the Standard Anthelmintics

It was found that with both Batch 6 and 7 larvae (Source: *ARGL*), the Water-MeOH Fraction had the highest nematocidal efficacy of all the crude solvent Fractions ($P<0.05$). Upon further separation of the Water-MeOH to Water and MeOH Fractions, the efficacy of the separated Water and MeOH Fractions were lower than the parent Water-MeOH Fraction ($P<0.05$) with Batch 6 larvae. However, with Batch 7 larvae, the Water Fraction had a much higher efficacy than the parent Water-MeOH Fraction ($P<0.05$), but the efficacy of the MeOH Fraction was found to be lower than the parent fraction ($P<0.05$). The efficacy of the Water Fraction with Batch 7 was slightly lower than IVM ($P<0.05$) but the difference in efficacy was not significant when compared to AB ($P>0.05$, NS).

The result with mixed larval population of Batch 8 was observed to be different. It was found that the efficacy of the Water-MeOH Fraction, the Water Fraction and the MeOH were comparable and similar when compared to one another ($P>0.05$, NS). Interestingly, the efficacy of the Water-MeOH, Water and MeOH Fractions were higher than both BZ and AB ($P>0.05$).

KK leaf sample	NLA tests with	Nematocidal efficacy (Mean of % larval mortality ± SEM)								
		Hexane Fraction	EtOAc Fraction	Water-MeOH Fraction	Water Fraction	MeOH Fraction	BZ	AB	IVM	PBS
KK-Dec19	Batch 6	9.0 ± 0.5^{g}	14.3 ± 0.3^{f}	36.3 ± 1.4^{b}	9.7 ± 0.3^{g}	17.7 ± 0.3^{e}	22.0 ± 0.5^{d}	26.3 ± 0.3^{c}	87.3 ± 0.6^{a}	0
	Batch 7	11.3 ± 0.3^{g}	17.0 ± 0.5^{f}	59.3 ± 0.3^{c}	81.7 ± 0.3^{b}	41.0 ± 0.5^{d}	36.3 ± 0.3^{e}	80.3 ± 0.3^{b}	88.0 ± 0^{a}	0
	Batch 8	-	-	69.3 ± 0.3^{a,b}	64.0 ± 1.0^{b}	67.7 ± 0.8^{b}	41.0 ± 3.2^{d}	54.0 ± 0.5^{c}	75.5 ± 3.5^{a}	0
KK-May20	Batch 9	1.0 ± 1.0^{e}	2.3 ± 2.0^{e}	32.3 ± 0.5^{d}	77.6 ± 2.0^{a}	72.3 ± 1.5^{b}	3.0 ± 2.6^{e}	77.6 ± 2.5^{a}	56.3 ± 1.5^{c}	0
KK-Oct20	Batch 11	0.7 ± 0.3^{e}	3.3 ± 0.8^{e}	18.0 ± 1.1^{c}	33.7 ± 1.7^{b}	40.3 ± 1.4^{a}	0	-	11.7 ± 1.2^{d}	0
KK-Dec20	Batch 12	2.3 ± 0.3^{e}	4.3 ± 0.6^{d,e}	12.7 ± 0.3^{d}	85.3 ± 1.6^{a}	45.0 ± 1.7^{b}	28.3 ± 4.9^{c}	26.7 ± 0.6^{c}	33.7 ± 0.3^{c}	0

Table 7.6. Efficacy (Mean of % larval mortality) of KK leaf crude solvent Fractions and controls with SEM=Standard Error of Mean. Superscript (a, b, c) within each row represents statistical significance difference from Tukey's test (P<0.05). Efficacy values in red represent the most effective KK fraction for that batch of larvae.

7.3.2.2. Nematocidal Efficacy of the Crude Solvent Fractions of KK-May20-leaf Sample and Comparison with the Standard Anthelmintics

It was found that the efficacy of the Water-MeOH Fraction was considerably higher than the Hexane and EtOAc Fractions ($P<0.05$) against Batch 9 larvae (Source: *SA* farm). The efficacy of Water Fraction was found to be comparable with AB ($P>0.05$, NS) and higher than the efficacy of IVM and BZ ($P<0.05$). The efficacy of MeOH Fraction was slightly lower than AB ($P<0.05$) but marginally higher than both IVM and BZ ($P<0.05$). Therefore, this result clearly indicated that the further separation of the KK-May20 leaf Water-MeOH into Water and MeOH Fractions, drastically improved the efficacy against this batch of larvae. From the dead larval species identifications (result presented in **Appendix 7.2.10 under Experiment 7.10**), it was found that the Water-MeOH Fraction mostly affected the LT, and *Trichostrongylus* spp. present in the larval population of Batch 9. However, the majority of the *H. contortus* present in the larval population survived. With the Water Fraction, it was found to kill all the LT, *Trichostrongylus* spp. and *T. circumcincta* but not the *H. contortus* species present in the larval population. A similar observation was recorded with the MeOH Fraction. AB was somewhat effective against the *H. contortus* present in the population but was not able to kill all of them.

7.3.2.3. Nematocidal Efficacy of the Crude Solvent Fractions of KK-Oct20-leaf Sample and Comparison with the Standard Anthelmintics

It was found that the Hexane Fraction did not have any effect on the larvae present in the population of Batch 11 (Source: *SA* farm) and the EtOAc Fraction had a very little efficacy. The Water-MeOH Fraction was more effective than both of these fractions ($P<0.05$). Additionally, both the Water and MeOH Fractions were found to be more effective than the parent Water-MeOH Fraction ($P<0.05$). The MeOH Fraction had slightly higher efficacy than the Water Fraction ($P<0.05$). The efficacy of IVM with this batch of larvae was found to be lower than all the Water and MeOH Fractions ($P<0.05$). BZ was completely ineffective against this batch of larvae. From the dead larval species identifications (result presented in **Appendix 7.2.18 under Experiment 7.18**), it was found that IVM only targeted the LT species present in the larval population of Batch 11. A similar observation was found with the Water-MeOH Fraction, *H. contortus* species present in the population were unaffected. However, the separated MeOH and Water Fractions affected a few of the *H. contortus* present in the population.

7.3.2.4. Nematocidal Efficacy of the Crude Solvent Fractions of KK-Dec20-leaf Sample and Comparison with the Standard Anthelmintics

It was found that the Water-MeOH Fraction of KK-Dec20 sample was more effective than the Hexane Fraction ($P<0.05$) against Batch 12 larvae (Source: *AR* farm). Upon further separation of the Water-MeOH, the separated Water Fraction had a significant increase from the efficacy found with the parent Water-MeOH Fraction ($P<0.05$). The MeOH Fraction had higher efficacy than the parent fraction ($P<0.05$) but lower than the Water Fraction ($P<0.05$). The efficacies of both the Water and MeOH Fractions were higher than all of the standard anthelmintics, BZ, AB and IVM ($P<0.05$). From the dead larval species identifications (result presented in **Appendix 7.2.21 under Experiment 7.21**). It was found that with the Water-MeOH Fraction, the majority of the *H. contortus* present in the population were unaffected. It was found that with the Water Fraction, the majority of the *Cooperia* spp. present in the larval population were dead. The total mortality count of *H. contortus* with this fraction was higher than the parent Water-MeOH fraction.

Therefore, from the above presented results of 4 groups of KK-leaf-sample, the Water and MeOH Fractions were found to be the most effective solvent fractions. Hence, further chromatographic separation was conducted with these two fractions with each group of KK-leaf samples (except KK-Dec19) in order to obtain separated fractions and evaluating the nematocidal efficacies thereof. Separation of the Hexane and EtOAc Fractions of KK-May20 samples was also conducted.

7.3.3. Summary of the NP-FC and RP-FC Separation Studies of the Various KK-leaf Fractions

The KK-May20-leaf-Hexane and KK-May20-EtOAc crude solvent Fractions were subjected to NP-FC separation. The results are described in detail in **Appendix 7.1.1** and **7.1.2** respectively. Initially, a total of 40 fractions were obtained from the NP-FC run of Hexane Fraction. These fractions were mixed with one another based on their TLC profiling to obtain 12 combined fractions. LC-MS analysis of these 12 samples resulted in 10 final fractions. With the EtOAc Fraction, initially 62 fractions were obtained. These sub-fractions were mixed with one another based on their TLC profiling to obtain 14 combined sub-fractions. LC-MS analysis of these 14 fractions resulted in 10 final fractions.

The separation of the KK-May20-leaf-Water Fraction was first attempted with a Sephadex LH-20 column chromatography which was found to be unsuccessful. It was found that throughout the process of the elution with the gradient of water and MeOH, the sample presented in column did not travel through the analyte unlike the QWF fraction with which the separation was found to be successful. The separation was successful with RP-FC separation. Initially, 75 fractions were obtained from the flash separation run (note that the accumulation of the flash chromatogram from this run was not possible due to a software failure). The fractions obtained from the RP-FC run were combined based on the peak areas in the flash chromatogram after the TLC analysis of the fractions found to be unsuccessful (see discussion). The combination work was performed following the hypotheses approach presented in Section 2.3.9.1. Firstly, the fractions were mixed with one another to obtain 10 initial combined fractions. LC-MS analysis was performed with these 10 samples to obtain 7 final fractions and the dry matter yield of each fraction was recorded. Separation of all the other MeOH and Water Fractions of various KK-leaf samples was achieved following the same technique.

The RP-FC separation of the various MeOH and Water Fractions was performed using two types of columns, *SEPACORE RP C18 4 g* column which was equivalent with the *TELOS RP C18 4 g* column, and a *TELOS RP C18 23 g* column. The summary of the results of the RP-FC separation of various KK-leaf-Water Fractions are presented in Table 7.8. The summary of the result of the RP-FC separation of various KK-leaf-Water Fractions is presented in Table 7.9.

Sample	***Sample quantity for the RP-FC study***	***Column used for the RP-FC study***	***Number of final separated fractions obtained***	***Fraction with the highest yield***	***Fraction with the second highest yield***
KK-May20-leaf-Water	800 mg	SEPACORE 4 g	7	Fraction 2; 336 mg	Fraction 1; 96 mg
KK-Oct20-leaf-Water	1130 mg	SEPACORE 4 g	6	Fraction 1; 902 mg	Fraction 2; 67 mg
KK-Dec20-leaf-Water (RUN 1)	817 mg	TELOS 4 g	6	Fraction 1; 596 mg	Fraction 2; 8 mg
KK-Dec20-leaf-Water (RUN 2)	928 mg	TELOS 4 g	5	Fraction 1; 718 mg	Fraction 2; 8 mg

Table 7.8. Summary of the results of RP-FC separation of the different KK-leaf-Water Fractions.

Sample	***Sample quantity for the RP-FC study***	***Column used for the RP-FC study***	***Number of final separated fractions obtained***	***Fraction with the highest yield***	***Fraction with the second highest yield***
KK-May20-leaf-MeOH	800 mg	SEPACORE 4 g	8	Fraction 1; 215 mg	Fraction 4; 51 mg
KK-Oct20-leaf-MeOH	1300 mg	SEPACORE 4 g	8	Fraction 1; 597 mg	Fraction 2; 78 mg
KK-Dec20-leaf-MeOH (RUN 1)	3250 mg	TELOS 23 g	9	Fraction 1; 1478 mg	Fraction 2; 265 mg
KK-Dec20-leaf-MeOH (RUN 2)	1015 mg	TELOS 4 g	8	Fraction 1; 648 mg	Fraction 4; 57 mg
KK-Dec20-leaf-MeOH (RUN 3)	928 mg	TELOS 4 g	8	Fraction 1; 602 mg	Fraction 5; 56 mg

Table 7.9. Summary of the results of RP-FC separation of the different KK-leaf-MeOH Fractions.

For most of the Water Fractions, the Fraction-1 contained >90% of the sample, as found from the dry matter yield of various fractions. This was only found to be different with the May20-Water Fraction where Fraction-2 was found to have the highest dry matter yield. Interestingly, there were occurrences of more peaks for the KK-Oct20-Water than KK-Dec20-Water in their respective flash chromatograms (see **Appendix 7.1.5** and **Appendix 7.1.10** respectively).

The separation of the MeOH Fractions of KK-Dec20, KK-Oct20 and KK-May20 were performed using a *TELOS 4 g*, *TELOS 23 g* and a *SEPARACORE 4 g* columns. Interestingly, the tall peak that was present for the Oct20 MeOH Fraction representing the Fraction 2, it was absent in the chromatogram of the Dec20 MeOH Fraction. The number of final fractions and LC-MS analysis of these fractions indicates that these fractions were identical to one another. This is presented in Section 7.3.9.

7.3.4. Dose Response NLA Tests of the Water and MeOH Solvent Fractions

The dose response curve (% larval mortality v drug concentration (mg/mL)) of the various concentration of the KK-May20-leaf-Water and KK-Ma20-leaf-MeOH Fractions with Batch 9 larvae are presented in Figure 7.3 and 7.4 respectively.

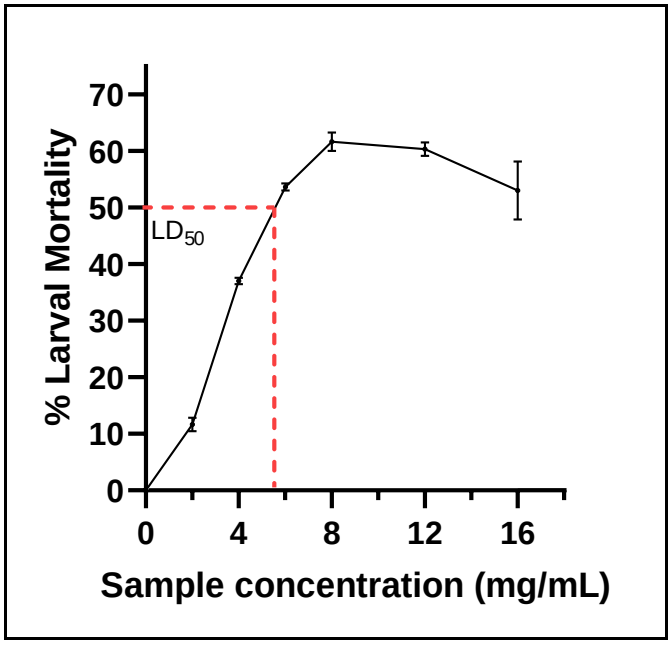


Figure 7.3. Dose response curve of the KK-May20-leaf-Water-Fraction with Batch 9 larvae; Red dotted lines on the x and y axis represents the LC_{50} concentration.

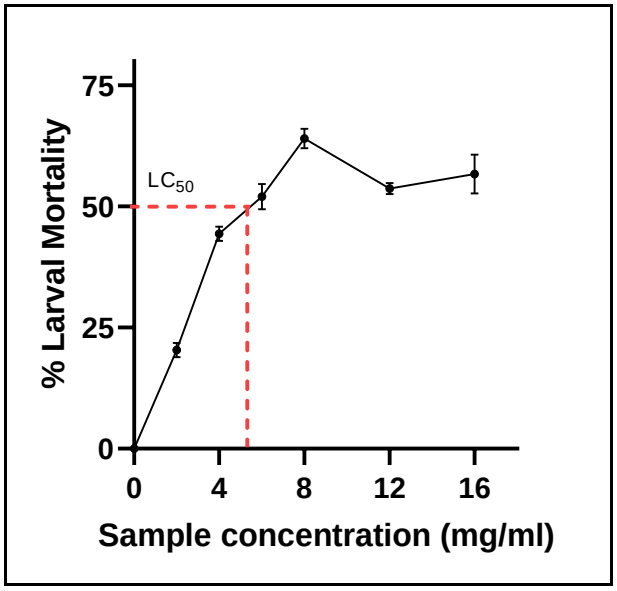


Figure 7.4. Dose response curve of the KK-May20-leaf-MeOH-Fraction with Batch 9 larvae; Red dotted lines on the x and y axis represents the LC_{50} concentration.

It was found that the efficacy of the KK-May20-leaf-Water Fraction at 6 mg/mL was higher than 4 mg/mL and 2 mg/mL concentration (P<0.05) (full result presented in **Appendix 7.2.11 under Experiment 7.11**). However, the difference in efficacy of 6 mg/mL and 8 mg/mL concentration was not significant (P=0.2), the P value was quite low from the Tukey's test. The efficacy of the Water Fraction did not increase beyond the 8 mg/mL concentration. LC_{50} was achieved at 6 mg/mL concentration. Based on this observation, 8 mg/mL was found to be the optimal concentration for Water Fraction.

It was found that the efficacy of KK-May20-leaf-MeOH Fraction at 8 mg/mL was higher than that the MeOH Fraction at 6 mg/mL, 4 mg/mL and 2 mg/mL concentration (P<0.05) (full result presented in **Appendix 7.2.12 under Experiment 7.12**). LC_{50} was achieved at 6 mg/mL. It was found that beyond 8 mg/mL, the efficacy did not increase. Hence, 8 mg/mL was found to be the optimal concentration for the MeOH Fraction.

Based on the result of Experiment 7.11 and 7.112, concentrations of the Water and MeOH Fractions (and the other crude solvent Fractions) for the other leaf samples were chosen to 8 mg/mL for their respective NLA tests.

7.3.5. Dose Response NLA Test of KK-May20-leaf-Water-Fraction-3

The dose response curve (% larval mortality v drug concentration (mg/mL)) of the various concentration of KK-May20-leaf-Water-Fr-3 with Batch 9 larvae is presented in Figure 7.5.

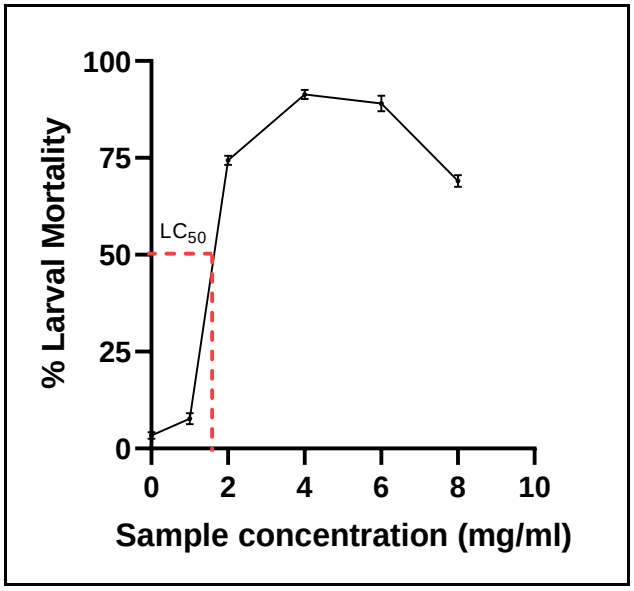


Figure 7.5. *Dose response curve of the KK-May20-leaf-Water-Fraction-3 with Batch 9 larvae; Red dotted lines on the x and y axis represents the LC_{50} concentration.*

It was found that at 4 mg/mL, the efficacy of the sample was significantly higher than the parent crude Water Fraction ($P<0.05$) against this batch of larvae (full result presented in Appendix 7.2.17 under Experiment 7.17). At 6 mg/mL, the difference in efficacy at 4 mg/mL was not significant ($P>0.05$). However, the efficacy of this sample at both 6 mg/mL and 4 mg/mL was higher than the efficacy at 8 mg/mL ($P<0.05$). This indicated that the KK-May20-leaf-Water-Fr-3 was more effective at 4 mg/mL than 8 mg/mL. Hence, the concentration of all the MeOH and Water separated fractions was chosen to be 4 mg/mL for the NLA tests as this concentration was found to be the optimal.

7.3.6. Summary of the Results of the Nematocidal Efficacies of the Most Effective Separated Fractions from MeOH and Water Solvent Fractions

In the Table 7.10, the most effective fractions from the 6 different KK leaf crude solvent Fractions with their efficacies are presented.

Solvent Fraction	*NLA performed with*	*Efficacy of the parent fraction (8 mg/mL)*	*Most effective fraction*	*Efficacy of the most effective fraction (4 mg/mL)*
KK-May20-leaf-MeOH	Batch 10	32.3 ± 0.8%[a]	Fraction 2	91.3 ± 1.7%[b]
KK-May20-leaf-Water	Batch 9	77.6 ± 2.0%[a]	Fraction 3	91.3 ± 1.2%[b]
KK-Oct20-leaf-MeOH	Batch 11	40.3 ± 1.4%[a]	Fraction 4	75.3 ± 2.6%[b]
KK-Oct20-leaf-Water	Batch 11	33.7 ± 1.7%[a]	Fraction 1	75.3 ± 5.4%[b]
KK-Dec20-leaf-MeOH	Batch 13	25.3 ± 1.4%[a]	Fraction 4	43.3 ± 3.2%[b]
KK-Dec20-leaf-Water	Batch 13	44.3 ± 3.2%[a]	Fraction 2	39.3 ± 4.8%[a]

Table 7.10. Efficacies of the parent and the most effective fractions of the various KK leaf solvent Fractions. Superscript (a, b, c) within each row represents statistical significance difference from Tukey's test (P<0.05).

Therefore, it was found that with the crude Water and MeOH Fractions, further separation of each resulted in fractions with higher nematocidal efficacy when studied against the same batch of larvae, except KK-Dec20-leaf-Water Fraction in which the efficacies of the parent fraction and its most effective was found to be comparable (P>0.05, NS).

7.3.7. Comparative Nematocidal Analysis of the Various KK-leaf MeOH and Water Fractions Obtained from Different Seasonal Periods

NLA tests of the KK-Oct20-leaf-Water-MeOH, KK-Dec20-leaf-Water-MeOH, KK-Oct20-leaf-MeOH, KK-Oct20-leaf-Water, KK-Dec20-leaf-MeOH, and KK-Dec20-leaf-Water Fractions were performed with Batch 12 (Source: *AR* farm*)* larvae. The result of Experiment 7.25 is presented in Table 7.11.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec20-leaf-Water	87	87	82	85.3 ± 1.7^{a}
KK-Dec20-leaf-MeOH	45	42	48	45.0 ± 1.7^{b}
KK-Oct20-leaf-Water	82	85	82	83.0 ± 1.0^{a}
KK-Oct20-leaf-MeOH	59	50	47	52.0 ± 3.6^{b}
KK-Dec20-leaf-Water-MeOH	12	12	9	11.0 ± 1.0^{d}
KK-Oct20-leaf-Water-MeOH	13	8	14	11.7 ± 1.8^{d}
IVM	33	34	34	33.7 ± 0.3^{c}
AB	26	28	26	26.7 ± 0.7^{c}
BZ	38	22	25	28.3 ± 4.9^{c}
PBS	0	0	0	0

Table 7.11. Result of Experiment 7.25: Mean of % mortality of the different solvent fractions of KK-Dec20 and KK-Oct20 samples (n=3; T1-3) against Batch 12 larvae with SEM=Standard Error of Mean. Concentration of each sample was 8 mg/mL. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

It was found that with Batch 12 larvae, the MeOH and Water Fractions of KK-Oct20 and KK-Dec20 leaf sample had very similar and comparable nematocidal efficacies (P>0.05, NS). Both the KK-Oct20-leaf-Water and the KK-Dec20-leaf-Water had remarkably high nematocidal efficacy, significantly higher than the positive control anthelmintics and MeOH Fractions (P<0.05). The Water-MeOH Fractions of both samples had similar efficacy as well (P>0.05, NS).

7.3.8. Results of Combination Strategy: Combining the Effective Solvent Fractions with Standard Anthelmintics

The summary of the NLA tests of combination formulations performed with three batches of larvae is presented in Table 7.12.

NLA tests with	*Nematocidal efficacy (Mean of % larval mortality ± SEM)*									
	BZ	**KK-W +BZ**	**KK-Me+ BZ**	**AB**	**KK-W+ AB**	**KK-Me+ AB**	**IVM**	**KK-W+ IVM**	**KK-Me+ IVM**	**BZ+AB**	**PBS**
Batch 6	21.7 ± 2.8^{f}	39.3 ± 0.8^{d}	16.7 ± 0.8^{f}	25.7 ± 0.7$^{e, f}$	59.3 ± 1.2^{c}	32.7 ± 2.1$^{d, e}$	88.0 ± 0^{a}	82.0 ± 0^{a}	74.0 ± 3.7^{b}	37.0 ± 2.0^{d}	0
Batch 7	36.7 ± 1.8^{e}	57.3 ± 0.3^{d}	24.3 ± 0.8^{f}	80.3 ± 0.3^{b}	80.0 ± 0.5^{b}	72.0 ± 1.5^{c}	88.0 ± 0^{a}	85.0 ± 2.3$^{a, b}$	77.0 ± 1.5$^{b, c}$	83.0 ± 1.1$^{a, b}$	0
Batch 8	41.0 ± 3.2^{d}	43.0 ± 1.0^{d}	64.0 ± 2.6$^{b, c}$	-	-	-	75.5 ± 3.5^{b}	92.0 ± 1.5^{a}	85.0 ± 3.0^{a}	-	0

Table 7.12. Efficacy (Mean of % larval mortality) of combination formulations against Batch 6, 7 and 8 larvae with SEM=Standard Error of Mean. Concentration of each KK sample was 8 mg/mL. Concentration of BZ: 3 mg/mL, AB and IVM was 1 mg/mL. Superscript (a, b, c) within each row represents statistical significance difference from Tukey's test (P<0.05).

The formulation, KK-W+BZ was found to have higher efficacy than BZ ($P<0.05$) with Batch 6, and 7 larvae. The KK-W+AB was found to be more effective than AB ($P<0.05$) with Batch 6 larvae. Additionally, with Batch 8 (mixed larvae batch) larvae, both the formulations KK-W+IVM and KK-Me+IVM had higher efficacy than that of IVM ($P<0.05$). Therefore, these results indicated that the combination of some of the KK-Dec19 leaf Water and MeOH Fractions with the standard anthelmintic resulted towards improved efficacy when combined with the latter.

7.3.9. Results of the LC-MS Analysis of the Various Kawakawa Leaf Fractions

7.3.9.1. Result of the LC-MS Analysis of the Crude Water Fractions

Chromatograms of the LC-MS analysis of the Water Fractions of KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 are presented in **Appendix 7.3.1**. It was found that the Water Fractions of KK-Dec19 and KK-Dec20 samples had identical trace using both (+) and (-) ionisations modes. Similar observations were found with the Water Fraction of KK-May20 when compared with the Water Fractions from other samples, as its LC-MS profile was found to be similar with the other seasonal samples.

7.3.9.2. Result of the LC-MS Analysis of the Crude MeOH Fractions

Chromatograms of the LC-MS analysis of the MeOH Fractions of KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 are presented in **Appendix 7.3.2**. It was found that the MeOH Fractions of KK-Dec19 and KK-Dec20 samples had identical traces using both (+) and (-) ionisations modes. However, the LC-MS results of the MeOH Fractions from KK-May20 and KK-Oct20 were found to be somewhat different than KK-Dec20/KK-Dec19 samples. It was observed that there were occurrences of more individual peaks in the KK-May20 sample than the KK-Oct20 sample in both (+) and (-) ionisation modes. The MeOH Fractions of the KK-Dec19/KK-Dec20 sample found to have the highest numbers of individual peaks than the other two samples in both (+) and (-) ionisation modes.

7.3.9.3. Result of the LC-MS Analysis of the Most Effective Separated MeOH Fractions

Chromatograms of the LC-MS analysis of the most effective fractions of the parent MeOH-Fraction from the KK-May20, KK-Oct20 and KK-Dec20 sample are presented in **Appendix 7.3.3**. It was found the KK-May20-leaf-MeOH-Fraction-2, the most effective

separated fraction of the crude KK-May20-MeOH, had a completely different result than the other most effective separated fractions of the crude MeOH Fractions (KK-Oct20 and KK-Dec20) in both (+) and (-) ionisation modes. A similar observation was recorded with the KK-Oct20-leaf-MeOH-Fraction-4, which was the most effective separated fraction of KK-Oct20-MeOH sample. Interestingly, with the (-) ionisation mode analysis of the KK-Oct20-leaf-MeOH-Fr-4 and KK-Dec20-leaf-MeOH-RUN1-Fr-4, they were found to have similar group of peaks. However, the KK-Dec20-leaf-MeOH-RUN1-Fr-4 found to have more individual peaks in both (+) and (-) ionisation mode when compared with KK-Oct20-leaf-MeOH-Fr-4.

7.3.9.4. Result of the LC-MS Analysis of the Most Effective Separated Water Fractions

Chromatograms of the LC-MS analysis of the most effective fractions of the Water Fractions from the KK-May20, KK-Oct20 and KK-Dec20 sample are presented in **Appendix 7.3.4**. It was found that the (+) ionisation mode resulted towards different results with the most effective fractions of Water samples from three periods (May20, Oct20 and Dec20). However, there were presence of similar peaks in these three samples. The (-) ionisation mode revealed similar observations with these samples. Interestingly, upon conducting the LC-MS analysis of KK-Oct20-leaf-Water-Fr-1, and KK-Dec20-leaf-Water-Fr-2 with the HILIC column (result in **Appendices 7.3.5**), there were occurrences of more peaks with the KK-Oct20-leaf-Water-Fr-1 in both (+) and (-) ionisation mode.

7.4. Discussion

In this chapter, the bioassay-guided fractionation and chromatographic separation of the KK leaf has been presented. The KK leaf was found to be the most effective component of the plant with the highest nematocidal efficacy in the previous chapter. KK is an evergreen plant and its leaf is found throughout the year (Briggs, 1941). Thus, collection of its leaf was not a challenging task. The sampling period of the KK leaf was from three different seasonal periods, summer (December 2019 and 2020), winter (May 2020) and spring (October 2020). This was to observe the seasonal effects (if any) on its PSM and subsequent nematocidal efficacies. In the literature, there are reports of studies where effectiveness of a plant fraction has varied with the bioavailability of the PSM in different parts of the plant, season and sampling periods, producing divergent results within the same plant species (Fonseca et al., 2014). Firstly, the crude solvent fractions were obtained using the Soxhlet extraction and liquid-liquid solvent partition techniques. From the dry matter yield of various fractions, it was observed that unlike the solvent fractions obtained from the KK fruit component (results presented in Section 5.3.1), which had a higher quantity of low polar components than highly polar components, the KK leaf consisted of more highly polar components than low polar components. The dry matter yields of various solvent fractions were similar with all the different KK-leaf samples, which indicated that there was no seasonal effect with the availability of PSM in the different solvent fractions of KK-leaf.

The nematocidal efficacies of the solvent fractions of KK-Dec19 were studied with three batches of larvae which revealed a very interesting result. From the result obtained with Batch 6 and 7, the efficacy of the Water-MeOH, Water and MeOH Fractions were different with both batches of larvae. Therefore, the hypothesis (**Hypothesis 7.1**) that the efficacy of the KK leaf crude solvent fractions would be irrespective of the larval population was found to be **not correct**. Nonetheless, the efficacy of a few positive control anthelmintics was found to be different with these two batches of larvae as well. However, as these were opportunistic larval populations and were mostly collected during an investigation of likely anthelmintic resistance, care needs to be taken when interpreting the relative efficacy of standard anthelmintics. For example, it was found that AB was more effective against *T. circumcincta* which were present in the population of Batch 7 than *H. contortus* which were present in the population of Batch 6. However, *H. contortus* are known to be very susceptible to AB when

anthelmintic resistance is not present. Nevertheless, based on this observation, not only the efficacy of the KK leaf fractions varied but also the standard anthelmintics varied as well. This indicated that the components that were present in the KK leaf Water-MeOH Fraction, were selective against the species of larvae present in the larval population. With this widely diverse result, it was difficult to conclude a specific statement. It was evident that the Water-MeOH Fraction was the most effective out of 3 crude solvent fractions. However, upon the further separation into the Water and MeOH Fractions, there was a decreased efficacy compared to the parent fraction as found with the larvae of Batch 6. On the contrary, the efficacy of the Water Fraction was higher than the parent fraction with larvae of Batch 7, and the efficacy remained the same with Batch 8. This result suggested that the efficacy of each was very dependent on the species of larvae present in the larval population.

The nematocidal study of the crude solvent fractions of KK-May20 with Batch 9 revealed that upon further separation of the Water-MeOH Fraction into individual Water and MeOH Fractions, the efficacy was significantly improved. It was observed that with this batch of larvae, the Water-MeOH Fraction was effective against the LT and *Trichostrongylus* spp. and ineffective against the *H. contortus* species present in the larval population. After separation into the Water and MeOH Fractions, both the samples were effective against the LT and *Trichostrongylus* spp. and the efficacy improved against the *H. contortus* species present in the population as well. More interestingly, comparing this result with the result of Experiment 7.1, the KK-Dec-19-Water-Fraction had ~10% efficacy against the *H. contortus* that were present exclusively in Batch 6 which was a lot less ($P<0.05$) than the efficacy of KK-May20-Water-Fraction against the *H. contortus* present in Batch 9 with 64% efficacy. The source of Batch 6 was different (*ARGL*) than the source of Batch 9 (*SA* Farm). This indicated that the efficacy of a KK leaf sample was not only dependent on the species of the larvae present in the population, but also the source of the species, as the resistance of a particular species was found to be reliant on the source. With the crude solvent fractions of the KK-Oct20 sample, it was found that upon further separation of the Water-MeOH Fraction, the efficacy improved with both Water and MeOH Fractions studied against larvae of Batch 11 (Source: *SA* farm). With the crude solvent fractions of the KK-Dec20 sample, it was found that the Water Fraction had a remarkably higher efficacy compared to the other solvent fractions and the positive control standard anthelmintics studied against the larvae of Batch 12

(Source: *AR* farm). The Dec20-Water Fraction was able to target all the species of larvae present in the population more effectively than IVM. Note that the exact resistance of these larval species was not known or assessed (these batches of larvae were all opportunistically found as previously discussed). Upon further separation, the Water and MeOH Fractions of both leaf samples were effective against the *H. contortus* present in both larval populations. IVM did not affect the *H. contortus* species present in the population of both batches of larvae. Hence, based on all these observations, it may be concluded that *H. contortus* from the *SA* and *AR* farms were quite resistant to IVM and difficult to kill. Additionally, it was observed that the Water and MeOH Fractions had better efficacy against these resistant *H. contortus* than the standard anthelmintics. Nevertheless, all these NLA tests were performed with different batches of larvae with different larval populations, it was very difficult to conclude whether a solvent fraction from one particular season was more or less effective than the other season. It was found that the nematocidal efficacies of all the samples was very dependent on the larval species present in the larval population. Therefore, a comparative study was performed with the 3 crude solvent fractions from KK-Oct20 and KK-Dec20 with a batch of larvae (Batch 12; Source: *AR* farm) to determine if the crude solvent from any season was more effective than the other. However, it was found that the efficacy of the solvent fractions from these two seasonal samples were similar with one another. This indicated that the fractions from these two leaf samples had similar nematocidal efficacy and there was no observed seasonal variation from Autumn to the Winter sample. Therefore, the hypothesis (**Hypothesis 7.2**) that the nematocidal efficacy will be irrespective of the seasonal variation and the samples collected from two different seasons will have similar efficacy, was found to be **correct**.

The nematocidal efficacies of the different solvent fractions were heavily reliant on the batches of larvae that were studied. The highly polar solvent fractions were found to be not effective against *H. contortus* species present in the population but were found to be quite effective against the other species of larvae present. Therefore, these highly polar fractions were strong candidates to do further research with. The efficacies of the highly polar fractions were either comparable or higher than the standard anthelmintics which were also found to be not effective against the *H. contortus* species.

The separation study of the Water and MeOH Fractions was difficult. The Sephadex LH-20 column chromatography with the KK-May20-leaf-Water Fraction was found to be unsuccessful as the analyte did not travel through the column. The column turned black upon the loading of the KK-Water-Fraction. It was thought that the KK leaf fraction reacted with the Sephadex LH-20 which caused the occurrence of black precipitation. This was an unexpected outcome as the Sephadex LH-20 is a widely accepted and reported technique (Mbopi et al., 2021, Liao et al., 2021) to separate any highly polar, water-soluble sample and it was previously successful with the separation of the Quebracho Fractions, as discussed in Chapter 3. The RP-FC with the pre-packed C18 column was found to be successful in separating the Water Fraction. However, the RP-TLC work with the initial fractions obtained from the separation was found to be unsuccessful. The spots of majority of the fractions did not travel on the RP-TLC plate with the series of different mobile phase used for the study. For other fractions, the spots were either condensed together or travelled too high on the plate. However, the pink coloured spots indicated the presence of tannins. The observation on the TLC plate with each mobile phase for respective fractions is presented in **Appendix 7.1.3**. Therefore, combination of the initial fractions with TLC study was found to be unachievable which was achieved via the combination work based on peak areas in the chromatogram. From the flash chromatography runs of every sample, there was occurrence of a very tall and sharp peak in the left-hand side of the chromatogram. This peak was obtained within the first 1-2 minute of the separation which corresponded to the elution of 100 % water (solvent A). This indicated that most of the very water-soluble highly-polar components present in the sample, flushed out at once during that period which were all UV absorbent. Thus, resulting in a very intense peak. The fractions representing this peak in the far-left hand side of the chromatogram had the highest dry matter yield. However, it was found that a tall or sharp peak did not always correspond to a large quantity of the sample present in a fraction obtained from the RP-FC run. For example, the Fractions 37-38 were combined from the KK-Oct20-leaf-MeOH Fraction separation which had a sharp peak in the chromatogram. This was the initial-combined-Fraction-7 and the final-Fraction-5. However, the dry matter yield of this fraction was found to be only 6 mg, whereas the final-Fraction-1 of the KK-Oct20-leaf-MeOH Fraction which had a similar peak height, had dry matter yield of 597 mg. It was found that the separation of the various KK-leaf-MeOH Fractions had more distinctive peaks than the Water Fractions in their respective flash chromatograms. The combination of the fractions

obtained from the separation was performed using the hypotheses approach. However, for most of the Water Fractions, the final-Fraction 1 contained >90 % of the sample, as found from the dry matter yield. This was only found to be different with May20-Water Fraction where the final-Fraction 2 was found to have the highest dry matter yield. Interestingly, there were occurrences of more peaks during the KK-Oct20-Water Fraction separation than the KK-Dec20-Water. It could be due to the reason that the Water Fractions of KK-Dec20 and KK-Oct20 were from two different seasons. As a result, they had different phytoconstituents present in them, which led to a different separation. This was investigated with the LC-MS analysis of the various KK leaf samples. However, the LC-MS analysis of the various Water Fractions from different periods revealed no divergence of PSM with each sample. Therefore, this phenomenon was quite difficult to explain as the column chosen (*SEPACORE 4 g* and *TELOS 4 g)* both had similar packing size and material) and the method used for both separations were the same. It was found that even while using a same type of column and method for the separation of a sample, the process was not repeatable, as observed with the different results of RUN 2 and 3 of KK-Dec20-leaf-MeOH and RUN 1-2 of KK-Dec20-leaf-Water Fractions. Overall, the results indicated that the RP-FC separation technique was effective in getting discrete fractions from the Water and MeOH Fractions. However, separation of the latter was found be to be more effective than the former. Nevertheless, the very high polar water-soluble components present in a sample flushed out at once and it was difficult to separate them into further components. It was found that neither use of a longer column with higher surface area nor the usage of a variable flow rate was successful towards a better separation of these high-polar components (see the result of RP-FC separation of the KK-Dec20-leaf-Water-Fr-1-1 in **Appendix 7.1.12**). Hence, the hypothesis (**Hypothesis 7.3**) that if the flow rate was variable and if it was gradually increased, a better separation of the high-polar water-soluble components present in the sample would likely to take place, was found to be **not correct**. Therefore, this RP-FC separation technique was found to be not effective in separating the very high-polar water-soluble components present in a fraction.

From the LC-MS investigation of the crude Water Fractions from the four-leaf samples, it was found that the Water Fractions of KK-Dec19 and KK-Dec20 samples had identical trace using both (+) and (-) ionisations modes. Hence, this indicated that the components found in the Water Fraction of KK-leaf, they were found to be similar going from one year to the next.

The Water Fractions of KK-Oct20, KK-May20 and KK-Dec20 samples were found to be very similar when compared to one another. This indicated that there were no apparent phytochemical changes for the Water Fractions between the course of winter (KK-May20), spring (KK-Oct20) and summer (KK-Dec20). However, the LC-MS analyses of the MeOH Fractions of these four samples was found to be bit different. It was found that the MeOH Fractions of KK-Dec19 and KK-Dec20 samples had identical trace using both (+) and (-) ionisations modes. This indicated that these two samples had same group of phytoconstituents present in them which was similar to the observation with the Water Fractions from these two samples. Therefore, not only this indicated that the components found in the MeOH, and Water Fractions of KK-leaf were comparable going from one year to the next, but also this further validated the method of bioassay-guided separation method used in the research. However, the LC-MS results of the MeOH Fractions from KK-May20 and KK-Oct20 were found to be different than KK-Dec20/KK-Dec19 samples. It was observed that there were occurrences of more individual peaks in the KK-May20 sample than the KK-Oct20 sample in both (+) and (-) ionisation modes. This indicated that there were more phytoconstituents present in the KK-May20 sample than the KK-Oct20 which might be associated due to the seasonal change. Overall, the MeOH Fractions of the KK-Dec19/KK-Dec20 sample found to have the highest number of individual peaks than the other two leaf samples. This result indicated that for the components present in the MeOH Fractions of KK-leaf, there is a greater occurrence of them during the summer period compared to the winter and spring sample. For the most effective separated fractions of the MeOH Fraction, it was found the KK-May20-leaf-MeOH-Fr-2, had a completely different result than the other most effective fractions of the MeOH samples (KK-Oct20 and KK-Dec20) in both (+) and (-) ionisation modes. A similar observation was recorded with the KK-Oct20-leaf-MeOH-Fr-4, which was the most effective separated fraction of the KK-Oct20-MeOH sample. This indicated that even though these fractions had different profile of phytoconstituents, they were effective against the batch of nematode larvae that they were studied with. Interestingly, with the (-) ionisation mode analysis of the KK-Oct20-leaf-MeOH-Fr-4 and KK-Dec20-leaf-MeOH-RUN1-Fr-4, they were found to have similar group of components. Now, going back to the RP-FC separation comparison of the parent crude MeOH Fractions of KK-Oct20 and KK-Dec20, there were occurrences of this Fraction 4 with both of these samples. However, as the KK-Dec20-MeOH sample was found to have more PSM with its LC-MS

analysis, it was believed that the Fraction-4 of this sample had more peaks present than the Fraction 4 of KK-Oct20-MeOH. It should be noted that the RP-FC separation of the KK-Dec20-MeOH Fraction was performed three times using two different columns. RUN 1 was performed using a *TELOS 23 g column*, RUN 2 was performed using a *TELOS 4 g column* and RUN 3 was the repeat of RUN 2 as RUN 2 was encountered with potential bubble formation. It was found that the KK-Dec20-leaf-MeOH-RUN1-Fr-4 were similar to the KK-Dec20-leaf-MeOH-RUN2-Fr-4, but the former had more individual peaks than the latter. More interestingly, as found with the investigation of LC-MS analysis of fractions obtained during the RUN 3, the Fraction-5 from RUN 3 was found to be similar to the Fraction-4 of RUN 1 and 2. The Fraction-4 of RUN 3 was a lot different than the Fraction-4 obtained from RUN 1 and 2. Upon the comparison of LC-MS analysis of the KK-Oct20-MeOH-Fr-4 and the KK-Dec20-leaf-MeOH-RUN2-Fr-4, they had similar group of components. However, there were occurrences of more individual peaks in the former fraction than the latter. This was a contradiction from the above-mentioned result with the Fraction-4 of KK-Dec20-MeOH sample obtained from RUN 1. This result indicated that the RP-FC separation of the MeOH fraction using the C18 RP column was not perfectly repeatable or reliable. It was found to be dependent on the column used, the quantity of sample taken, and the other parameters such as bubble formation.

From the results presented in Section 7.3.6, the separated MeOH and Water fractions of KK-May20, KK-Oct20, and KK-Dec20 (except Water Fraction of KK-Dec20) were found to have higher nematocidal efficacy than their parent solvent fractions when studied with the same batch of larvae. Therefore, the hypothesis (**Hypothesis 7.4**), that the nematocidal efficacy of a few of these fractions would be higher than the parent fraction was found to be **correct**. Additionally, the optimal concentration, i.e., the lowest concentration with maximal efficacy of the separated fraction was found to be a lower (4 mg/mL) than the optimal concentration of its parent crude solvent Fraction (8 mg/mL) as found from their respective dose response tests with the same batch of larvae. Hence, the hypothesis (**Hypothesis 7.5**), that a separated fraction from a crude solvent fraction will reach its maximum efficacy at a lower concentration than the optimal concentration of its parent crude fraction, was found to be **correct**.

The results of the combination formulations made with the KK solvent fractions and standard anthelmintic showed that the combination of some of the KK-Dec19 leaf Water and

MeOH Fractions with standard anthelmintics demonstrated higher efficacy than was observed with the standard anthelmintics alone. Therefore, the hypothesis (**Hypothesis 7.6**), the combination of effective KK samples with the standard anthelmintic will improve the efficacy of the latter, was found to be **correct** in some instances. This was a very important observation as the combination of these KK extracts with existing anthelmintics may improve the efficacy of standard anthelmintics in the face of declining efficacy due to anthelmintic resistance. Refer to Chapter 12 for a more detailed discussion regarding this observation.

Therefore, in this chapter, it was found that the KK leaf had promising anthelmintic efficacy. The most effective compounds were found in its high-polar MeOH and Water Fractions. The separation using the RP-FC technique was not effective as several issues were observed, and repeatability of the process was found to be poor. Therefore, a version of an established chromatographic separation was developed in order to better separate the PSM present in an effective fraction of MeOH fraction. This is presented in the next chapter.

Chapter 8. Separation of the Kawakawa Leaf Fractions Using an Improvised Countercurrent Chromatography Separation Technique: HCCCS

Separation of the Kawakawa Leaf Fractions Using an Improvised Countercurrent Chromatography Separation Technique: HCCCS

8.1. Introduction

It was found in the previous chapter that the RP-FC technique was not very efficient for the separation of the highly polar fractions from the KK leaf samples. Therefore, it was necessary to look into other established separation techniques in order to better separate the components present in the highly polar KK leaf samples. Countercurrent chromatography is a separation process that is founded on the principles of liquid-liquid extraction (Jing et al., 2021). The basic principle of countercurrent chromatography is where a chemical compound is partitioned between two immiscible liquid phases according to its relative solubility between the two phases (Sun et al., 2020). A particular advantage of this technique is that it does not involve any solid support and the complications that arise from the use of one, such as adsorptive sample loss, denaturation, tailing of solute peaks as well as contamination that are mitigated in this chromatographic separation technique (Ito, 2019). This liquid-liquid separation technique was first developed by Martin, Synge, Craig and Post in the 1940's, and through years of further development, it has matured into the modern countercurrent chromatography separation (CCS) technique (Friesen et al., 2015). CCS utilises two immiscible solvent phases, and the partition process takes place in an open column space (Luca et al., 2019). In this column, one phase (stationary phase) is retained, and the other phase (mobile phase) continuously passes through the column space. The system uses acute combinations of various column configurations with an applied force field (gravitational or centrifugal) to maintain the optimum amount of the stationary phase in the column (Ito, 2019). The CCS instruments are fully automated and are designed for preparative work with the advantages of low pressures, high flow rates as well as the ability to use a wide variety of solvents (Ito and Conway, 1986). A single analyte can be targeted, or several analytes with a wide range of polarities can be separated with a suitable method developed with CCS.

A search of SciFinder from 2000 to 2021 revealed a total of 172 articles on 'countercurrent chromatography separation of natural products'. This process has been successfully implemented for the separation of many groups of PSM from many different plant extracts (Luca et al., 2019, Báthori et al., 2002, Pozzebon et al., 2001, Jing et al., 2021). In CCS, the separation efficiency is reliant on the partitioning ability (represented by the parameter partition coefficient) of the separated substance between the stationary phase and the mobile phase (Jing et al., 2021). The selection of a suitable solvent system plays the

most vital role in the separation of countercurrent chromatography. The usage of a two or three phase solvent system has been widely reported for the separation and isolation of different groups of PSM from many different plant extracts (Jing et al., 2021, Chen et al., 2021, Yuan et al., 2020, Luca et al., 2019). Some typical examples include: three novel glycosides from *Digitalis lanata* were isolated using the solvent systems $CHCl_3$-MeOH-H_2O (5:6:4) and CH_2Cl_2-MeOH-H_2O (5:6:4) (Krüger et al., 1983); four strophanthidin glycosides out of a total of eight isolated compounds from *Lophopetalum toxicum* were isolated using the solvent systems $CHCl_3$-MeOH-PrOH-H_2O (5:6:1:4) and $CHCl_3$-MeOH-PrOH-H_2O (45:70:5:40) (Wagner et al., 1984); Usnic and protocetratic acid were isolated *from Neuropogon aurantiaco-atra* using the solvent system $CHCl_3$-MeOH-Water (43:37:20) (Pozzebon et al., 2001).

At the time of conducting this research, Massey University did not have a CCS instrumental facility. Therefore, an improvised technique was developed to apply the liquid-liquid separation technique of the CCS for further separation of the KK fractions. This technique was applied to a wide selection of solvent systems that are typically used during a CCS process. The name '*Hand Controlled Countercurrent Chromatography Separation*' (HCCCS) was coined by Gupta due to the reason that the principle of CCS was applied in this technique, but this was done manually rather than using an automated CCS instrument. The solvent systems were designed by Gupta and Xu, and the research was conducted by Gupta. The KK-Dec20-leaf-MeOH-Fr-4 was the KK fraction chosen for this research as from Experiment 7.22, it was found to be the most effective KK fraction. This fraction was subjected to the HCCCS analysis with the different solvent systems that were developed. The most suitable solvent systems were chosen based on the dry matter yield of the sample on the top and bottom layer of the solvent system and LC-MS analysis of sample collected from each layer. A nematocidal analysis was performed with the fractions collected from both layers of the most suitable solvent system(s). Moreover, a combination formulation was made with IVM together with the dry sample of KK-Dec20-leaf-MeOH-Fr-4 collected from the solvent layer of the most suitable solvent system. This combination was studied against a known anthelmintic resistant batch of mixed nematode larvae. It was hypothesised that the combination formulation will have higher efficacy than the individual components. In this chapter, the methodology of the HCCCS and the results of the chemical and nematocidal analysis are presented.

8.2. Materials and Methods

8.2.1. Method of the HCCCS Analysis with the KK-Dec20-leaf-MeOH-Fr-4

A wide range of solvents were chosen and mixed in specific amounts to create immiscible layers in a 1.5 mL cryogenic vial. The solvents in the vial had the presence of a highly polar and medium-low polar layers which were immiscible. In total, 15 solvents systems were designed. The list of solvent mixtures, mixture ratio, and the mixture quantities are presented in Table 8.1. The dried KK-Dec20-leaf-MeOH-Fraction-4 (4 mg) from RUN 1 was added to each vial of the respective solvent system and thoroughly shaken and left for settling for around 30 mins. The top and bottom solvent layers of each solvent system were then carefully pipetted out (*Pasteur glass pipette length 230 mm*) to individual 5 mL round-bottom flasks and subjected to freeze-drying (*FTS Systems Flexi-Dry; Stoneridge, NY, USA*). After drying, the samples were weighed and subjected to LC-MS analysis following the method described in Section 2.3.11.1.2.

# HCCCS solvent system	*Solvents taken*	*Mixture ratio*	*Mixture quantity*
1	$CHCl_3$-MeOH-H_2O	5:6:4	250 µL-300 µL-200 µL
2	Hex-EtOAC-MeOH-H_2O	1:1:1:1	200 µL-200 µL-200 µL-200 µL
3	$CHCl_3$-MeOH-H_2O	6:5:4	300 µL-250 µL-200 µL
4	Hex-EtOAC-MeOH-H_2O	2:2:1:1	300 µL-300 µL-150 µL-150 µL
5	$CHCl_3$-MeOH-H_2O	4:6:5	200 µL-300 µL-250 µL
6	$CHCl_3$-MeOH-H_2O	1:2:2	150 µL-300 µL-300 µL
7	$CHCl_3$-MeOH-H_2O	7:4:4	350 µL-200 µL-200 µL
8	$CHCl_3$-MeOH-H_2O	8:3:4	400 µL-150 µL-200 µL
9	$CHCl_3$-MeOH-H_2O-Dil. HCl (0.1N)	5:6:4:1	250 µL-300 µL-200 µL-50 µL
10	$CHCl_3$-MeOH-H_2O-Dil. NaOH (0.1M)	5:6:4:1	250 µL-300 µL-200 µL-50 µL
11	BuOH-H_2O	3:2	600 µL-400 µL
12	$CHCl_3$-MeOH-PrOH-H_2O	5:6:1:4	250 µL-300 µL-50 µL-200 µL
13	CH_2Cl_2-MeOH-H_2O	8:5:4	400 µL-250 µL-200 µL
14	CH_2Cl_2-MeOH-H_2O-Dil. NaOH (0.1M)	4:2:2:1	400 µL-200 µL-200 µL-100 µL
15	$CHCl_3$-MeOH-H_2O-Dil. NaOH (0.1M)	3:2:2:1	300 µL-200 µL-200 µL-100 µL

Table 8.1. List of solvents, mixture ratios and quantities for the HCCCS with the KK-Dec20-leaf-MeOH Fraction 4; Dil.=Dilute.

8.2.2. Nematocidal Analysis of the Dry Samples Acquired from HCCCS Analysis

8.2.2.1. Sample Preparation

The HCCCS solvent systems 1, 10 and 15 were prepared in 10 mL cryogenic vials. The quantity of solvent taken for each layer is described below in Table 8.2.

# HCCCS solvent system	*Mixture ratio*	*Mixture quantity*
1: $CHCl_3$-MeOH-H_2O	5:6:4	2.0 mL-2.4 mL-1.6 mL
10: $CHCl_3$-MeOH-H_2O-Dil. NaOH (0.1M)	5:6:4:1	2.0 mL-2.4 mL-1.6 mL-0.4 mL
15: $CHCl_3$-MeOH-H_2O-Dil. NaOH (0.1M)	3:2:2:1	2.4 mL-1.6 mL-0.8 mL

Table 8.2. The HCCCS solvent gradients, mixture ratio and quantity for NLA tests; Dil.=Dilute.

The KK-Dec20-leaf-MeOH-Fr-4 (12 mg) was added to each 5 mL cryogenic vial containing the designated solvent system. The vials were then vigorously shaken and left for settling for 30 minutes. The top and bottom layers of each gradient were then carefully pipetted out to individual 5 mL round bottom flasks. After that they were concentrated to dryness by rotary-evaporation (*BÜCHI Rotavapor R-200 with BÜCHI Heating Bath B-490; BÜCHI Labortechnik AG, Flawil, Switzerland*) and subjected to freeze-drying (*FTS Systems Flexi-Dry; Stoneridge, NY, USA)*. The dry matter yield of the sample present in the respective gradient layers was recorded.

8.2.2.2. Experiment 8.1: Nematocidal Experiment with Batch 13 Larvae

The dried samples obtained from each solvent layer were then dissolved in PBS and subjected to NLA test with Batch 13 larvae. The concentration of each sample for NLA tests, and number of experiments done are given in Table 8.3. These studies are detailed as Experiment 8.1.

HCCCS Sample	*Concentration in PBS*	*Number of experiments (n)*
KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-t.l.	4 mg/mL	2
KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l.	1 mg/mL	2
KK-Dec20-leaf-MeOH-Fr4-HCCCS-10-t.l.	4 mg/mL	2
KK-Dec20-leaf-MeOH-Fr4-HCCCS-10-b.l.	1 mg/mL	1
KK-Dec20-leaf-MeOH-Fr4-HCCCS-15-t.l.	4 mg/mL	2
KK-Dec20-leaf-MeOH-Fr4-HCCCS-15-b.l.	1 mg/mL	1

Table 8.3. HCCCS samples, concentration in PBS and the number of experiments performed for the NLA test with Batch 13 larvae. t.l.=top layer, b.l.=bottom layer.

8.2.3. Experiment 8.2: Combination Strategy: Combining the Effective HCCCS Sample with Standard Anthelmintic and Performing NLA Tests

The KK-Dec20-leaf-MeOH-Fraction 4 and KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-t.l. were mixed with IVM and NLA tests were performed with Batch 13 larvae in duplicate (n=2, T=1,2). The drug mixtures, and concentration of KK sample in mixtures are given in Table 8.4. The concentration of the KK-Dec20-leaf-MeOH-Fr4 crude was 4 mg/mL in the drug mixture and for the KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l. was 1 mg/mL. These studies are detailed as Experiment 8.2.

# Formulation	***Formulation ID***	***Concentration of each active in mixture (mg/mL)***
Formulation 1	IVM+ KK-Dec20-leaf-MeOH-Fr4	1 + 4
Formulation 2	IVM + KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l.	1 + 1

Table 8.4. Combination formulations and the concentration of the active in each mixture (mg/mL); b.l.=bottom layer.

8.2.3.1. Dead Larval Species Identifications

For the NLA experiments conducted with T1 and T2 of the Formulation 2, the identification of the larvae which died were made using the method described in Section 2.4.5.

8.2.4. Calculations and Statistics

The nematocidal efficacy of each fraction was specified as % larval mortality using the equation described in Section 2.4.3.1. The efficacy of each sample was compared with one another using an ANOVA and post-hoc Tukey's test.

8.3. Results

8.3.1. The Dry matter Yield of the Sample Present in the Layers of HCCCS Solvent Systems

The dry matter yield of sample present in the top and bottom layers of the 15 solvent systems used for the HCCCS analysis are presented in Table 8.5.

Solvent system layer ID	***Layer***	***Dry matter yield of the KK sample present in the respective layer***
1 - top layer: HCCCS-1-t.l.	Water	3.0 mg
1 - bottom layer: HCCCS-1-b.l.	Chloroform	1.0 mg
2 - top layer: HCCCS-2-t.l.	Hexane	0.1 mg
2 - bottom layer: HCCCS-2-b.l.	Water	3.9 mg
3 - top layer: HCCCS-3-t.l.	Water	3.3 mg
3 - bottom layer: HCCCS-3-b.l.	Chloroform	0.7 mg
4 - top layer: HCCCS-4-t.l.	Hexane	<0.1 mg
4 - bottom layer: HCCCS-4-b.l.	Water	3.9 mg
5 - top layer: HCCCS-5-t.l.	Water	3.5 mg
5 - bottom layer: HCCCS-5-b.l.	Chloroform	0.5 mg
6 - top layer: HCCCS-6-t.l.	Water	3.8 mg
6 - bottom layer: HCCCS-6-b.l.	Chloroform	0.2 mg
7 - top layer: HCCCS-7-t.l.	Water	3.3 mg
7 - bottom layer: HCCCS-7-b.l.	Chloroform	0.7 mg
8 - top layer: HCCCS-8-t.l.	Water	3.3 mg
8 - bottom layer: HCCCS-8-b.l.	Chloroform	0.7 mg
9 - top layer: HCCCS-9-t.l.	Water	3.6 mg
9 - bottom layer: HCCCS-9-b.l.	Chloroform	0.4 mg
10 - top layer: HCCCS-10-t.l.	Water	3.6 mg
10 - bottom layer: HCCCS-10-b.l.	Chloroform	0.4 mg
11 - top layer: HCCCS-11-t.l.	Water	3.1 mg
11 - bottom layer: HCCCS-11-b.l.	Butanol	0.8 mg
12 - top layer: HCCCS-12-t.l.	Water	3.2 mg
12 - bottom layer: HCCCS-12-b.l.	Chloroform	0.7 mg
13 - top layer: HCCCS-13-t.l.	Water	3.4 mg
13 - bottom layer: HCCCS-13-b.l.	DCM	0.6 mg
14 - top layer: HCCCS-14-t.l.	Water	3.8 mg
14 - bottom layer: HCCCS-14-b.l.	DCM	0.2 mg
15 - top layer: HCCCS-1-t.l.	Water	3.7 mg
15 - bottom layer: HCCCS-15-b.l.	Chloroform	0.3 mg

Table 8.5. The dry matter yield of the sample present in the respective solvent layer in the different HCCCS solvent systems; t.l.=top layer, b.l.=bottom layer.

From the results, it was found that the solvent system HCCCS-1 had the highest quantity of dry sample present in its two layers. All these samples were subjected to LC-MS analysis.

8.3.2. Result of the LC-MS Analysis of the Fractions Present in the Layers of HCCCS Solvent Systems

From the results of the LC-MS analysis (presented in **Appendix 8.1**), it was found that in all the chloroform based solvent systems (HCCCS-1, 3, 5, 6, 7, 8, 9, 10, 12, and 15), there were presence of divergent peaks in the respective top and bottom layers. The *m/z* of the peaks present in top and bottom layers of these solvent systems were different from one another which indicated good separation. Of note, there was a single peak at RT = 12.13 (*m/z* of base peak = 165.97) in the top layer (hexane layer) of solvent system HCCCS-4 using the (-) ionisation mode (result presented in **Appendix 8.1.4**). This peak at RT = 12.13 was also present at the bottom layer (chloroform layer) of the chloroform based solvent systems in (-) ionisation mode. From the LC-MS analysis of the sample present in the solvent layers of HCCCS-1 (result presented in **Appendix 8.1.1**), HCCCS-10 (result presented in **Appendix 8.1.10**) and HCCCS-15 (result presented in **Appendix 8.1.15**), it was evident that there was presence of similar groups of components in each solvent system at their bottom layer (chloroform layer). The peak at RT = 14.01 min (base *m/z* = 420.25) was present in the bottom layer of HCCCS-1, HCCCS-10, and HCCCCS-15 in (-) ionisation mode. However, the peak at RT = 1.23 (base *m/z* = 316.95/452.92) present in the bottom layer of HCCCS-1, HCCCS-10 and HCCCCS-15 in (-) ionisation mode, was more intense in HCCCS-10/15 than in HCCCS-1. This indicated that the isolate corresponding to this peak was predominantly present in HCCCS-10 and HCCCS-15 than in HCCCS-1. There were occurrences of more distinct peaks in the bottom layer of HCCCS-1 than in both HCCCS-10 and HCCCS-15. This indicated that the HCCCS-1 bottom layer contained more isolates than the bottom layers of both the HCCCS-10 and HCCCS-15. The HCCCS-10 and HCCCS-15 were based on HCCCS-1 with the addition of dilute NaOH. The presence of dilute NaOH did not have any effect on the analytes present in the bottom layer. However, the difference in *m/z* ratios of base peaks on the top layer of HCCCS-10 and HCCCS-15 in (-) ionisation mode, indicated that the addition of NaOH affected the analytes present in the top layer as observed by the divergence of their *m/z* ratios of base peaks from those of HCCCS-1.

Based on these two above observations, the dry matter yield and LC-MS investigation, the HCCCS solvent systems 1, 10 and 15 were chosen for the nematocidal analysis.

8.3.3. Result of the Sample Preparation for Nematocidal Analysis

The result of the dry matter yield of sample present in the top and bottom layer of the solvent systems HCCCS-1, HCCCS-10 and HCCCS-15 are presented in Table 8.6.

Solvent system layer ID	***Layer***	***Dry matter yield of sample present in the layer***
1 – top layer: HCCCS-1-t.l.	Water	9.5 mg
1 – bottom layer: HCCCS-1-b.l.	Chloroform	2.5 mg
10 – top layer: HCCCS-10-t.l.	Water	10.8 mg
10 – bottom layer: HCCCS-10-t.l.	Chloroform	1.2 mg
15 – top layer: HCCCS-15-t.l.	Water	11.1 mg
15 – bottom layer: HCCCS-15-b.l.	Chloroform	0.9 mg

Table 8.6. The dry matter yield of the sample present in the respective solvent layer in the different HCCCS solvent systems subjected to nematocidal analysis; t.l.=top layer, b.l.=bottom layer.

Similar to the observation presented in Table 8.5, the HCCCS-1 had the higher quantity of sample in its top layer compared to the other solvent systems.

8.3.4. Result of Experiment 8.1: NLA Tests of the Dry Samples Acquired from HCCCS Analysis

The result of Experiment 8.1, the mean of % larval mortality with SEM of each sample is presented in Table 8.7.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-t.l.	55	51	-	53.0 ± 2.0[a]
KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l.	51	53	-	52.0 ± 1.0[a]
KK-Dec20-leaf-MeOH-Fr4-HCCCS-10-t.l.	24	25	-	24.5 ± 0.5[c]
KK-Dec20-leaf-MeOH-Fr4-HCCCS-10-b.l.	7	-	-	7.0
KK-Dec20-leaf-MeOH-Fr4-HCCCS-15-t.l.	52	55		53.5 ± 1.5[a]
KK-Dec20-leaf-MeOH-Fr4-HCCCS-15-b.l.	9	-	-	9.0
KK-Dec20-leaf-MeOH-Fr4	37	45	48	43.3 ± 3.2[b]
IVM	42	42	46	43.3 ± 1.3[b]
AB	25	24	-	24.5 ± 0.5[c]
BZ	12	14	12	12.7 ± 0.7[d]
PBS	0	0	0	0

Table 8.7. *Results of Experiment 8.1: Mean values of % mortality of the HCCCS samples and the standard anthelmintics against Batch 13 larvae with SEM =Standard Error of Mean. Letters in superscript (a, b, c) next to the mean values represent the order of the statistical significance (P<0.05); t.l.=top layer; b.l.=bottom layer.*

It was observed that at the concentrations used the KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-t.l. and the KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l. samples had similar nematocidal efficacy (P>0.05, NS). However, the concentration of these two samples in PBS during the NLA tests were different (as presented in Table 8.3). It was found that the dried sample from the bottom layer (chloroform) of the HCCCS-1, the KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l. had 52.0 ± 1.0% efficacy at 1 mg/mL concentration against Batch 13 larvae, which was higher than any standard anthelmintics, BZ (P<0.05), AB (P<0.05) and IVM (P<0.05). The dried sample from the top layer (water) of the HCCCS-1, the KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-t.l., had 53.0 ± 2.0% efficacy at 4 mg/mL concentration. The efficacies of both top and bottom were higher than the parent Fraction-4 (P<0.05). This indicated that the sub-fractions obtained from the HCCCS separation of the Fraction-4 were more effective than the parent fraction.

8.3.5. Result of Experiment 8.2: NLA Tests of the Combination Formulations

The result of Experiment 8.2, the mean of % larval mortality with SEM of each sample is presented in Table 8.8.

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
Formulation 2	91	96	-	93.5 ± 3.5^{a}
Formulation 1	65	70	-	67.5 ± 3.5^{b}
KK-Dec20-leaf-MeOH-Fr4-HCCCS-1-b.l.	51	53	-	52.0 ± 1.0^{c}
KK-Dec20-leaf-MeOH-Fr4	37	45	48	43.3 ± 3.2^{d}
IVM	42	42	46	43.3 ± 1.3^{d}
PBS	0	0	0	0

Table 8.8. Results of Experiment 8.2: Mean values of % mortality of the combination formulations against Batch 13 larvae with SEM =Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference (P<0.05).

It was found that both the formulations had quite impressive nematocidal efficacy against Batch 13 larvae, which were significantly higher than IVM (P<0.05). However, the efficacy of the Formulation 2 was much higher than both IVM and the parent Fraction-4.

From the dead larval identifications with T1 and T2 of the Formulation 2, the only surviving larvae in the larval population were *H. contortus*. However, the surviving larvae had weak condition and appeared to be moving very slowly. Thus, it was observed that combining the HCCCS fractions with IVM improved the efficacy dramatically. The combination mixtures were found to be very effective against this batch of larvae with which the standard anthelmintic did not demonstrate to possess a high efficacy.

8.4. Discussion

In this chapter, a chromatographic separation technique, HCCCS, based on the fundamental of CCS is presented. CCS has been used to separate and purify PSM from many plant extracts (Friesen et al., 2015). The technique was developed applying the same principles of CCS. Nevertheless, this separation was performed manually in contrast to the usual procedure for CCS which is to use an automated instrument (Ito, 2019, Luca et al., 2019). The name '*Hand Controlled Countercurrent Chromatography Separation*' (HCCCS) was coined by Gupta. This technique was used to separate the KK-Dec20-leaf-MeOH-Fr-4 which was the most effective separated fraction of the KK-Dec20-MeOH Extract from the previous chapter. A series of solvent systems consisting of layers of immiscible solvents were designed, and the dried sample of Fraction-4 was suspended in equal amount in each. This method was similar to that used in the two-phase solvent layer systems in the automated CCS instruments (Luca et al., 2019). From the dry matter yield of sample from both layers of the individual solvent system, it was found that the solvent system HCCCS-1 had the highest quantity of sample present in both solvent layers. From the LC-MS investigation, the top and bottom layers of all the solvent systems had a different group of components which was represented by their different *m/z* ratio. This indicated a good separation between the two solvent layers. The solvent system HCCCS-1 was found to be the most suitable solvent system to assess with a nematocidal assay. Another two solvent systems, HCCCS-10 and HCCCS-15 were also selected as these were based on HCCCS-1 but with the presence of dilute NaOH. It was found that the dilute NaOH affected the components present in the top layer of HCCCS-10 and HCCCS-15, but the components present in the bottom layers were unaffected. Upon upscaling the volume of the solvent system and quantity of the sample (from 4 mg to 16 mg) suspended in these solvent systems, it was found that a similar ratio of fraction distribution took place in the respective layers of each solvent system. This indicated the good repeatability and reliability of the HCCCS process unlike with the RP-FC where the repeatability was found to be poor as presented in the previous chapter.

From the nematocidal experiment preformed with Batch 13 larvae, the sample collected from the top and bottom layer of HCCCS-1 was found to be more effective than the parent MeOH-Fraction-4. This indicated that the phytoconstituents present in these separated sub-fractions had higher nematocidal efficacy than the parent Fraction-4. More

importantly, the formulation made with the fraction collected from the bottom layer of HCCCS-1 and IVM had a remarkable efficacy close to ~95% against the resistant larvae present in the population of Batch 13 at a very low concentration (1 mg/mL) of the HCCCS-1-b.l. Fraction. The efficacy of this fraction was found to be much higher than the combination of IVM and the parent Fraction 4 ($P<0.05$) and the individual component. Therefore, the hypothesis, that that the combination formulation will have higher efficacy than the individual components, was found to be **correct**.

The manual application of CCS for separation as used here (HCCCS) was successful in obtaining single or double isolates from the original Fraction-4 in contrast to the lack of success using RP-FC. The use of HCCCS developed a single peak in the upper layer (Hexane layer) of the HCCCS-4. This peak represented a single isolate which was exclusively in the upper layer of this solvent system. This peak was also present in the chloroform-based solvent systems. This was indicative that this isolate was present exclusively in the upper layer of HCCCS-4, and it was mixed with other components in the chloroform-based solvent systems where these other components potentially belonged to different classes of compounds (see **Chapter 9** for a detailed qualitative chemical investigation of the components in the top and bottom layer of the chloroform-based system HCCCS-1). Of note, the dry matter yield of the HCCCS-4 Fraction was very low (<0.1 mg), which indicated the presence of a single isolate. The identification of this peak and a prediction of the structure of this compound are presented in **Chapter 10**.

Therefore, this HCCCS process was found to be very effective for the separation of the highly-polar fraction obtained from the separation of original MeOH Extract. The process was found to be repeatable, and upscaling of the process was noted to be transferrable. These were some of the major issues arose from the RP-FC separation which were mitigated in the HCCCS process. The additional advantages of HCCCS were no loss of the parent sample, faster separation time, and more effective distribution of PSM. The separated sub-fraction obtained from the HCCCS analysis was found to be very effective against the resistant Batch 13 larvae when mixed with IVM.

In the next chapter, the qualitative chemical investigation of the KK-leaf crude solvent extract, the separated fractions, and the sub-fractions from the HCCCS analysis are presented

to further understand the classes of compounds of the PSM present in each sample. In Chapter 10, the study towards evaluating the chemical structure of a few isolates from HCCCS analysis is presented.

Chapter 9. Qualitative Chemical Investigation of the Kawakawa Leaf Fractions

Qualitative Chemical Investigation of the Kawakawa Leaf Fractions

9.1. Introduction

In this chapter, the qualitative chemical investigation of the KK MeOH and Water Fractions and their separated fractions is presented. The qualitative chemical investigation of plant extracts is a widely reported tool to obtain a preliminary idea of the phytochemicals present in the plant extract (Shalini and Ilango, 2021, Jaradat et al., 2021, Bednarska et al., 2020). In this investigation, a series of qualitative chemical tests is performed with a crude solvent extract or/and their separated fractions which helps to a) identify the group of phytoconstituents present in the crude solvent extract or the separated fractions thereof, b) evaluate the therapeutic potential of the plant, and c) develop phytochemical standards for the medicinal plant materials for quality control purposes. These chemical investigations lead towards the detection of different classes of compounds such as carbohydrates, amino acids, proteins, glycosides, tannins, cholesterol and alkaloids present in the plant extract (Rabab et al., 2020, Ji et al., 2019). However, no comprehensive knowledge on the phytochemical analysis of the KK leaf extract was available prior to this research. The studies performed in this chapter helped to provide a preliminary understanding towards the compound detection and identification of the effective KK solvent extracts and their separated fractions. Moreover, by performing the same series of chemical tests with the parent solvent extract and its separated fraction helped understand the pattern of chromatographic separation as to how the phytoconstituents were separated into individual fractions from the parent sample.

9.2. Materials and Methods

These KK leaf samples were subjected to the identification tests: Water-Fraction, MeOH-Fraction, MeOH-Fr-1, MeOH-Fr-4, MeOH-Fr4-HCCCS-1-t.l., and MeOH-Fr4-HCCCS-1-b.l. The crude solvent extracts were from KK-May20, KK-Oct20 and KK-Dec20 samples. All the separated fractions were from KK-Dec20 sample. The materials that were used and the methodology of the various tests that were followed for the qualitative investigation are presented below.

Preparation of test solution: The test solution (where mentioned) was made by dissolving the KK dried sample in water at 8 mg/mL concentration.

9.2.1. Tests for Carbohydrates

9.2.1.1. Fehling's Test

Equal amount of Fehling's A and Fehling's B solutions were mixed and boiled for 2-3 minute in a water bath. An equal volume of the test solution was added to the mixture. Then, the mixture was heated in boiling water bath for 5-10 min. It was observed as a yellow, then brick red precipitate.

9.2.1.2. Benedict's Test

Equal amounts of Benedict's reagent and the test solution in a test tube were mixed. It was heated in a boiling water bath for 5 min. The solution might appear green, yellow, or red depending on amount of reducing sugar present in test solution.

9.2.1.3. Iodine Test

The test solution (2 mL) was mixed with a few drops of dilute iodine solution. It was observed as a blue colour which disappeared on boiling and reappeared on cooling.

9.2.1.4. Tannic Acid Test

A few drops of 20% tannic acid solution were added to 2 mL of test solution. It was observed as a precipitate indicating the presence of starch.

9.2.2. Tests for Proteins and Amino Acids

9.2.2.1. Biuret Test (General test)

To the test solution (2 mL), a few drops of 4% NaOH and 1% $CuSO_4$ were added. It was observed as a violet or mauve colour.

9.2.2.2. Ninhydrin Test (General test for primary and secondary amines)

A few drops of 5% ninhydrin were added to 2 mL of test solution and boiled in water bath for 10 min. It was observed as a purple of blueish colour.

9.2.2.3. Xanthoproteic Test (Test for tryptophan or tyrosine)

Concentrated HNO_3 (1 mL) was added to 2 mL of the test solution. It was then heated in a water bath for 5-6 minute. The solution was cooled under tap water, observed for yellow colour. NaOH (40%) was added dropwise to the cold solution, observed for an orange colour solution.

9.2.2.4. Hopkins-Cole Test (Test for tryptophan)

A few drops of glyoxylic acid were added to 2 mL of the test solution and drops of H_2SO_4 were added slowly to observe a reddish violet ring at the junction of the two layers.

9.2.2.5. Millon's Test (Test for tyrosine)

A few drops of Millon's reagent were added to 2 mL of test solution and it was heated in a water bath for 2-3 minutes to observe for a reddish brown or red precipitate.

9.2.2.6. Test for Cysteine

A few drops of 40% NaOH and 10% $Pb(OAC)_2$ were added to 2 mL test solution to observe for a black precipitate.

9.2.3. Tests for Steroids

9.2.3.1. Salkowski's Test (Test for Cholesterol)

The test solution (2 mL) was mixed with 2 mL $CHCl_3$ and 2 mL concentrated H_2SO_4. It was shaken well and observed for the chloroform layer to appear red and the acid layer to show a greenish yellow fluorescence.

9.2.3.2. Liebermann-Burchard Test

The test solution (2 mL) was mixed with 2 mL $CHCl_3$ and 1-2 mL of acetic anhydride and 2 drops of concentrated H_2SO_4 from the side of test tube to observe for first red, then blue and finally a green colour.

9.2.4. Tests for Flavonoids and Tannins

9.2.4.1. Lead Acetate Test

The test solution (1 mL) was mixed with 1-2 drops of $Pb(OAc)_2$ to observe a yellow precipitate.

9.2.4.2. Ferric Chloride Test

A few drops of $FeCl_3$ were added to 1 mL of test solution to observe for a greenish black colouration.

9.2.4.3. Dilute Nitric Acid Test

A few drops of dilute HNO_3 were added to 1 mL of test solution to observe for a red colouration.

9.2.5. Tests for Alkaloids

9.2.5.1. Dragendorff's Test

To 2-3 mL of the test solution, a few drops of Dragendorff's reagent were added to observe for an orange or orange-red precipitate.

9.2.5.2. Mayer's Test

A few drops of Mayer's reagent were added to 2 mL of the test solution to observe a for precipitate.

9.2.6. Test for Saponin Glycosides

9.2.6.1. Foam Test

The test solution (2 mL) was shaken vigorously with water. Observed for persistent foam.

9.2.7. Tests for Cardiac Glycosides

9.2.7.1. Baljet's test (for unsaturated lactone ring)

The test sample (6-8 mg) was dissolved in 2 mL hydro-alcoholic solution. A few drops (1-2) of picric acid were added carefully to the solution. Then, a few drops of NaOH (1 M) were added to observe for a yellow to orange colour.

9.2.7.2. Keller-Killiani test (for deoxy sugars)

The test sample (6-8 mg) was added in 1 mL glacial acetic acid. A few drops of $FeCl_3$ and 2 mL concentrated H_2SO_4 were added carefully to the side of test tube. It was observed for a reddish-brown colour at the junction of the two liquids and the top layer for a bluish green colour.

9.2.8. Test for Anthraquinone Glycosides

9.2.8.1. Modified Borntrager's Test

To the test solution (2 mL), an equal amount of 5% $FeCl_3$ and dilute HCl were added. It was heated for 5 min in a boiling water bath. It was then cooled down under tap water and once cold, an equal volume of benzene (6 mL) was added. It was shaken vigorously, and the organic layer was separated. An equal volume of ammonia was added to the organic layer. It was observed for ammoniacal layer showing a pinkish red colour.

9.3 Results

The summary of the results of the qualitative investigation are presented below in Table 9.1.

Chemical test	***Kawakawa leaf fraction***					
	KK-May20-Water KK-Oct20-Water KK-Dec20-Water	KK-May20-MeOH KK-Oct20-MeOH KK-Dec20-MeOH	KK-Dec20-MeOH-Fraction-1	KK-Dec20-MeOH-Fraction-4	KK-Dec20-MeOH-Fr4-HCCCS-1-t.l.	KK-Dec20-MeOH-Fr4-HCCCS-1-b.l.
Tests for carbohydrates						
Tests for reducing sugars						
Fehling' s test	+	+	+	+	+	+
Benedict' s test	+	+	+	+	+	+
Tests for non-reducing polysaccharides (starch)						
Iodine test	-	-	-	-	-	-
Tannic acid test	-	-	-	-	-	-
Tests for proteins and amino acids						
Biuret test	+	+	-	-	-	-
Ninhydrin test	+	+	+	+	+	+
Xanthoprotein test	+	+	+	+	+	+
Hopkins-Cole test (tryptophan)	-	+	+	-	-	-

(Contd. on next page)

Chemical test	***Kawakawa leaf fraction***					
	KK-May20-Water KK-Oct20-Water KK-Dec20-Water	KK-May20-MeOH KK-Oct20-MeOH KK-Dec20-MeOH	KK-Dec20-MeOH-Fraction-1	KK-Dec20-MeOH-Fraction-4	KK-Dec20-MeOH-Fr4-HCCCS-1-t.l.	KK-Dec20-MeOH-Fr4-HCCCS-1-b.l.
Tests for proteins and amino acids						
Millon' s test (tyrosine)	+	+	-	+	+	+
Test for Cysteine	-	-	-	-	-	-
Tests for steroids						
Salkowski' s test	-	-	-	-	-	-
Liebermann-Burchard Test	-	-	-	-	-	-
Tests for flavonoids and tannins						
Lead acetate test	+	+	-	+	+	-
$FeCl_3$ test	+	+	-	+	+	-
Dilute HNO_3 test	+	+	-	+	+	-

Chemical test	*Kawakawa leaf fraction*				
	KK-May20-Water KK-Oct20-Water KK-Dec20-Water	KK-May20-MeOH KK-Oct20-MeOH KK-Dec20-MeOH	KK-Dec20-MeOH-Fraction-1	KK-Dec20-MeOH-Fraction-4	KK-Dec20-MeOH-Fr4-HCCCS-1-t.l.	KK-Dec20-MeOH-Fr4-HCCCS-1-b.l.
Tests for alkaloids						
Dragendorff' s test	-	-	-	-	-	-
Mayer' s test	-	-	-	-	-	-
Test for saponin glycosides						
Foam test	+	+	-	+	+	-
Tests for cardiac glycosides						
Baljet' s test	+	+	-	+	+	+
Keller-Killiani test	+	+	-	+	+	+
Test for anthraquinone glycosides						
Modified Borntrager's test	-	-	-	-	-	-

Table 9.1. Results of the qualitative investigation of the Kawakawa leaf fractions, '+' corresponds to positive observation, '- 'corresponds to negative observation.

9.3.1. Result of the Qualitative Investigation of the Kawakawa Leaf Water-Fractions

The KK leaf Water-Fractions were positive for the tests of cardiac glycosides, reducing sugars, amino acids, tyrosine, flavonoids and tannins, and saponin glycosides.

9.3.2. Result of the Qualitative Investigation of the Kawakawa Leaf MeOH-Fractions

The KK leaf MeOH-Fractions were positive for the tests of reducing sugars, saponins, amino acids: tests of both tyrosine and tryptophan, flavonoids and tannins, and cardiac glycosides.

9.3.3. Result of the Qualitative Investigation of the KK-Dec20-MeOH-Fraction-1

The KK-Dec20-leaf-MeOH-Fraction-1 was found to be positive for tests of reducing sugars, and amino acids.

9.3.4 Result of the Qualitative Investigation of the KK-Dec20-MeOH-Fraction-4

The KK-Dec20-leaf-MeOH-Fraction-4 was found to be tested positive for tests of flavonoids and tannins, reducing sugars, amino acids, saponins and cardiac glycosides

9.3.5. Result of the Qualitative Investigation of the KK-Dec20-MeOH-Fr4-HCCCS-1-t.l.

The KK-Dec20-MeOH-Fr4-HCCCS-1-t.l. tested positive for tests of flavonoids and tannins, reducing sugars, amino acids, saponins and cardiac glycosides.

9.3.6. Result of the Qualitative Investigation of the KK-Dec20-MeOH-Fr4-HCCCS-1-b.l.

The KK-Dec20-MeOH-Fr4-HCCCS-1-b.l. tested positive for tests of reducing sugars, amino acids, and cardiac glycosides.

9.4. Discussion

This qualitative investigation study was very useful towards finding the preliminary idea of the phytoconstituents in the fractions obtained from the bioassay-guided fractionation of chromatography separation of the KK leaf. The same group of phytoconstituents were present in both the Water and MeOH-Fractions. However, there was the presence of only tyrosine in the Water-Fractions, but the MeOH-Fractions were found to be positive for both tyrosine and tryptophan.

The crude MeOH-Fraction showed the presence of reducing sugars and amino acids (both tyrosine and tryptophan), with cardiac glycosides, flavonoids and tannins. The MeOH-Fraction-1 was found to have reducing sugars and amino acids (only tryptophan). However, there was no presence of cardiac glycosides and flavonoids in this fraction. On the other hand, the MeOH-Fraction-4 was found to have cardiac glycosides, and flavonoids. From the result of **Experiment 7.22** presented in **Chapter 7**, the NLA test of the KK-Dec20-leaf MeOH samples with Batch 13 larvae, it was found that the crude MeOH Extract had an efficacy of 25.3%, the Fraction-1 had efficacy of 10.3% and Fraction 4 had an efficacy of 43.3%. This indicated that the Fraction-4 was the most effective fraction ($P<0.05$) which had presence of cardiac glycosides, flavonoids and tannins. The Fraction-1 was not nearly as effective as the Fraction-4 when studied with Batch 13 larvae. This indicated that the phytoconstituents found in the Fraction-1 (reducing sugars and amino acids) did not possess a high nematocidal property compared to the phytoconstituents (cardiac glycosides, flavonoids and tannins) present in the Fraction-4.

From the result of Experiment 8.1 in Chapter 8, the MeOH-Fr4-HCCCS-1-t.l. and Fr4-HCCCS-1-b.l. had similar efficacy (higher than the parent Fraction-4). However, the concentration of Fr4-HCCCS-1-b.l. was 1 mg/mL and Fr4-HCCCS-1-t.l. was 4 mg/mL, which indicated that the former was effective at a much lower concentration than the latter.

Therefore, this qualitative chemical investigation also was able to provide the preliminary idea about the classes of compounds present in the KK leaf samples. In the next chapter, compound identification and structure prediction from the LC-MS/MS analysis of Fr4-HCCCS-1-b.l. is presented.

Chapter 10. LC-MS/MS Investigation of the Kawakawa Leaf MeOH-HCCCS Fractions and Compounds Identification

LC-MS/MS Investigation of the Kawakawa Leaf MeOH-HCCCS Fractions and Compounds Identification

10.1 Introduction

In this chapter, the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) investigation of the separated HCCCS Fractions of the KK-Dec20-leaf-MeOH-Fraction-4 is presented. LC-MS/MS is a widely-reported, powerful analytic tool used to identify and quantify the phytoconstituents present in plant samples (Boga et al., 2021, Razgonova et al., 2021, Sinaga et al., 2021, El-Hawary et al., 2021, Kabkrathok et al., 2021, Singh et al., 2021). Mass spectrometry (MS) is based on the production of gaseous, positively or negatively charged ions from chemical constituents of a sample that previously are separated by Liquid Chromatography (LC) according to their mass-to-charge (*m/z*) ratio. A second MS can be used to provide tandem mass spectrometry (Glish and Vachet, 2003). The chemical constituents based on their *m/z* ratio can be analysed and identified using compound detection/discovery software that is designed to detect the constituents present in a sample (Guo et al., 2018). The software Compound Discoverer™ is equipped with mzCloud™ and ChemSpider™ online chemical databases which work simultaneously to predict the name, formula, and chemical structure of the phytoconstituents if they are available in these two databases (Reddy et al., 2020, Cocconi et al., 2018). This software detects and identifies all compounds in a single sample (with MS/MS), even compounds with very low abundance. This software works in tandem with the Thermofisher UPLC system, the LC system used in this research. The compound information is predicted with >95% confidence by the software (Scarpone et al., 2020). Thus, it can be a good tool to get a preliminary understanding of the phytoconstituents present in a sample (Scarpone et al., 2020) before confirming the structures with nuclear magnetic resonance (NMR) analysis. However, a pure isolate is necessary for the NMR analysis. A crude plant extract or its separated fractions have many constituents present which restrict NMR analysis (Liu et al., 2022, Pesek et al., 2021, Njanpa et al., 2021). Using both MS and NMR methods, the definite structures of phytoconstituents are elucidated (Vanhaverbeke et al., 2021, Lee and Cho, 2021, Hellal et al., 2021). During the present research with Kawakawa samples there was no attempt to obtain purified fractions/pure isolates as only LC-MS/MS investigations were carried out with the separated HCCCS fractions of the KK-Dec20-leaf-MeOH-Fraction-4. In Chapter 8, the HCCCS-1-t.l. and HCCCS-1-b.l. had impressive nematocidal efficacy and were found be more effective than the parent MeOH-Fraction-4. The objective of this chapter was to propose/elucidate structures of compounds

isolated from these fractions using the chromatographic, analytical and spectroscopic methods that are offered by LC-MS/MS and the Compound Discoverer™ software.

10.2. Materials and Methods

The LC-MS/MS analysis of the HCCCS Fractions were performed using the usual method described in Section 2.3.11.1.2 using both (+) ([M+H]+) and (-) ([M-H]-) ionisation modes. The selected fractions were: HCCCS-1-t.l. (water layer), HCCCS-1-b.l. (chloroform layer), and HCCCS-4-t.l. (hexane layer). The chromatograms of each fraction were analysed using the Compound Discoverer 2.1™ software (*ThermoFisher Scientific Inc.*). The workflow '*Max ID - Detect Unknowns with ID Using Online Database Searches Single Sample*' was selected. The compounds (based on the molecular weight) having ≥85% best match in both mzCloud™ (*HighChem LLC*) and ChemSpider™ (*Royal Society of Chemistry*) databases were selected. The structures of the predicted compound were exported from the software and redrawn in ChemDraw Ultra™ (*PerkinElmer Inc.)* software.

10.3. Results

A total of 34 compounds were detected from HCCCS-1-t.l., HCCCS-1-b.l. and HCCCS-4-t.l. Fractions with ≥85% match in mzCloud™ and ChemSpider™ databases. In Table 10.1, the list of these compounds with their molecular weight, formula, name, structure, and the origin fraction(s) specifying the ionisation mode are presented.

ID	Molecular weight	Formula	Name	Structure	Fraction(s)
1	129.16	$C_8H_{19}N$	Octodrine		HCCCS-1-t.l. (+)
2	132.06	$C_8H_8N_2O_3$	Asparagine		HCCCS-1-t.l. (+)
3	166.98	$C_7H_5NS_2$	2-Mercaptobenzothiazole		HCCCS-1-t.l. (-) HCCCS-4-t.l. (-) HCCCS-1-b.l. (-)
4	180.06	$C_6H_{12}O_6$	D-(-)-Fructose		HCCCS-1-t.l. (-)
5	188.10	$C_9H_{16}O_4$	Azelaic acid		HCCCS-1-t.l. (-)

(Contd. on the next page)

ID	Molecular weight	Formula	Name	Structure	Fraction(s)
6	192.07	$C_{11}H_{12}O_3$	Myristicin		HCCCS-1-b.l. (+)
7	194.08	$C_{11}H_{14}O_3$	Butylparaben		HCCCS-1.b.l. (-)
8	198.05	$C_9H_{10}O_5$	Syringic acid		HCCCS-1-t.l. (-)
9	204.08	$C_{11}H_{12}N_2O_2$	D-(+)-Tryptophan		HCCCS-1-t.l. (-)
10	214.13	$C_{12}H_{25}NO_2$	12-Aminododecanoic acid		HCCCS-1-t.l. (+) HCCCS-4-t.l. (+) HCCCS-1-b.l. (+)

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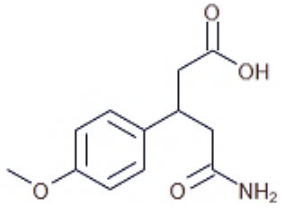
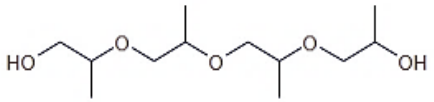
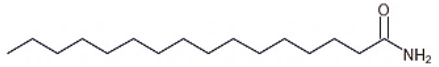
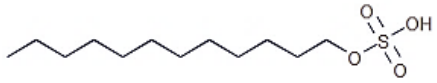
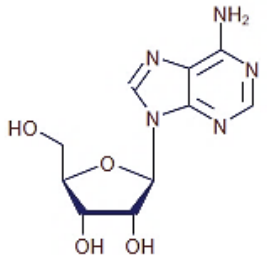
ID	Molecular weight	Formula	Name	Structure	Fraction
11	237.20	$C_{12}H_{15}NO_4$	5-Amino-3-(4-methoxyphenyl)-5-oxopentanoic acid		HCCCS-1-b.l. (+)
12	250.17	$C_{12}H_{26}O_5$	2-{2-[2-(2-Hydroxypropoxy)propoxy]propoxy}-1-propanol		HCCCS-1-t.l (+) HCCCS-1-b.l (+)
13	255.26	$C_{16}H_{33}NO$	Hexadecanamide		HCCCS-1-t.l (+) HCCCS-4-t.l. (+) HCCCS-1-b.l (+)
14	266.15	$C_{12}H_{26}O_4S$	Dodecyl hydrogen sulfate		HCCCS-1-t.l. (+)
15	267.10	$C_{10}H_{13}N_4O_4$	Adenosine		HCCCS-1-t.l. (+) HCCCS-1-b.l. (+)

ID	Molecular weight	Formula	Name	Structure	Fraction
16	281.28	$C_{18}H_{35}N0$	Oleamide		HCCCS-1-t.l. (+) HCCCS-4-t.l. (+) HCCCS-1-b.l. (+)
17	284.26	$C_{18}H_{37}NO$	Stearamide		HCCCS-1-t.l. (+) HCCCS-4-t.l. (-) HCCCS-1-b.l. (+)
18	308.22	$C_{15}H_{32}O_6$	2,5,8,11-Tetramethyl-3,6,9,12-tetraoxapentadecane-1,14-diol		HCCCS-1-b.l. (+) HCCCS-4-t.l. (+)
19	326.18	$C_{18}H_{30}O_3S$	4-Dodecylbenzenesulfonic acid		HCCCS-1-t.l. (-)
20	337.34	$C_{22}H_{43}NO$	Erucamide		HCCCS-1-t.l. (+)

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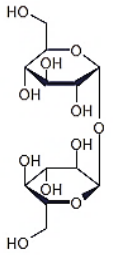
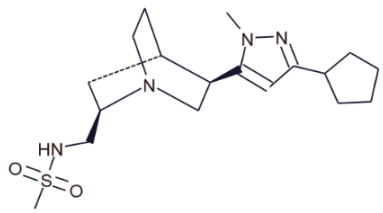
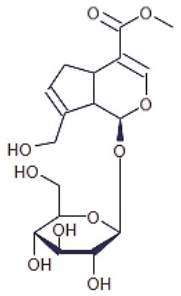
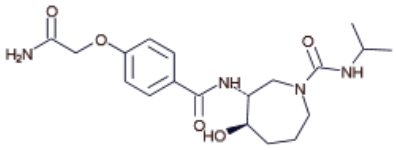
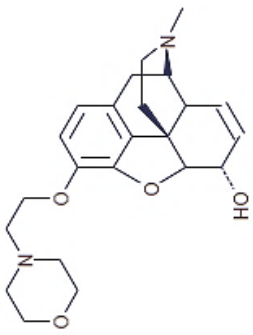
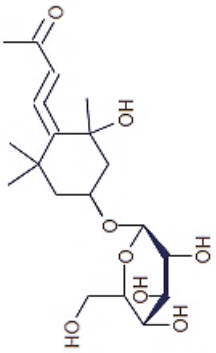
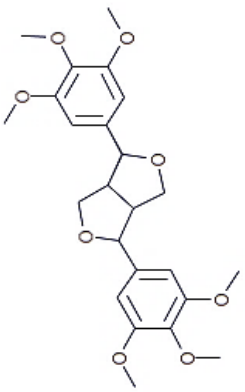
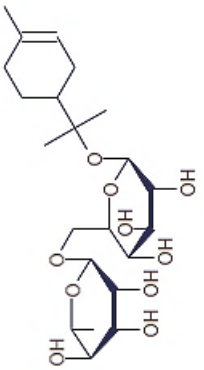
ID	Molecular weight	Formula	Name	Structure	Fraction
21	342.11	$C_{12}H_{22}O_{11}$	α,α-Trehalose		HCCCS-1-t.l. (-)
22	366.20	$C_{18}H_{30}N_4O_2S$	N-{[(2R,4S,5R)-5-(3-Cyclopentyl-1-methyl-1H-pyrazol-5-yl)-1-azabicyclo[2.2.2]oct-2-yl]methyl}-1-sulfonamide		HCCCS-1-t.l. (+)
23	388.13	$C_{17}H_{24}O_{10}$	Geniposide		HCCCS-1-t.l. (-)
24	392.29	$C_{19}H_{28}N_4O_5$	(3R,4R)-3-{[4-(2-Amino-2-oxoethoxy)benzoyl]amino}-4-hydroxy-N-isopropyl-1-azepanecarboxamide		HCCCS-1-t.l. (+) HCCCS-1-b.l. (+)

ID	Molecular weight	Formula	Name	Structure	Fraction
25	398.22	$C_{23}H_{30}N_2O_4$	Pholcodine		HCCCS-1-t.l. (+)
26	432.19	$C_{19}H_{30}O_8$	3-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-buten-1-ylidene)cyclohexyl β-D-glucopyranoside		HCCCS-1-t.l. (-)
27	446.20	$C_{24}H_{30}O_8$	Diayanagambin		HCCCS-1-b.l. (+)
28	462.24	$C_{22}H_{38}O_{10}$	2-(4-Methyl-3-cyclohexen-1-yl)-2-propanyl 6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside		HCCCS-1-t.l. (-)

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ID	Molecular weight	Formula	Name	Structure	Fraction
29	474.16	$C_{21}H_{30}O_{12}$	4-Allyl-2-(β-D-glucopyranosyloxy)phenyl β-D-glucopyranoside		HCCCS-1-t.l. (-) HCCCS-4-t.l. (-)
30	594.15	$C_{27}H_{30}O_{15}$	5,7-Dihydroxy-2-(4-hydroxyphenyl)-6,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]-4H-chromen-4-one		HCCCS-1-t.l. (-)
31	594.16	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside		HCCCS-1-t.l. (+)

(Contd. on the next page)

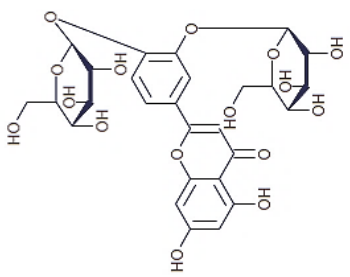
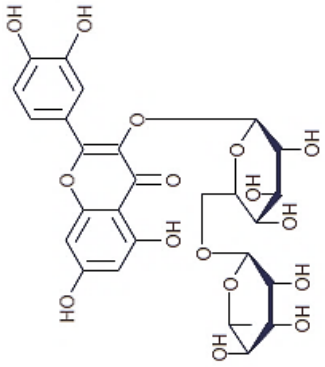
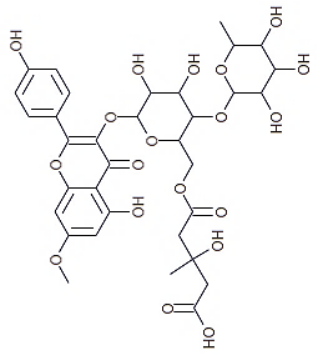
ID	Molecular weight	Formula	Name	Structure	Fraction
32	610.16	$C_{27}H_{30}O_{16}$	4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-(β-D-glucopyranosyloxy)phenyl β-D-glucopyranoside		HCCCS-1-t.l. (+)
33	610.15	$C_{27}H_{30}O_{16}$	Rutin		HCCCS-1-t.l. (-)
34	752.21	$C_{34}H_{40}O_{19}$	5-Hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4-oxo-4H-chromen-3-yl 6-O-(4-carboxy-3-hydroxy-3-methylbutanoyl)-4-O-(6-deoxyhexopyranosyl)hexopyranoside		HCCCS-1-t.l. (-)

Table 10.1. The list of compounds with their molecular weight, formula, name, structure, and the origin HCCCS fraction(s) with ionisation mode used (specified in parenthesis).

In Table 10.2, the compounds are specified accordingly to their chemical classes. The number of compounds detected by their classes of compounds were: 6 amides, 6 glycosides, 5 flavonoids, 4 amino acids, and 2 carbohydrates.

Compound ID	***Chemical name***	***Chemical class***
2	Asparagine	Amino acid
9	D-(+)-Tryptophan	
10	12-Aminododecanoic acid	
11	5-Amino-3-(4-methoxyphenyl)-5-oxopentanoic acid	
15	Adenosine	
13	Hexadecanamide	Amide
16	Oleamide	
17	Stearamide	
20	Erucamide	
22	N-{[(2R,4S,5R)-5-(3-Cyclopentyl-1-methyl-1H-pyrazol-5-yl)-1-azabicyclo[2.2.2]oct-2-yl]methyl}-1-sulfonamide	
24	(3R,4R)-3-{[4-(2-Amino-2-oxoethoxy)benzoyl]amino}-4-hydroxy-N-isopropyl-1-azepanecarboxamide	
4	D-(-)-Fructose	Carbohydrate
21	α,α-Trehalose	
8	Syringic acid	Flavonoid
12	2-{2-[2-(2-Hydroxypropoxy)propoxy]propoxy}-1-propanol	
17	2,5,8,11-Tetramethyl-3,6,9,12-tetraoxapentadecane-1,14-diol	
30	5,7-Dihydroxy-2-(4-hydroxyphenyl)-6,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]-4H-chromen-4-one	
34	5-Hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4-oxo-4H-chromen-3-yl 6-O-(4-carboxy-3-hydroxy-3-methylbutanoyl)-4-O-(6-deoxyhexopyranosyl)hexopyranoside	

(Contd. on the next page)

Compound ID	***Chemical name***	***Chemical class***
26	3-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-buten-1-ylidene)cyclohexyl β-D-glucopyranoside	Glycoside
28	2-(4-Methyl-3-cyclohexen-1-yl)-2-propanyl 6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside	
29	4-Allyl-2-(β-D-glucopyranosyloxy)phenyl β-D-glucopyranoside	
31	Kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside	
32	4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-(β-D-glucopyranosyloxy)phenyl β-D-glucopyranoside	
33	Rutin	
1	Octodrine	Alkylamine
25	Pholcodine	Alkaloid
3	2-Mercaptobenzothiazole	Thioamide
5	Azelaic acid	Dicarboxylic acid
6	Myristicin	Terpene
23	Geniposide	Terpene glycoside
7	Butylparaben	Parahydroxybenzoate
14	Dodecyl hydrogen sulfate	Anionic surfactant
19	4-Dodecylbenzenesulfonic acid	Sulfonic acid
27	Diayanagambin	Furan

Table 10.2. The list of detected compounds classified by their chemical class.

Figure 10.6. LC-MS chromatogram of the HCCCS-4-t.l. with (-) ionisation mode. The highlighted number above the peak represents the compound detected from Table 10.1. The number above the peaks specifies the mass of the base peak present in the fragment.

10.4. Discussion

In this chapter, the LC-MS/MS investigation to identify the compounds of the HCCCS Fractions of the KK-Dec20-leaf-MeOH-Fraction-4 is presented. A total of 34 compounds were identified: 6 amides, 6 glycosides, 5 flavonoids, 4 amino acids, 2 carbohydrates and 1 alkylamine, alkaloid, thioamide, dicarboxylic acid, terpene, terpene glycoside, parahydroxybenzoate, anionic surfactant, sulfonic acid, and furan. From the literature, the compounds that were already reported to be present in KK are as follows: Myristicin (**6**), Stearamide (**17**), Erucamide (**20**), 4-Dodecylbenzenesulfonic acid (**19**), Kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside (**31**), Diayanagambin (**27**), and 4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-(β-D-glucopyranosyloxy)phenyl β-D-glucopyranoside (**32**) (Briggs, 1941, Lei et al., 2015, Butts et al., 2019b). From the results of **Chapter 8**, both the HCCCS-1-t.l. and HCCCS-1-b.l. Fractions had better nematocidal efficacies than IVM and the parent Fraction-4. It was found that the HCCCS-1-b.l. when combined with IVM, had high efficacy (~95%) against the resistant larval population of Batch 13. The HCCCS-1-b.l. was found to contain the previously reported compounds **6**, **17**, and **27**. Nevertheless, there was no previous report of the anthelmintic efficacy of any of these compounds or for the newly detected compounds in this study. However, some of these compounds have many reported applications in biomedical and other fields. For example, Myristicin (**6**) has been found to have insecticidal properties and has been reported to enhance the efficacy of other insecticides in combination (Lichtenstein and Casida, 1963); Diayangambin (**27**) has been studied to have immunosuppressive and anti-inflammatory effects (De León et al., 2002); Oleamide (**16**) has been shown to accumulate in the cerebrospinal fluid during sleep deprivation and induces sleep in animals (Ahn et al., 2009); Adenosine (**15**) has been used for cardiac related issues in both humans and animals (Mitchell and Lazarenko, 2008). From the literature, there is no report of the compound 2-Mercaptobenzothiazole (**3**), commonly known as MBT, present in any plant extract. This compound is used widely for vulcanisation of rubber (Engels et al., 2004). MBT also has reported usage in veterinary dermatology and is also one of the active ingredients in some topical medications at 1-2% concentrations applied in wide variety of canine dermatoses (Zeerak et al., 2016, Fisher, 1975, Rossoff, 1974). MBT (**3**) was detected in the HCCCS-1-b.l. and HCCCS-4-t.l. Fractions using (-) ionisation mode. It was present in the HCCCS-1-t.l. Fraction but in very low abundance as evidenced from the peak height. However,

in HCCCS-4-t.l. it had a very sharp and tall peak, which indicated a high abundance of this compound in this fraction. MBT can be found in many rubber related products (Crepy, 2016) such as rubber tips, gloves, rubber bands. As the author was aware of this use there is confidence that the peak corresponding to MBT was definitely from the KK-HCCCS Fractions while using the (-) ionisation mode and not due to contamination during sample preparation. This was confirmed using the LC-MS/MS investigation of a blank sample (water) in (-) ionisation mode which did not exhibit this peak (result presented in **Appendix 10.1**). Thus, the occurrence of MBT In KK Fraction was a very interesting and novel observation from this study.

Therefore, the HCCCS Fractions were found to have many compounds which have many reported biomedical and other applications. However, many of these compounds have not previously been reported in the literature and are novel to the present research. Further NMR studies are needed to confirm the identity of the compounds. Nonetheless, this LC-MS/MS investigation has given a preliminary indication of the compounds present. This is a valuable finding which can be used towards future anthelmintic development and overcoming the drug resistance issue as this has provided information about compounds having anthelmintic efficacy which have not been previously reported. In the next chapter, **Chapter 11**, the cytotoxicity evaluation of the KK-Dec20-MeOH-Fraction 4 and a few other KK fractions are presented.

Chapter 11. Cytotoxic Effects of a Few Selective Kawakawa Leaf Fractions on Small Intestinal Epithelial Cells

Cytotoxic Effects of a Few Selective Kawakawa Leaf Fractions on Small Intestinal Epithelial Cells

11.1. Introduction

KK is a medicinal plant with no known toxic effects (Briggs, 1941, Butts et al., 2019b). Previously, the amides derived from KK fruit were reported to have complete lack of cytotoxicity when tested with immortalized bone marrow-derived mesenchymal stem cell line but exhibited selective cytotoxicity towards colon cancer cell line (Lei et al., 2015). Nevertheless, this fact did not necessarily provide any concrete evidence as to whether crude solvent fractions and the separated fractions of the KK leaf which exhibited nematocidal activity throughout the previous chapters, will possess any toxicity effect to the biological cells. Therefore, the cytotoxicity assessment of the KK leaf fractions needed to be evaluated. The MTT and lactate dehydrogenase (LDH) assays for cell viability and proliferation assessment are two widely reported techniques to evaluate the cytotoxicity of plant extracts (Mehrbod et al., 2021, Sophia et al., 2022, Siddiqui et al., 2021, Yeap et al., 2021, Tumewu et al., 2021). The MTT reagent, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, is a light-sensitive, water-soluble tetrazolium dye (Rooney et al., 2000). LDH is a soluble cytoplasmic enzyme found in cells. It gets released into extracellular space if the plasma membrane is damaged (Burd and Usategui-Gomez, 1973). In the MTT and LDH assay, the nicotinamide-adenine-dinucleotide (NADH) coenzyme and dehydrogenases from the biological cells reduce the tetrazolium salts to strongly coloured and lipophilic formazan products, which are quantified by absorbance. The intensity of the colour is measured at 570 nm (Stockert et al., 2018, Chan et al., 2013). The MTT assay is used to detect the metabolic activity of the cells and the LDH assay is used to evaluate the plasma membrane damage of the cell (Stockert et al., 2018).

The toxicity assessment of the solvent fractions and their separated fractions from the KK leaf were conducted using these two assays. In this chapter, the methodology of the cytotoxicity assays and the results are presented. Note that the cytotoxicity assessment of the KK leaf fractions was not performed in Massey University by Gupta. The research was accomplished in collaboration with *Trinity Bioactives, Wellington, New Zealand* led by Davis. The KK leaf fractions, and the standard anthelmintics were provided by Gupta. The study design, and various protocols of the assays mentioned in the methodology were established by Davis and Gupta. The research was conducted, and the data were supplied by Davis.

11.2. Materials and Methods

11.2.1. Activation of Epithelial Cells

Epithelial cells have been shown to respond to stimulators and to be activated to an inflammatory state (Kelly et al., 2019). Epithelial cells derived from the small intestine of human were cultured in the presence of the test samples for a defined period. After this period, the effects on cell viability and on toxicity were determined by MTT and lactate dehydrogenase (LDH) assays respectively.

11.2.2. Preparation of the Kawakawa Leaf fractions and the Reference Standards

A series of KK leaf fractions was taken for their cytotoxicity assessment along with the reference standards IVM and BZ. Each of the KK fractions and the reference standards were suspended in PBS at their respective concentration as presented in Table 11.1.

Test sample	***Stock solutions (in PBS)***	***Final concentration***
KK-Dec20-leaf-MeOH-Fr-1	7 mg/mL	0.7 mg/mL
KK-Dec20-leaf-MeOH-Fr-4	7 mg/mL	0.7 mg/mL
KK-Oct20-leaf-MeOH-Fr-1-4	7 mg/mL	0.7 mg/mL
KK-Oct20-leaf-Water-Fr-1	7 mg/mL	0.7 mg/mL
KK-Dec20-leaf-Water	7 mg/mL	0.7 mg/mL
KK-Dec20-leaf-MeOH	7 mg/mL	0.7 mg/mL
BZ	1 mg/mL	0.1 mg/mL
IVM	1 mg/mL (in 15% EtOH)	0.1 mg/mL

Table 11.1. Preparation of the test samples in stock PBS solutions and the final concentrations.

11.2.3. Characterisation of the Test System

The human small intestine epithelial cells (FHs 74 Int) (ATCC, Cat. No. CCL241) were stored in liquid nitrogen. The hybri-Care Medium (ATCC, Cat. No. 46-X) supplemented with 30 ng/mL epidermal growth factor (EGF), foetal bovine serum (FBS) (10%), penicillin (100 U) and streptomycin (100 µg/mL). The epidermal growth Factor (EGF) (Sigma Aldrich, Cat. No. E9644) was stored at -20°C. The foetal bovine serum (FBS) (Moregate, Cat. No. FBSF) was stored at -

20 °C. The Penicillin-streptomycin (Gibco, Cat. No. 15140122) was stored at -20 °C. Insulin (bovine) (Sigma, Cat. No. I-0156) was stored at -20 °C. The Hanks Balanced Salt Solution (HBSS) (Gibco, Cat. No. 14185-052) was stored at 4 °C. Phosphate buffered saline (PBS) (Gibco, Cat. No 10010-023) was stored at 4 °C. The Trypsin-EDTA solution was 0.5% Trypsin in EDTA (Invitrogen Cat. No. 15400054) (x10 in stock). The MTT reagent (Sigma, Cat. No. M2128) was dissolved in PBS at 5 mg/mL and stored at 4 °C as the working solution. The MTT lysis buffer: 10% SDS/45% dimethyl formamide was made by dissolving 20 g sodium dodecyl sulphate (SDS) in 100 mL double distilled water (DDW), added 90 mL of dimethyl formamide to SDS solution. The pH was adjusted to 4.7 with glacial acetic acid, and then DDW was added up to 200 mL). The lactate dehydrogenase assay kit (Abcam, Cat. No. AB65393) was stored at -20 °C.

11.2.4. Cell Preparation, Harvesting, and Culturing of Human Small Intestinal Epithelial Cells

The frozen stock cell line (FHs 74 Int cells) was removed from liquid nitrogen and immediately thawed in a 37°C water bath. The contents were then transferred to a 250 mL (75 cm^2) culture flask containing 20 mL Hybri-care medium containing 10% FBS and EGF (final concentration 30 ng/mL) plus 100 µg/mL penicillin and 100 µg/mL streptomycin (pre-warmed to 37°C). The cells were cultured at 37 °C in 95% air/5% CO_2 for 24 hours. The medium in the flask was removed and fresh medium added. The cells were then cultured until they reached approximately 80% density/confluence. The cells were examined daily for confluence under the microscope. Once 80% confluence was reached the cell culture was then split and fresh media added. The new flasks were grown at 37 °C in 95% air/5% CO_2 until the cells reached approximately 80% density/confluence. The medium was changed every 3-4 days.

11.2.4.1. Harvesting the Cells

To detach the adherent cells, the medium was removed from the culture flasks and the adherent cells washed with PBS to remove any traces of FBS. Then 5 mL of 0.25% Trypsin/EDTA solution was added and incubated at 37°C for 5 min until all the cells had detached. The trypsin was then neutralised by adding an equal volume of pre-warmed Hybri-care medium and supplements and centrifuged at 125 g (500 rpm) for 7 min at 4 °C. The supernatant from each flask was discarded and the cell pellets re-suspended with 10 mL of pre-warmed medium containing FBS, EGF, penicillin and streptomycin. The cell number was

counted and adjusted to 8 x 10^4 cells/mL. A total volume of approximately 6 mL of cells at 8 x 10^4 cells/mL was required.

11.2.4.2. Cell Culturing

A flat-bottomed 96-well plate was used to treat for cell adhesion. For the assay, 180 µl of the cell suspension (8 x 10^4 cells/mL) was plated into wells A1-6, B1-3, C-E1-6 maintaining approximately 1.52 x 10^4 cells/well. An aliquot (180 µL) of medium only was added to wells A7-12, B7-9, C-E7-12. The plate was then placed in the incubator for 18 hrs to allow the cells to adhere to the wells. After this period, the plate was removed from the incubator and centrifuged at 300 g for 5 minutes at 4 °C. The supernatants were discarded and replaced by 180 µL of the medium and 20 µL of the test samples. The plate was incubated at 37 °C in 95% air/5% CO_2 for a further 16 hrs. At the conclusion of the incubation, the supernatant was removed from each well and stored in a separate plate at -20 °C.

11.2.4.3. Cell Proliferation (MTT) Assay

At the end of the incubation, the cells were resuspended in 200 µL of the culture medium and 20 µL of MTT working solution (5 mg/mL) was added to the relevant wells and incubated for an additional 4 hours at 37 °C. MTT lysis buffer (100 µL) was then added to each of these wells and the plate incubated overnight at 37 °C. The absorbance of each well was read using the VersaMax microplate reader at 570 nm.

11.2.4.4. Cell Toxicity Assay

Aliquots of each culture media collected at the conclusion of the incubations was used for determining the lactate dehydrogenase activity. The instructions provided by the manufacturer of the kit was followed**.**

11.3. Results

11.3.1. Results of Cell Proliferation/Viability

The effects of the test and reference samples on the viability of the cells are presented below in Table 11.2.

Test sample	*Mean (OD 570 nm) ± SEM*	*P value*	*% Inhibition*
Cells Only (PBS)	0.94 ± 0.03	-	-
Cells Only (15% EtOH/PBS)	0.53 ± 0.01	-	-
IVM	0	<0.05	99.2
BZ	0.87 ± 0.02	NS	5.7
KK-Dec20-leaf-MeOH-Fr-1	0.96 ± 0.01	NS	-2.0
KK-Dec20-leaf-MeOH-Fr-4	0.70 ± 0.03	<0.05	26.3
KK-Oct20-leaf-MeOH-Fr-1-4	0.56 ± 0.006	<0.05	40.3
KK-Oct20-leaf-Water-Fr-1	0.69 ± 0.03	<0.05	26.1
KK-Dec20-leaf-Water	0.97 ± 0.009	NS	-3.6
KK-Dec20-leaf-MeOH	0.68 ± 0.04	<0.05	27.7

Table 11.2. Cell viability as measured with MTT assay. The % inhibition or stimulation of each test sample is compared with appropriate Cells Only. Statistical significance is P<0.05 compared to appropriate Cells Only. SEM=Standard Error of Mean, NS=Not Significant.

The KK-Dec20-leaf-MeOH-Fr-1), and KK-Dec20-leaf-Water had no effect on the viability/proliferation of the intestinal epithelial cells. The other four fractions inhibited the viability/proliferation of the cells. The inhibitions were between 26.1% and 27.7% for them except for the KK-Oct20-leaf-MeOH-Fr-1-4 which produced a 40.3% inhibition.

11.3.2. Results of Cytotoxicity

The cytotoxic effects of each of the test samples was determined by the level of LDH activity of the cells cultured in the presence of the test samples when compared with activity of cells cultured without any test samples. The LDH activities are presented below in Table 11.3.

Test sample	*Mean (OD 570 nm) ± SEM*	*P value*	*% Inhibition*	*% Stimulation*
Cells Only (PBS)	0.106 ± 0.006	-	-	-
Cells Only (15% EtOH/PBS)	0.01 ± 0.009	-	-	-
IVM	0.16 ± 0.007	<0.05	-	1516.2
BZ	0.04 ± 0.008	<0.05	58.9	-
KK-Dec20-leaf-MeOH-Fr-1	0.06 ± 0.01	<0.05	40.0	-
KK-Dec20-leaf-MeOH-Fr-4	0.04 ± 0.004	<0.05	55.1	-
KK-Oct20-leaf-MeOH-Fr-1-4	0.05 ± 0.009	<0.05	47.1	-
KK-Oct20-leaf-Water-Fr-1	0.01 ± 0.016	<0.05	85.2	-
KK-Dec20-leaf-Water	0.02 ± 0.015	<0.05	80.2	-
KK-Dec20-leaf-MeOH	0.06 ± 0.021	NS	43.6	

Table 11.3. Lactate dehydrogenase (LDH) activity expressed as absorbance units at 450 nm. The % inhibition or stimulation of each test sample is compared with appropriate Cells Only. Statistical significance is P<0.05 compared to appropriate Cells Only. SEM=Standard Error of Mean, NS=Not Significant.

None of the six KK fractions stimulated the activity of lactate dehydrogenase (LDH). This indicates that none of them were cytotoxic. In fact, all of them inhibited the activity. Between IVM and BZ, the former stimulated the activity of LDH to a considerable amount (1516.2% stimulation).

11.4. Discussion

KK has been widely reported to be used as a medicinal plant (Briggs, 1941, Butts et al., 2019b, Lei et al., 2015). However, the toxicity assessment of the fractions obtained from the bioassay-guided fractionation and chromatographic separation were necessary. These fractions were selected for this research: KK-Dec20-leaf-MeOH-Fr-1, KK-Dec20-leaf-MeOH-Fr-4, KK-Oct20-leaf-MeOH-Fr-1-4, KK-Oct20-leaf-Water-Fr-1, KK-Dec20-leaf-Water, and KK-Dec20-leaf-MeOH. The outcome from this study helped in evaluating the toxicity of the components present in the crude extract and its separated fractions from the KK leaf. This was the first step towards evaluating the applicability of the active components present in

the effective KK sample as an anthelmintic to carry out the in-vivo research with nematode infested ruminants. If the KK samples were found to be toxic against the mammalian biological cells, it would severely restrict their development and use as a drug for the future in-vivo study. Hence, the assessment of the cytotoxic effects of the KK leaf fractions when added to cultures of small intestinal epithelial cells was very essential research. The MTT and LDH assays have been widely used to assess the cytotoxicity effect of many plant extracts (Mehrbod et al., 2021, Siddiqui et al., 2021).

Each of the six KK leaf fractions was assessed for its effect on the proliferation of intestinal cells in culture. At the time of conducting this research, Trinity Bioactives did not have access to any ovine intestinal epithelial cells. Therefore, the available human intestinal epithelial cells were selected for the research. The cytotoxicity evaluation of isolates and fractions derived from plant extracts with mammalian epithelial cells using LDH and MTT assays have been previously reported (Cvetković et al., 2020, Kelly et al., 2019). The concentration of each KK leaf fraction was chosen to be 7 mg/mL for the cytotoxicity evaluation which was near the optimal concentration of the crude solvent fractions (8 mg/mL) and above the optimal concentration of the separated Water and MeOH Fractions (4 mg/mL), as found from the nematocidal experiment results in **Chapter 7**. Note that the LC_{50} cytotoxic concentration of any of these fractions was not observed. This research was a pilot study towards evaluating the toxicity of the fractions of KK leaf and more work needs to be performed in future. The toxic effects of the KK leaf fractions on the intestinal cells were investigated based on changes in LDH activity. When compared with the untreated cells, none of the KK fractions stimulated LDH activity. This suggested that they were not toxic. In fact, all of them inhibited the low level of activity noted for the reference culture. Two of the three KK leaf fractions had no effect on the proliferation of the cells from the MTT assay. These were the KK-Dec20-leaf-MeOH Fraction-1 and the KK-Dec20-leaf-Water. The other fractions were inhibitory of the cell growth. These were about 26 or 27% for each except for the KK-Oct20-leaf-MeOH-Fraction-1-4 which reduced the cell concentration by 40%. However, since there was no cytotoxic effect from any of these four fractions, these reductions in cell concentration were a consequence of inhibition of cell proliferation and not due to toxicity. Therefore, the KK leaf crude and separated fractions were found to be not toxic. Whereas IVM produced a substantial increase in LDH (1516.3%) indicating that it was toxic to these

cells at the concentration of 1 mg/mL, which was the concentration used throughout all the nematocidal experiments. In the previous experiment chapters of the KK leaf, it has been documented how several KK fractions, both the crude solvent extracts and their separated fractions, were more effective compared to IVM. This finding further strengthens the development and usage of the components present in KK leaf sample as anthelmintics. Moreover, IVM, the standard anthelmintic was found to be toxic as the concentration it was used but none of the KK leaf fractions were. Therefore, based on the study of this chapter, it was concluded that it will be very worthwhile to pursue further research with the KK leaf towards the development of the effective phytoconstituents present in drug forms.

Chapter 12. General Discussion and Future Directions

General Discussion and Future Directions

12.1 General Discussion

In this thesis, the overarching aim was to identify a novel plant compound with anthelmintic activity. Such a compound may have some use in addressing the growing problem of controlling anthelmintic resistant nematodes in livestock. The most likely role would be as an adjunct to enhance the efficacy of existing anthelmintics rather than as a 'standalone' anthelmintic product. The search for such a novel anthelmintic compound explored a number of plant extracts using bioassay-guided fractionation and chromatographic separation to obtain separated and purified fractions from the crude extract. There were three main hypotheses taken during this PhD research as discussed in Section 1.6.1 of **Chapter 1**. These were: **1**) The bioassay-guided fractionation and chromatographic separation of a crude plant extract will result in a series of fractions distinct from one another; **2**) Some of these separated fractions will possess higher anthelmintic efficacy than their parent crude plant extract. The major active phytochemical groups isolated from the active fractions may belong to plant secondary metabolites known for their anthelmintic activity, such as tannins, and alkaloids; and **3**) The efficacy of a sample will not vary depending on the larval population i.e., when tested with two or more batches of larvae having different larval populations. The research towards finding a novel anthelmintic compound was initiated with the Quebracho extract which was the only commercially available source of CT available. Quebracho extract has previously been reported to have both in-vitro and in-vivo anthelmintic properties (Athanasiadou et al., 2000b, Athanasiadou et al., 2000a, Paolini et al., 2005). Nevertheless, all the reported studies in the literature were with its crude extract. Attempts on bioassay-guided fractionation and chromatographic separation of this extract were not previously performed. Such an approach was undertaken in this thesis. It was found that the fractions obtained from the bioassay-guided fractionation of the Quebracho extract had higher efficacy than the parent extract ($P<0.05$). However, further chromatographic separation did not result in a separated fraction with higher anthelmintic efficacy. Moreover, the characterisation of the Quebracho extract and its separated fractions using LC-MS was unsuccessful. A series of different LC methods were created and performed using a Synchronis aQ Column with low particle size, a column designed to provide optimal retention of polar analytes (Jia et al., 2014, Thermofisher-Scientific, 2018). However, it was observed that the Quebracho fractions could not be distinguished from the LC-MS analysis,

and all were found to have comparable chromatograms. The efficacy of the Quebracho fractions were found to be dependent on the larval population. It was found that they were more effective against the *T. circumcincta* species present in a larval population than the *H. contortus* species. Therefore, the 1st (the bioassay-guided fractionation would result in discrete fractions) and 2nd hypotheses (the efficacy of a separated fraction would be higher than the parent fraction) from Section 1.6.1 were found to be correct but, the 3rd hypothesis (the efficacy of a sample will not vary depending on the larval population) was incorrect. Of note was the observation that the Quebracho Water Fraction (QWF), when combined with KK leaf fractions, and studied against 3 batches of larvae (Experiment 7.7, Experiment 7.8, and Experiment 7.9, results presented in **Appendix 7.2.7**, **Appendix 7.2.8** and **Appendix 7.2.9** respectively) was found to have higher efficacy than the QWF ($P<0.05$) alone. However, the efficacy of the combination was lower or only comparable with the KK leaf fractions ($P<0.05$) which indicated that the QWF did not contribute towards a higher efficacy when mixed with the KK leaf fraction. Moreover, the efficacy of the QWF and other Quebracho fractions were lower than the KK fruit and leaf fractions ($P<0.05$) when studied with the same batch of larvae using the same concentration (6 mg/mL). Therefore, despite previously published studies indicating Quebracho extract has useful anthelmintic activity, the present studies found superior activity in extracts of other plants. Consequently, no further investigation of the Quebracho extract was undertaken.

The focus of the research was subsequently shifted towards the endemic NZ medicinal plants. As discussed in **Chapter 1**, a series of NZ plants have been reported to have medicinal properties. However, they were not previously explored for anthelmintic efficacies in the literature. A series of components from these plants were collected and screened for their nematocidal efficacy as presented in **Chapter 4**. The concentration of all the extracts of these plant components was chosen to be 6 mg/mL in PBS as a media for the nematocidal experiments. This was an arbitrary choice later confirmed in dose response test to be an effective concentration. Of those examined at this time, the KK fruit was found to be the most effective. KK is a medicinal plant and has documented usage in traditional Māori medicines (Briggs, 1941). However, the anthelmintic efficacy of this plant has not been reported in the literature. The KK fruit was further subjected to chromatographic separation based on the hypotheses of Section 1.6.1 in **Chapter 5.** It was found that the bioassay-guided fractionation

of the KK fruit resulted in a fraction (KK-fruit-Hexane) having higher nematocidal efficacy than the parent crude MeOH extract ($P<0.05$). Further chromatographic separation of the KK-fruit-Hexane resulted in KK-fruit-Hexane-Fraction-5 having even higher nematocidal efficacy ($P<0.05$). However, further separation of this fraction, did not result in a sub-fraction with any higher nematocidal efficacy. It should be noted that the nematocidal study of many of the separated fractions from these fractions could not be performed as these fractions were not soluble in the PBS based medium, which was the most suitable medium for nematocidal experiments as found in **Chapter 6**. Consequently, any possible anthelmintic activity in these insoluble fractions was not explored and they may have as-yet unidentified activity. The following research was conducted with other plant components of the KK, as presented in **Chapter 6**, which were amenable to nematocidal assay. Screening studies of a medicinal plant through collection of its different components and evaluating their nematocidal efficacy by making extracts of each have been widely reported in the literature (Chhabra et al., 2014, Buza et al., 2020, Ahmed et al., 2020). (Chhabra et al., 2014, Buza et al., 2020, Ahmed et al., 2020). Some medicinal plants are reported to possess the most effective PSM in their root extracts (Baby and Regi Raphael, 2014, Bodke et al., 2013), stembark extracts (Wahab Obeng et al., 2021, De Amorim et al., 2021), leaf extracts (Jan et al., 2021, Hadi et al., 2021), fruit extracts (Wahab Obeng et al., 2021, Chennuru et al., 2021), and flower extracts (Turan and Mammadov, 2020, Ahmed et al., 2020). From the study conducted in Chapter 6, the KK leaf component was found to have higher nematocidal efficacy than any other plant component.

The KK leaf was subjected to further bioassay-guided separation in **Chapter 7**. The KK leaf samples were collected from four different periods covering three different seasons (Summer, Winter and Spring) periods to observe the seasonal effects (if any) on its PSM and subsequent nematocidal efficacies. In the literature, there were reports of studies where effectiveness of a plant fraction has varied with the bioavailability of the PSM in different parts of the plant, season and sampling periods, producing divergent results within the same plant species (Fonseca et al., 2014). However, no such attempt with KK was carried out previously. Throughout the nematocidal experiments conducted in **Chapter 7**, the MeOH and Water Fractions of the leaf sample were found to be the most effective crude solvent fractions. The efficacy of the MeOH and Water Fractions of the Spring and Winter samples when studied against a same batch of larvae were found to be comparable with each other.

However, the LC-MS profile of a few fractions were found to be slightly different. This could be due to the seasonal phytochemical change. Nevertheless, this did not affect the nematocidal efficacy as the efficacies of these fractions were found to be comparable. The separation of MeOH and Water Fractions was attempted first with the Sephadex LH-20 column chromatography. However, it did not result in any separation. The column turned black upon the loading of the KK-Water-Fraction. It is believed that the KK leaf fraction reacted with the Sephadex LH-20 which caused the occurrence of a black precipitation. The separation was achieved with the RP-FC. This separation resulted in poor analyte retention, particularly the highly polar components present in the fraction which flushed out at once during the water elution of the separation. Moreover, the RP-FC had poor repeatability and scale-up. Therefore, an improvised technique, HCCCS, based on CCS was developed which is presented in **Chapter 8**. CCS has been previously used extensively to separate and purify PSM from plant extracts (Friesen et al., 2015, Ito, 2019). A series of solvent systems was developed, and it was found that some of these systems effectively separated the group of phytoconstituents based on their polarity present in the KK leaf fraction. The fractions obtained from this separation were found to have better nematocidal efficacy than the parent fractions and the standard anthelmintics. Therefore, the 1st (the bioassay-guided fractionation and chromatographic separation will result towards discrete fractions) and 2nd (efficacy of a separated fraction will be higher than the parent fraction) hypotheses of Section 1.6.1 were found to be correct for the KK leaf fractions. However, the 3rd hypothesis (the efficacy of a sample will not vary depending on the larval population) was found to be incorrect as the efficacy of any leaf fraction was found to be dependent on the larval population of a batch of larvae.

A series of formulations was prepared with the KK leaf fractions and the standard anthelmintics. The nematocidal experiments were carried out with different batches of L3 nematode larvae. From the nematocidal experiment results of these formulations presented in **Chapter 7** and **Chapter 8**, it was found that these formulations had higher efficacy than the standard anthelmintics. The Formulation 2 in **Chapter 8**, the combination of HCCCS-1-b.l. and IVM, had high efficacy (~95%) against the resistant larval population of Batch 13. The efficacy of this formulation was considerably higher than IVM ($P<0.05$). This was a very important finding as it could lead towards the future anthelmintic development. The anthelmintic resistance issue (Hodgson and Mulvaney, 2017, Lecova et al., 2014, Shalaby, 2013, Waller,

1994, Kaplan, 2004) has been widely documented in **Chapter 1**. There have been no previously published studies with a formulation made with a combination of a plant fraction with a standard anthelmintic which has a higher efficacy than the anthelmintic alone.

The LC-MS/MS and compound identification investigation of HCCCS-1-b.l. in **Chapter 10** identified some compounds that had previously been reportedly found in KK (Briggs, 1941, Lei et al., 2015, Butts et al., 2019b). However, many compounds that had not been previously reported in KK were identified for the first time during this research. Of note is that none of these compounds have previously been reported to have anthelmintic properties making them novel compounds for anthelmintic research. In particular, it was found that Formulation 2 (HCCCS-1-b.l. + IVM) had a higher anthelmintic efficacy with a batch of larvae that was found to be relatively insusceptible to IVM during this study. Consequently, these compounds should be further isolated (see future direction for method) and studied for nematocidal activity in both in-vitro and in-vivo studies. KK has been reported to possess no toxic effect and is well regarded as a medicinal plant (Butts et al., 2019b, Briggs, 1941). Māori medicinal uses of Kawakawa include a range of ailments (eczema, boils, rheumatism, toothache), as a general tonic and for the treatment of various urinary, dermatological, gastrointestinal and respiratory complaints (Riley, 1994). Nevertheless, the toxicity assessment of these separated leaf fractions was necessary to evaluate their potential as a drug. From the cytotoxicity study in **Chapter 11**, none of the KK leaf fractions were found to be cytotoxic at the concentration of 7 mg/mL which was the optimal concentration for most fractions studied for nematocidal activity. This was an important finding as it may qualify the compounds present in the KK leaf fractions for future in-vivo research towards anthelmintic efficacy.

Earlier studies used a set standard dose for assessing nematocidal activity. In later studies a series of dose response studies were undertaken with varying concentrations of the crude solvent fractions and the separated fractions of the KK leaf. It was found that the optimal concentration of the separated fractions was lower than their parent fraction. It should be noted that any compound identified with these in vitro studies would still need to prove that it was effective in vivo. This would require it to survive standard digestion in the gastrointestinal tract and in particular survive passage through the rumen in ruminants. The latter is because few actives are absorbed in the region of the gastrointestinal tract prior to the stomach.

Overall, this thesis has paved the way for future anthelmintic development. More future works needs to be performed with the KK leaf which is addressed in the Future Directions section of this chapter. Below, the strength and limitations of the research performed in this PhD are presented.

12.2. Strength and Novelty of this Research

- The bioassay-guided fractionation and chromatographic separation studies of the Quebracho extract, KK Fruit, and KK leaf were performed. These had not been previously reported in the literature.
- Nematocidal efficacies of a series of NZ endemic medicinal plants were assessed which had not been previously reported.
- The Māori endemic medicinal plant KK was found to have anthelmintic properties which had not been previously reported.
- Development of an improvised chromatographic separation technique, Hand Controlled Countercurrent Separation (HCCCS), to better separate the highly polar, water soluble fraction of the KK leaf.
- Detection of 34 compounds from the KK leaf, only 8 of which were previously reported in the literature. None of these compounds had been previously reported to have anthelmintic property. This makes them novel compounds for future anthelmintic research.
- Development of the formulations with the combination of the effective KK leaf fractions and the standard anthelmintic ivermectin. The combination formulations were found to have better efficacy than the standard anthelmintic. This approach of combination formulations had not been previously reported with plant extracts.

12.3. Limitations of this Research

- Throughout the research, the nematocidal experiments were carried out with batches of larvae that were opportunistically found. Consequently, the different nematode species present in these batches generally varied from one to the other. In addition, as these were generally from investigations into field cases of anthelmintic resistance, it is likely that one or more species would be at least partly resistant to one or more standard anthelmintics. The exact resistance status of any of these batches was

unknown to the present researcher even if for confidentiality reasons this had been determined by these related studies. It is possible there are different efficacies against different species of nematode, and these may vary between fractions. A checkerboard dose response curve with separate nematode species needs to be performed to properly quantify the efficacy against different species.

12.4. Future Directions

- LC-MS/MS and NMR analyses of the purified isolates to obtain structure.
- In-vitro nematocidal study of the pure isolates.
- Cytotoxicity assessment of the pure isolates and LC_{50} determination.
- Disposition and efficacy of the pure isolates in ruminant infested with GIN.
- Chemical synthesis of the pure isolates.

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Appendices

Appendices

Appendix 3. Chapter 3 Appendix

Appendix 3.1. TLC Study of The Quebracho Sample

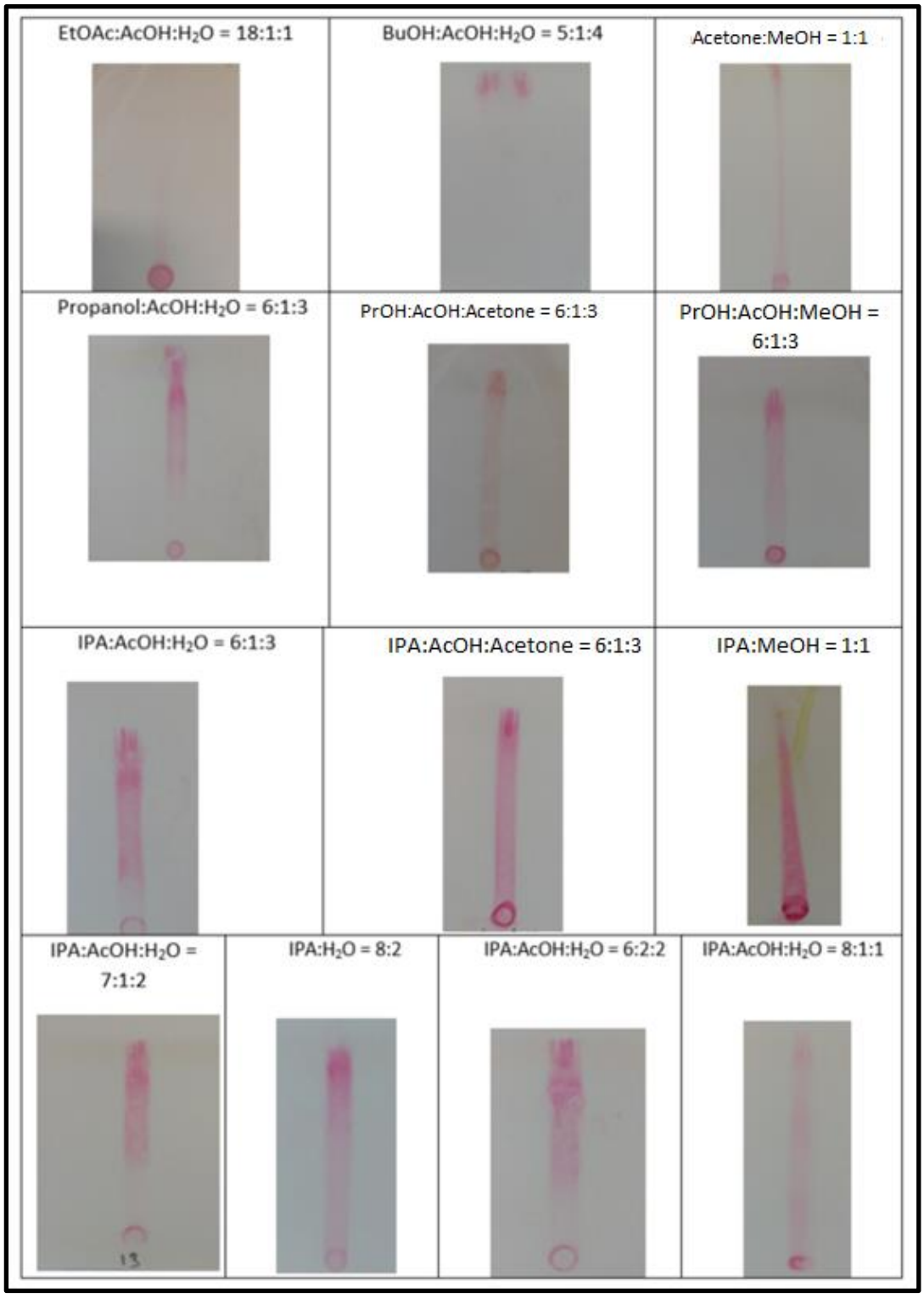


Figure A.1. Pictures of the TLC plate obtained from the respective mobile phases of the TLC analysis of the Quebracho sample.

Appendix 3.2. RP-FC Separation of the Quebracho Samples

Appendix 3.2.1. RP-FC Separation of the QWF Sample

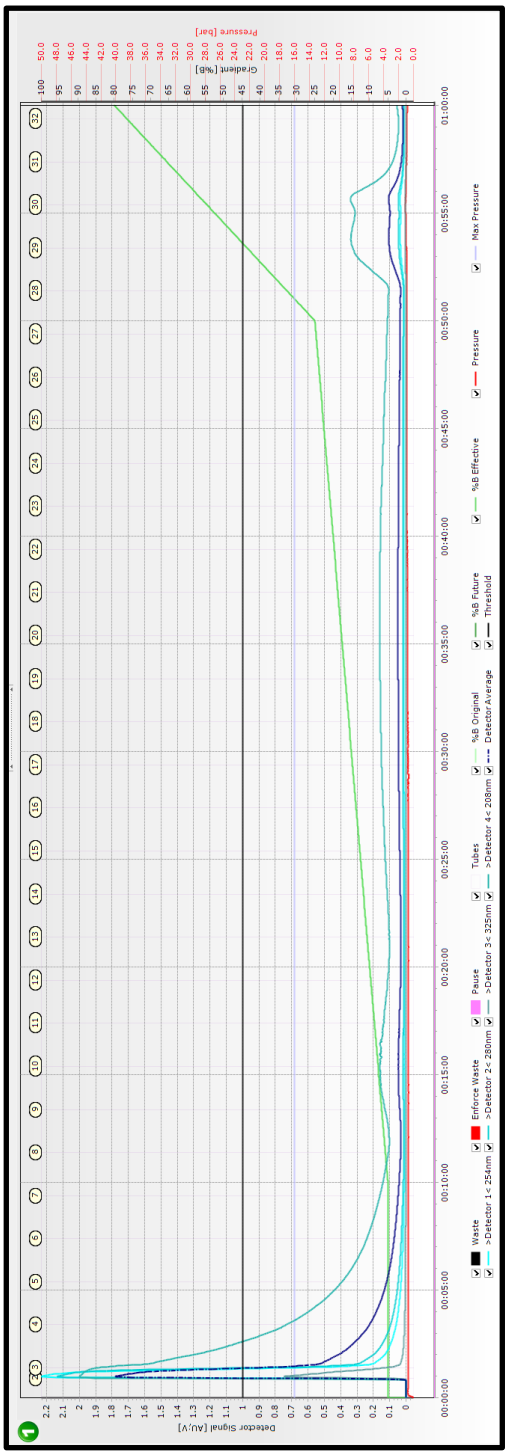


Figure A.2. Flash chromatogram of the RP-FC separation of the QWF.

Appendix 3.2.2. RP-FC Separation of the QBF Sample

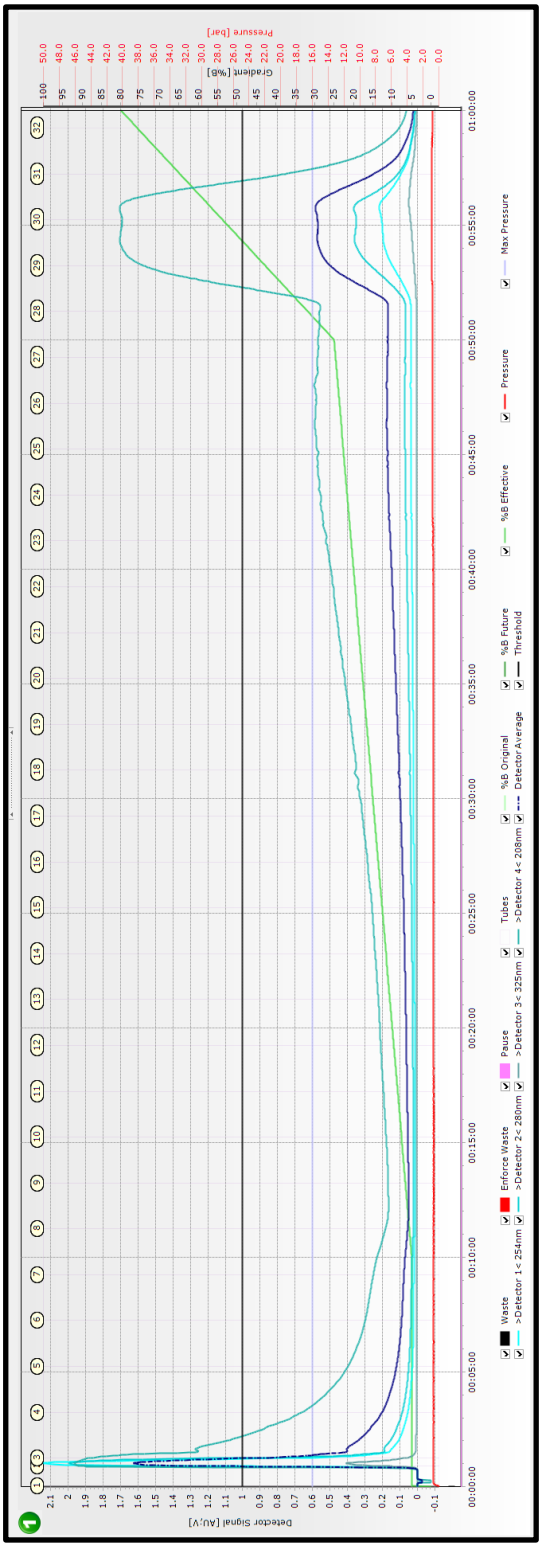


Figure A.3. Flash chromatogram of the RP-FC separation of the QBF.

Appendix 3.3. LC-MS Analysis of the Quebracho Samples

Appendix 3.3.1. LC-MS Analysis of the QWF Sample Using Different methods

(-) ionisation mode:

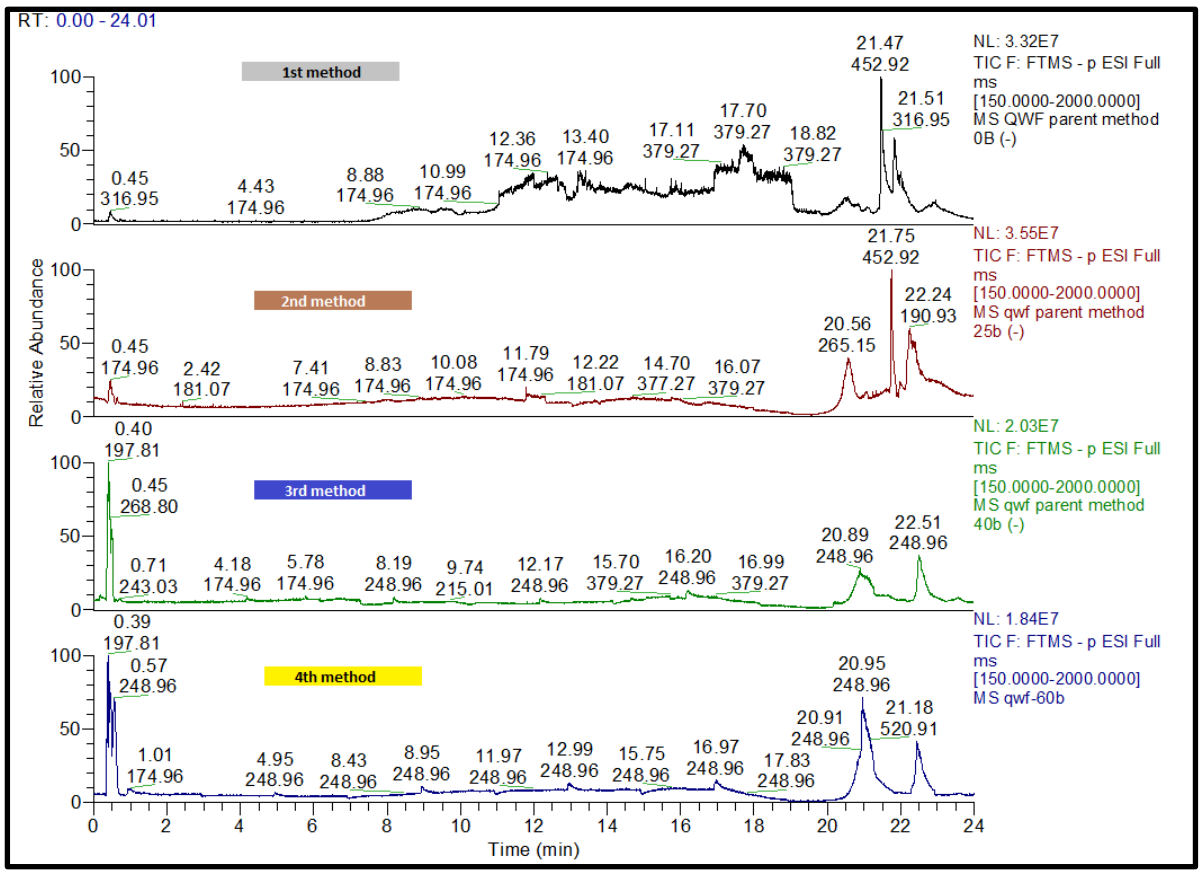


Figure A.4. LC-MS chromatograms of the QWF using different methods (Method 1-4). Labels above peak represent the RT and m/z of base peak.

Appendix 3.3.2. LC-MS Analysis of the QWF Sample Using 3rd and 4th method

(-) ionisation mode:

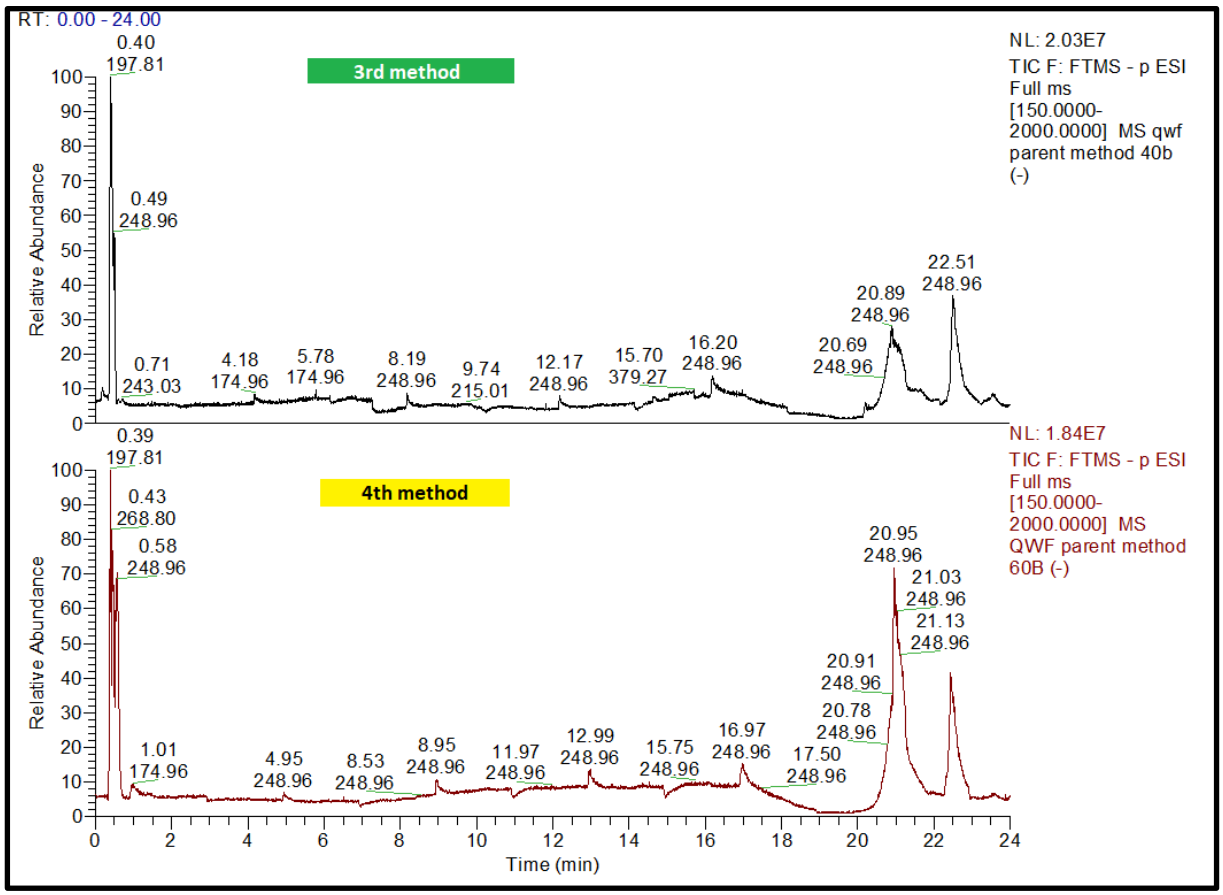


Figure A.5. LC-MS chromatograms of the QWF using different methods (Method 3-4). Labels above peak represent the RT and m/z of base peak.

Appendix 3.3.3. LC-MS analysis of the Different Quebracho Samples

(-) ionisation mode:

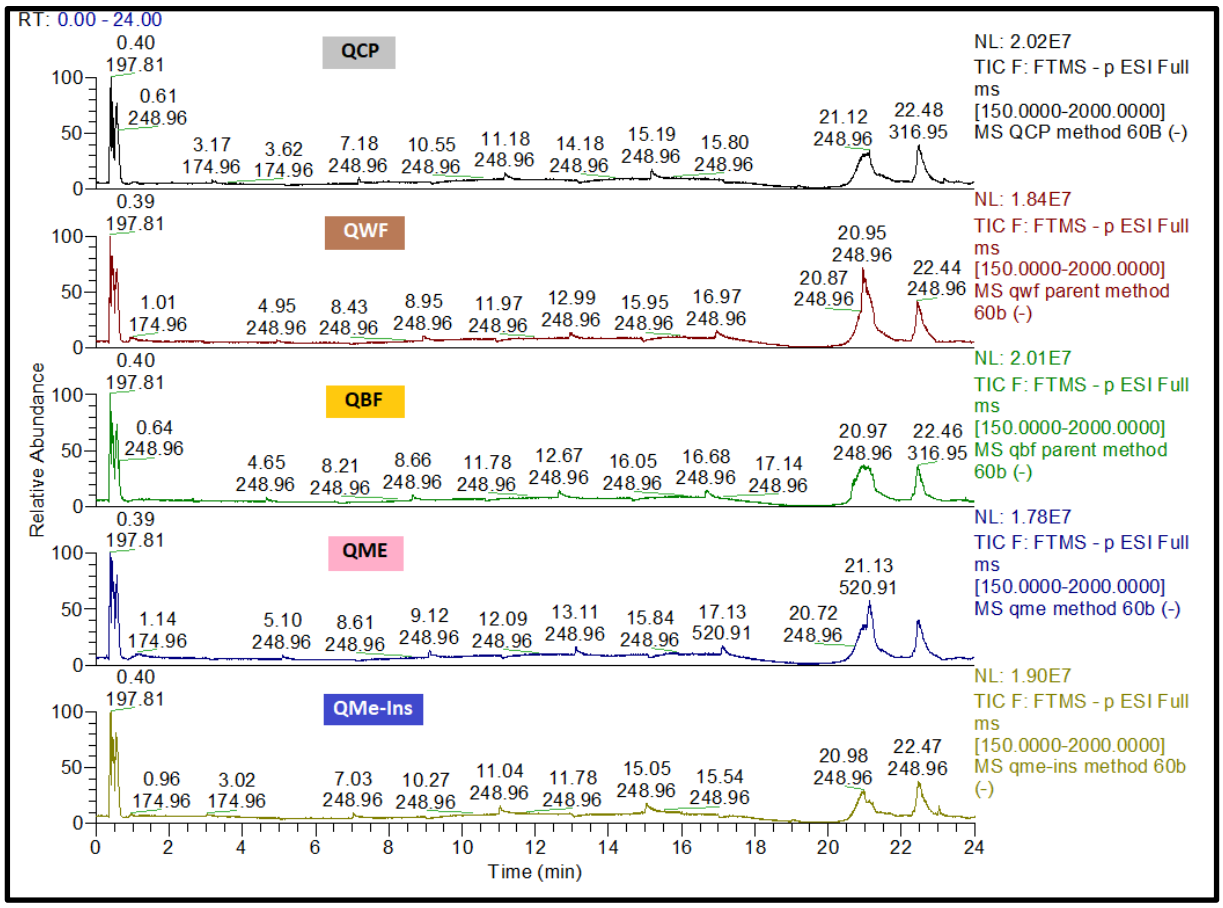


Figure A.6. LC-MS chromatograms of the different Quebracho samples using (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(+) ionisation mode:

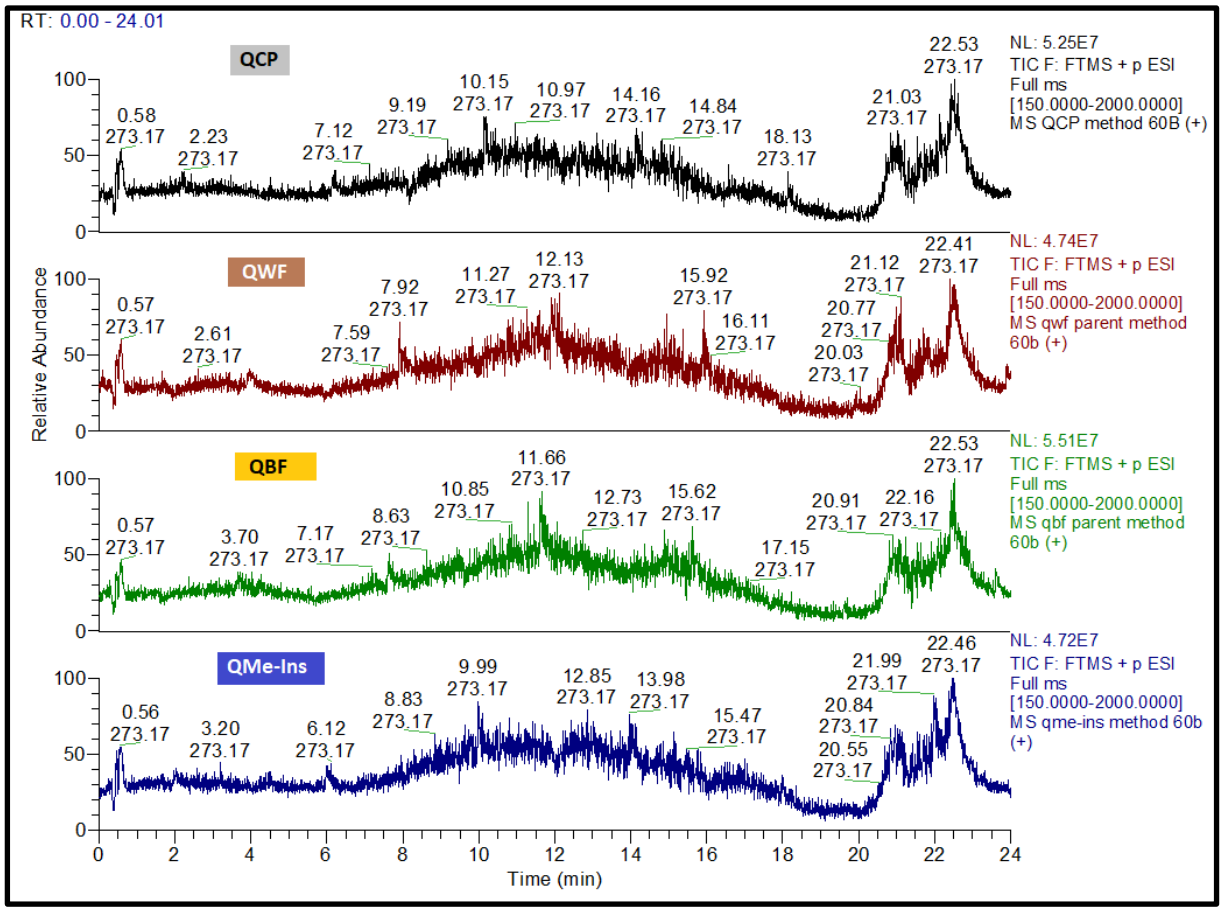


Figure A.7. LC-MS chromatograms of the different Quebracho samples using (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 3.3.4. LC-MS analysis of the QWF and its Separated Fractions from RP-FC

(-) ionisation mode:

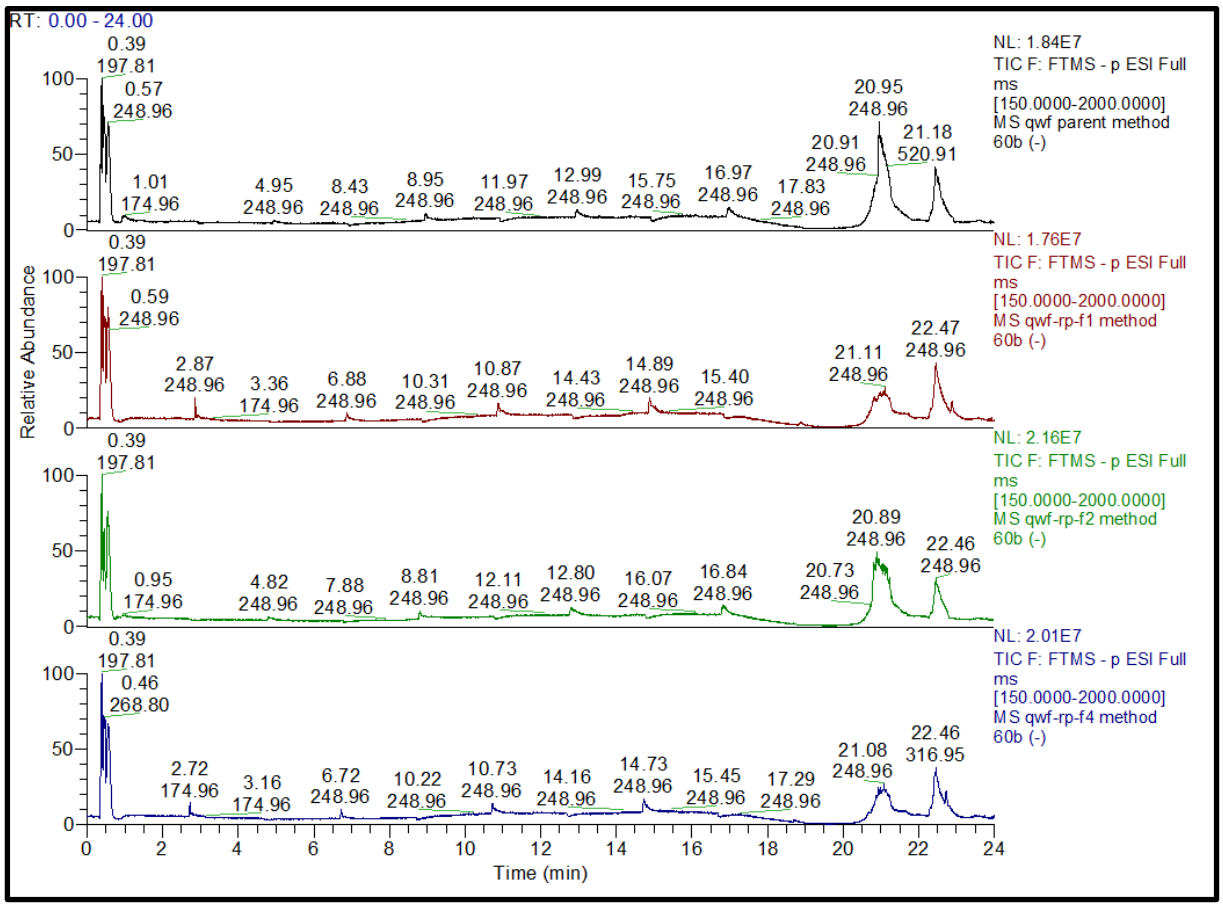


Figure A.8. LC-MS chromatograms of the QWF and its separated fractions from RP-FC using (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(+) ionisation mode:

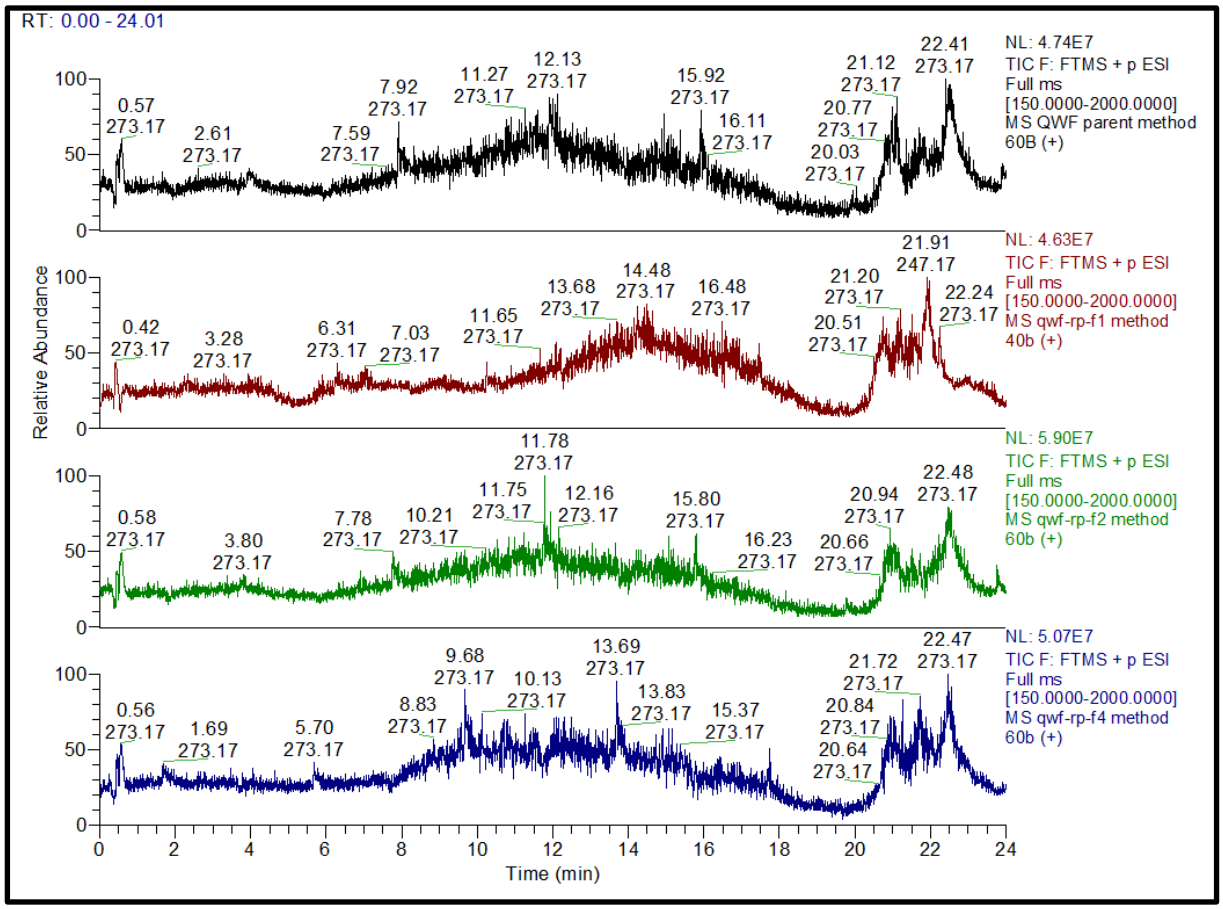


Figure A.9. LC-MS chromatograms of the QWF and its separated fractions from RP-FC using (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 3.3.5. LC-MS analysis of the QBF and its Separated Fractions from RP-FC

(-) ionisation mode:

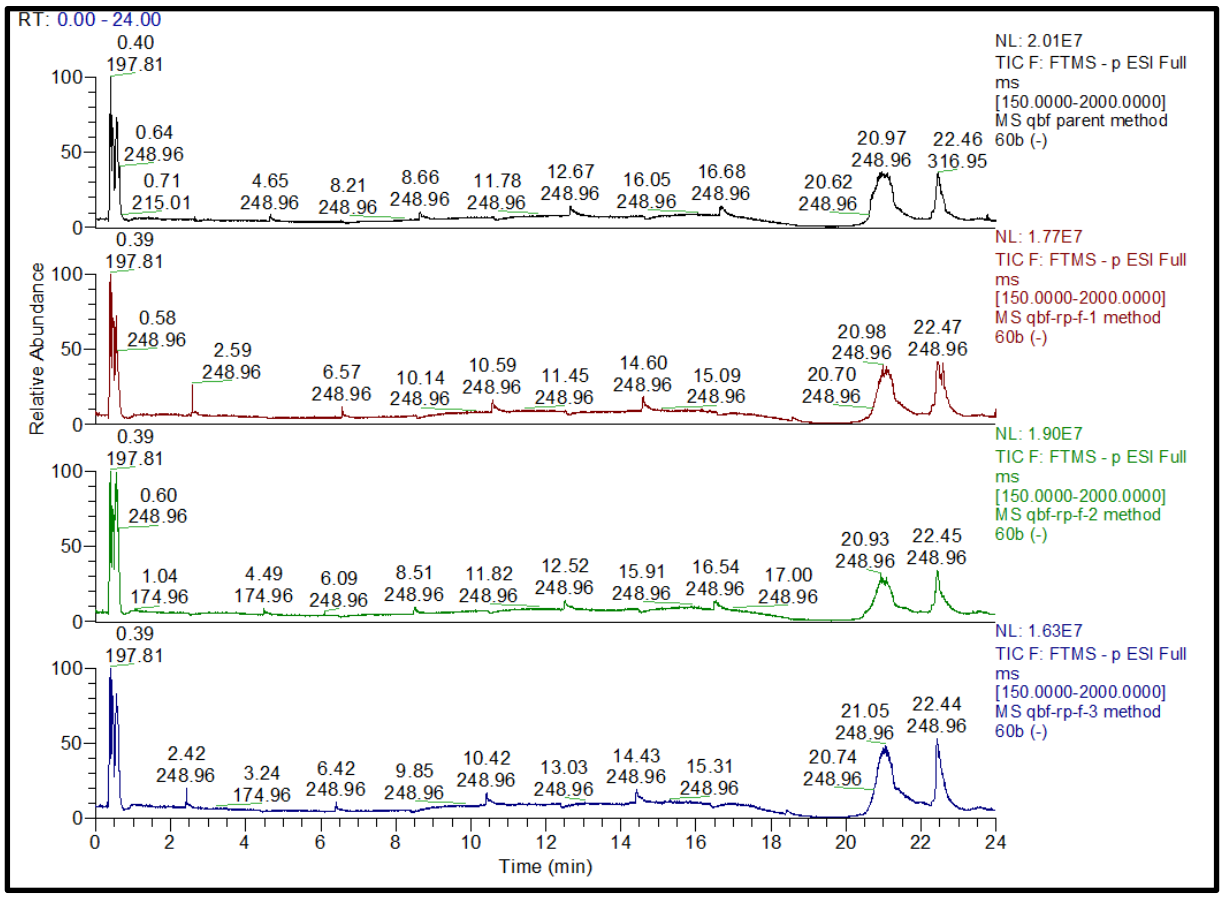


Figure A.10. LC-MS chromatograms of the QBF and its separated fractions from RP-FC using (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(+) ionisation mode:

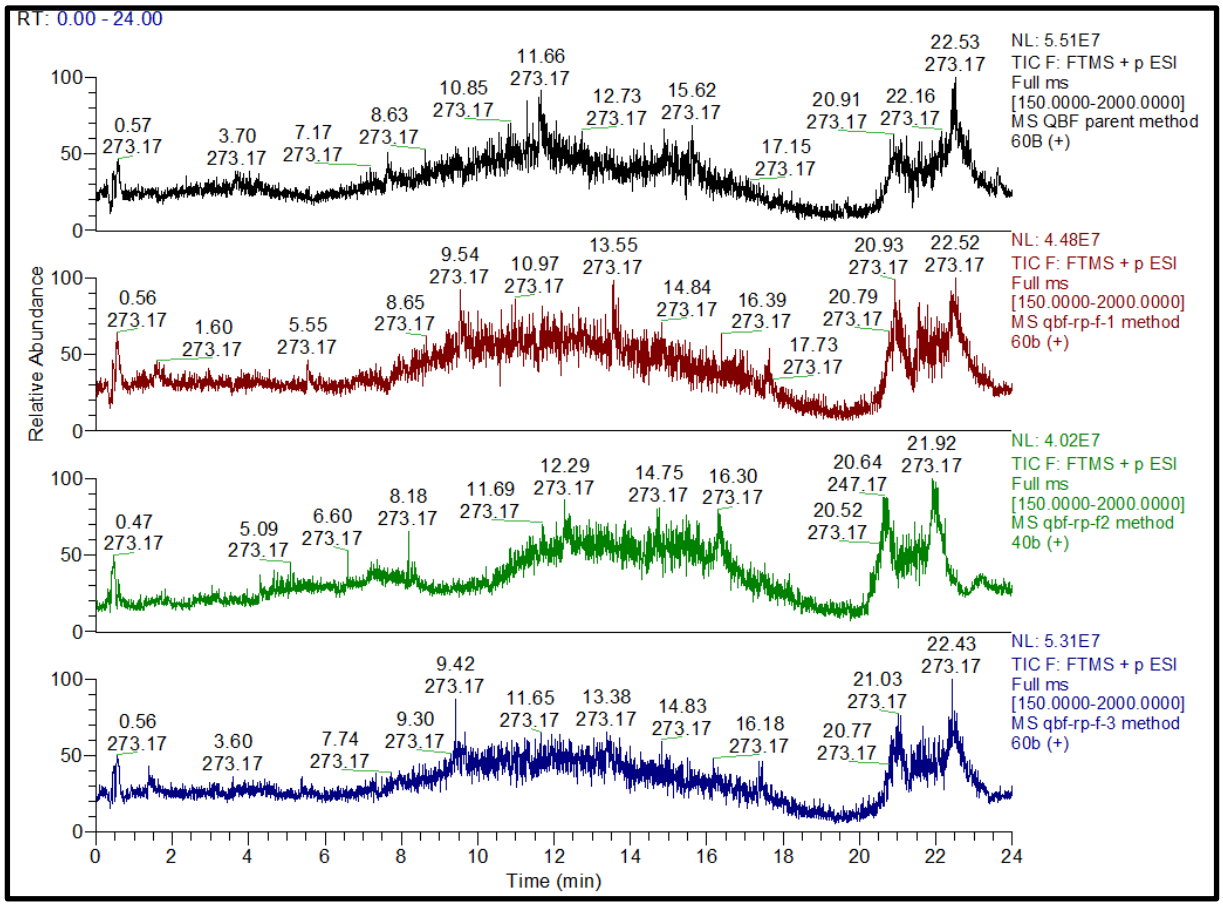


Figure A.11. LC-MS chromatograms of the QBF and its separated fractions from RP-FC using (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 3.3.6. LC-MS analysis of the QWF and its Separated Fractions from SLCC

(-) ionisation mode:

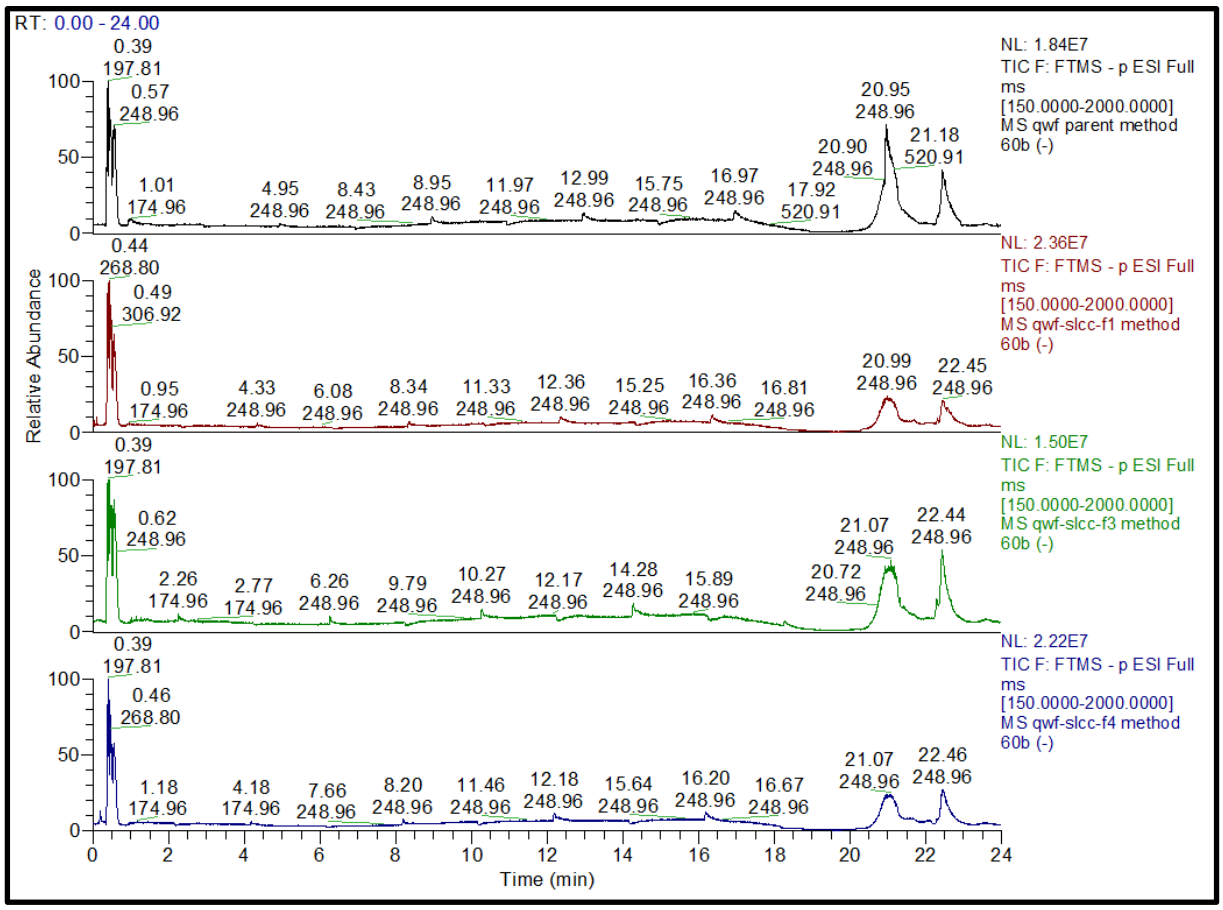


Figure A.12. LC-MS chromatograms of the QWF and its separated fractions from SLCC using (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(+) ionisation mode:

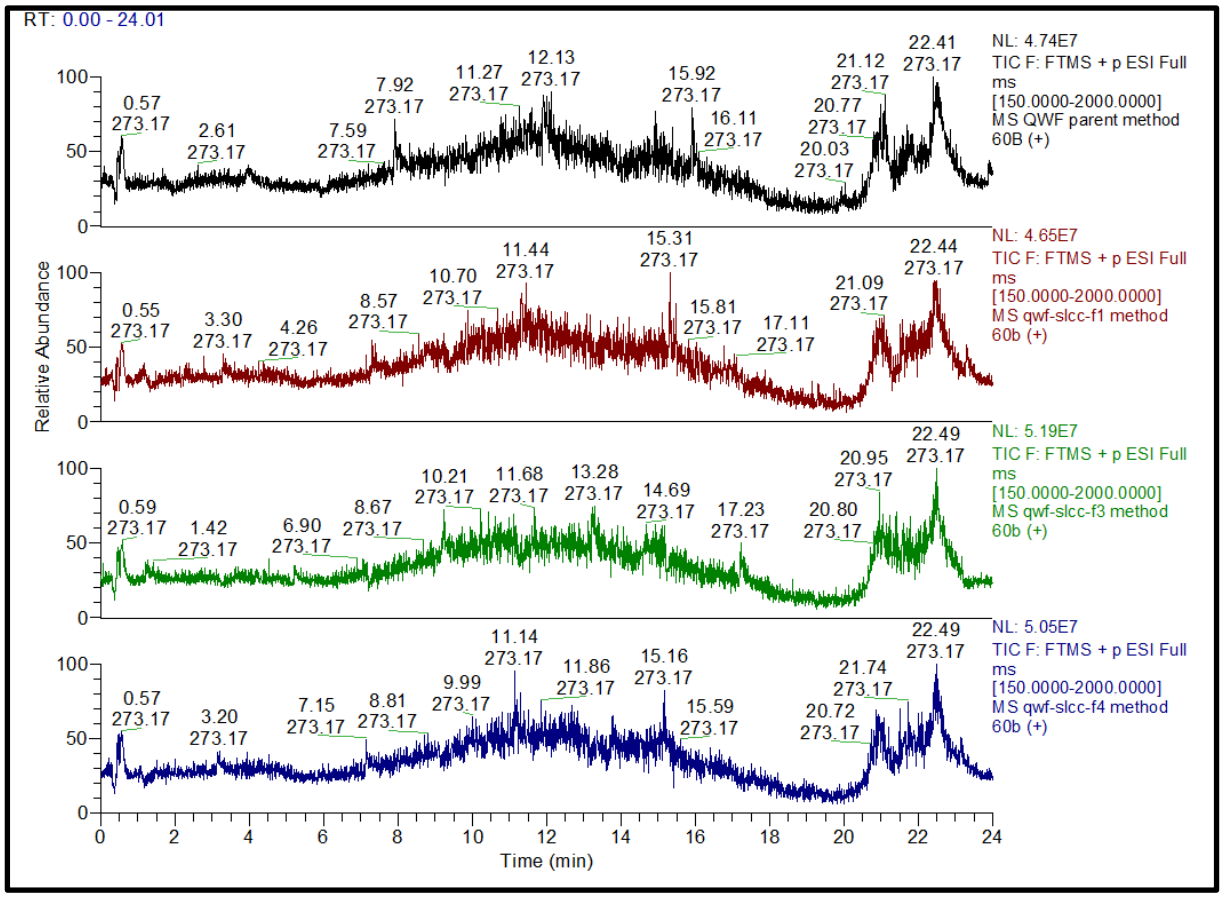


Figure A.13. LC-MS chromatograms of the QWF and its separated fractions from SLCC using (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7. Chapter 7 Appendix

Appendix 7.1. Chromatographic Analysis of Fractions of Kawakawa Leaf Samples

Appendix 7.1.1. Separation of KK-May20-leaf-Hexane-Fraction Using NP-FC

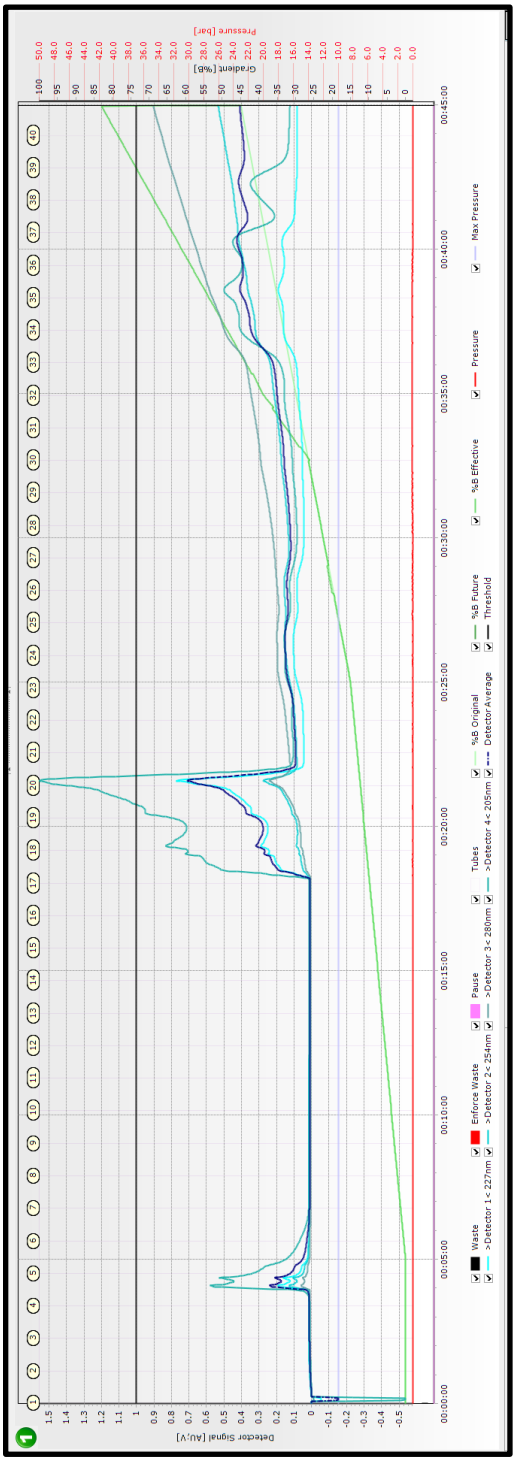


Figure A.14. Flash chromatogram of the NP-FC analysis of the KK-May20-leaf-Hexane-Fraction.

Combination of fractions based on TLC study to obtain initial combined fractions	1-3	Initial Fraction 1
	4-5	Initial Fraction 2
	6-15	Initial Fraction 3
	16-19	Initial Fraction 4
	20	Initial Fraction 5
	21-22	Initial Fraction 6
	25	Initial Fraction 7
	26-32	Initial Fraction 8
	33-34	Initial Fraction 9
	35-37	Initial Fraction 10
	38	Initial Fraction 11
	39-40	Initial Fraction 12

Table A.1. Combination of the fractions obtained from NP-FC run of the KK-May20-leaf-Hexane-Fraction based on the TLC study to obtain initial combined fractions.

Combination of initial fraction(s) having the same LC-MS result	***Final fraction***
1-2	KK-May20-leaf-Hexane Fr 1
3	KK-May20-leaf-Hexane Fr 2
4-5	KK-May20-leaf-Hexane Fr 3
6	KK-May20-leaf-Hexane Fr 4
7	KK-May20-leaf-Hexane Fr 5
8	KK-May20-leaf-Hexane Fr 6
9	KK-May20-leaf-Hexane Fr 7
10	KK-May20-leaf-Hexane Fr 8
11	KK-May20-leaf-Hexane Fr 9
12	KK-May20-leaf-Hexane Fr 10

Table A.2. Final fractions of the KK-May20-leaf-Hexane-Fraction through combination of initial fractions based on LC-MS analysis.

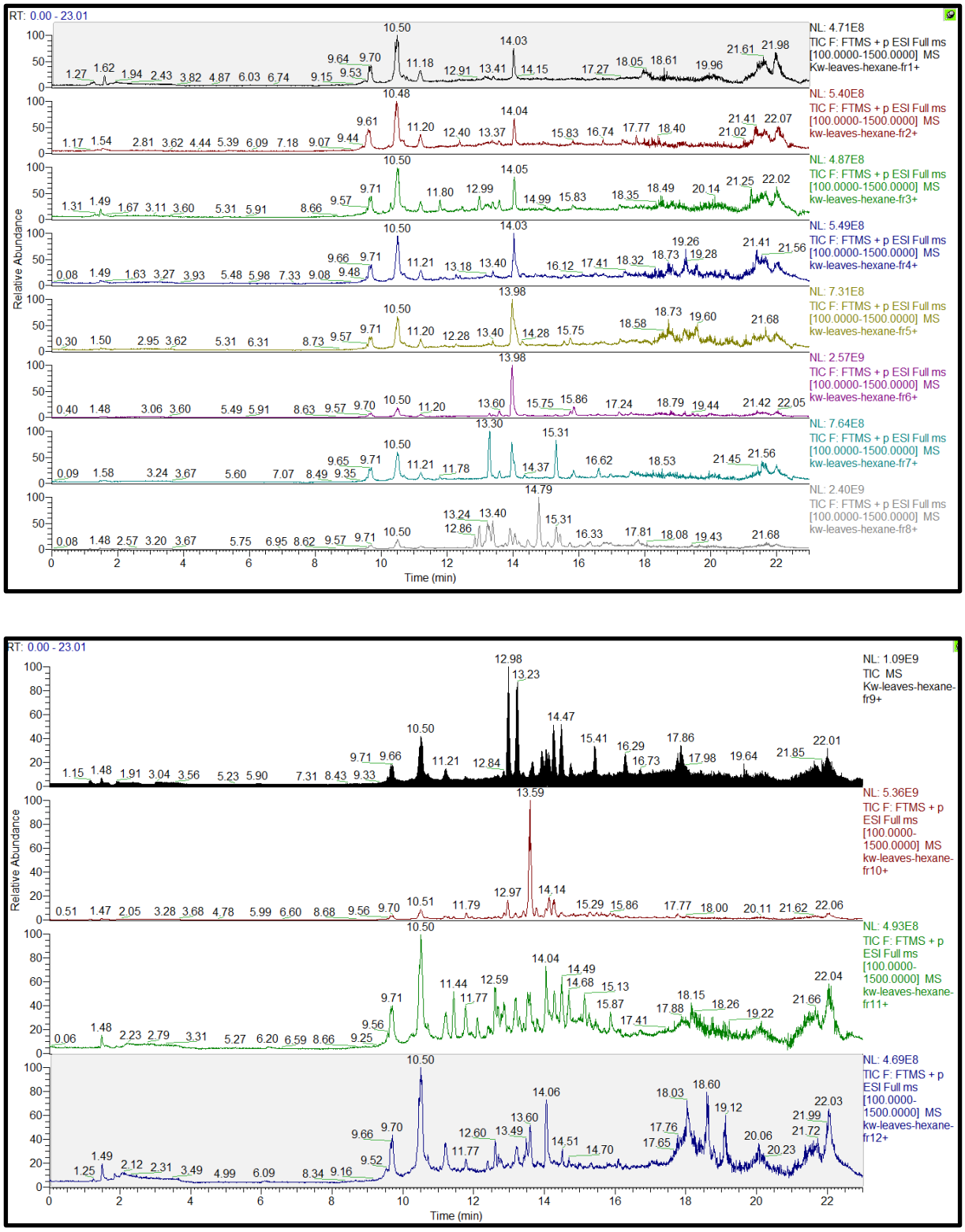


Figure A.15. LC-MS chromatograms of Initial Fractions 1-12 of the KK-May20-leaf-Hexane-Fraction with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.2. Separation of KK-May20-leaf-EtOAc-Fraction Using NP-FC

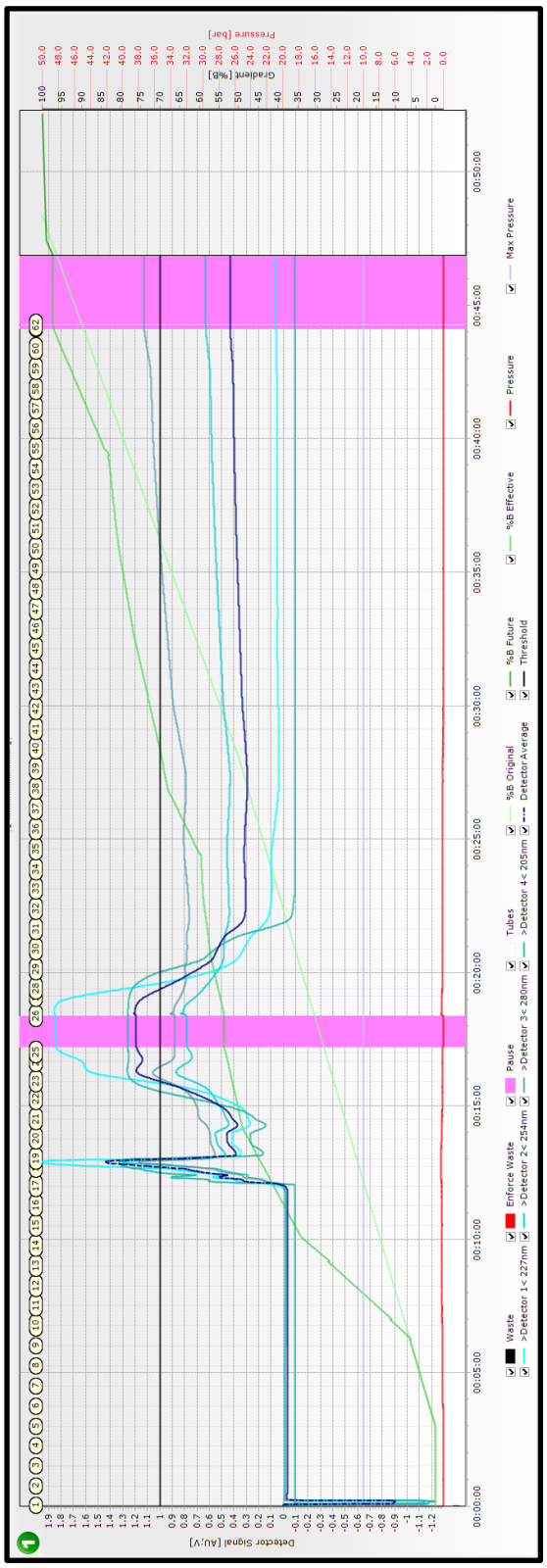


Figure A.16. Flash chromatogram of the NP-FC analysis of the KK-May20-leaf-EtOAc-Fraction.

Combination of fractions based on TLC study to obtain initial combined fractions	6-8	Initial Fraction 1
	16	Initial Fraction 2
	17	Initial Fraction 3
	18	Initial Fraction 4
	19	Initial Fraction 5
	20-21	Initial Fraction 6
	22	Initial Fraction 7
	23	Initial Fraction 8
	24-26	Initial Fraction 9
	27-29	Initial Fraction 10
	30-31	Initial Fraction 11
	32-34	Initial Fraction 12
	35	Initial Fraction 13
	36-62	Initial Fraction 14

Table A.3. Combination of fractions obtained from NP-FC run of the KK-May20-leaf-EtOAc-Fraction based on the TLC study to obtain initial combined fractions.

Combination of initial fraction(s) having the same LC-MS result	***Final fraction***
1-2	KK-May20-leaf-EtOAc Fr 1
3	KK-May20-leaf- EtOAc Fr 2
4-5	KK-May20-leaf- EtOAc Fr 3
6	KK-May20-leaf- EtOAc Fr 4
7-8	KK-May20-leaf- EtOAc Fr 5
9	KK-May20-leaf- EtOAc Fr 6
10-11	KK-May20-leaf- EtOAc Fr 7
12	KK-May20-leaf- EtOAc Fr 8
13	KK-May20-leaf- EtOAc Fr 9
14	KK-May20-leaf- EtOAc Fr 10

Table A.4. Final fractions of the KK-May20-leaf-EtOAc Fraction through combination of initial fractions based on LC-MS analysis.

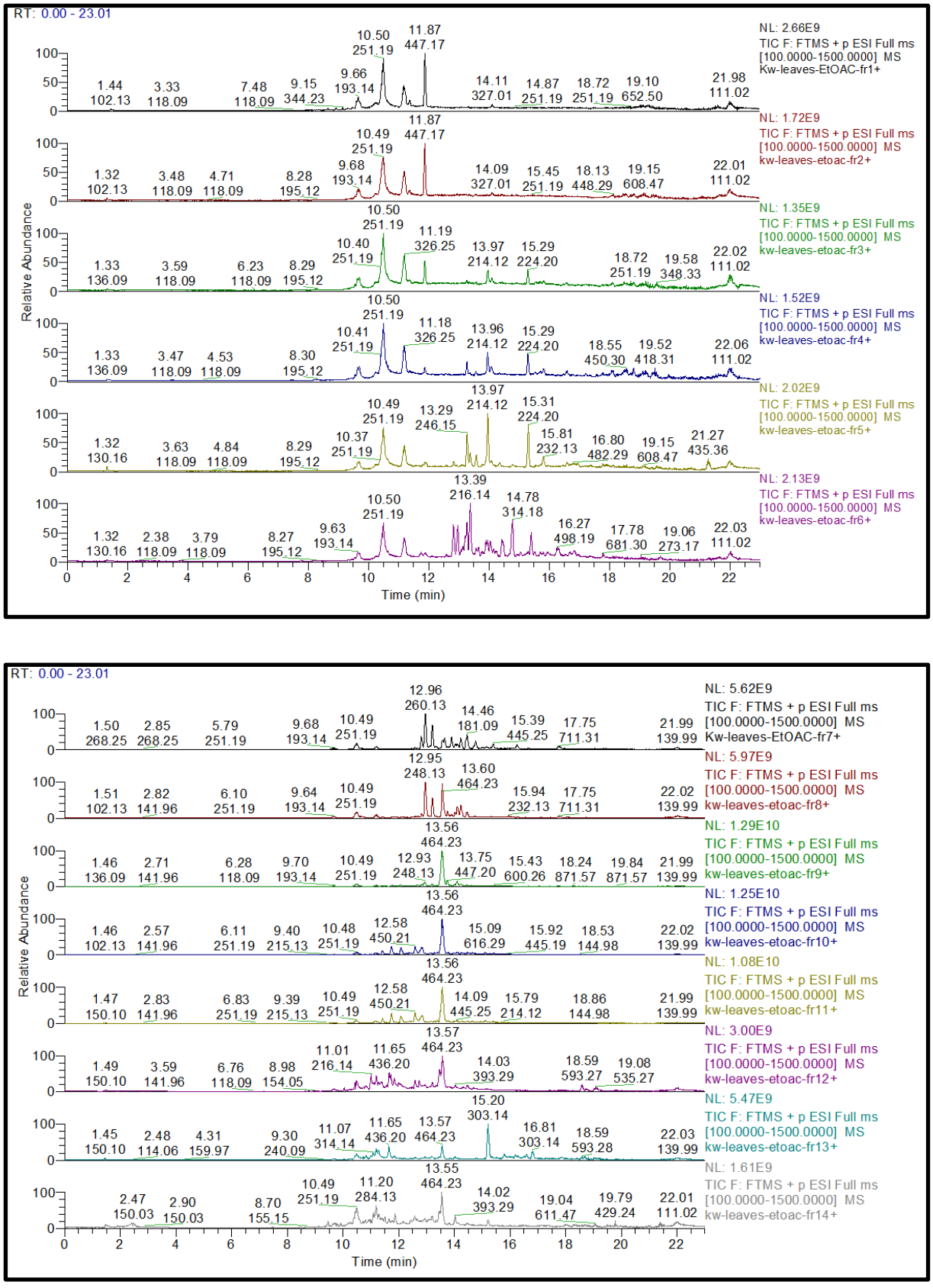


Figure A.17. LC-MS chromatograms of Initial Fractions 1-14 of the KK-May20-leaf-EtOAc-Fraction with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.3. Separation of the KK-May20-leaf-Water-Fraction Using RP-FC

Fractions	***Mobile phase of TLC study***	***Observation on TLC plate***
1-5	60% MeOH in Water	Pink and yellow coloured spots at the baseline, condensed together, no upward travel.
	70% MeOH in Water	Pink and yellow coloured spots condensed together.
	90% MeOH in Water	Pink and yellow coloured spots travelled too high, condensed together.
	EtOAc:AcOH:H_2O = 18:1:1	No spots.
	IPA:AcOH:H_2O = 7:1:2	No spots.
	BuOH:AcOH:H_2O = 5:1:4	Pink and yellow coloured spots at baseline, no upward travel.
6-25	60% MeOH in Water	Pale pink and yellow coloured spots at the baseline, condensed together, no upward travel.
	70% MeOH in Water	Spots condensed together.
	90% MeOH in Water	Spots too high, condensed together.
	EtOAc:AcOH:H_2O = 18:1:1	No spots.
	IPA:AcOH:H_2O = 7:1:2	No spots.
	BuOH:AcOH:H_2O = 5:1:4	Spots at baseline, no upward travel.
25-30	60% MeOH in Water	No spots.
	70% MeOH in Water	No spots.
	90% MeOH in Water	No spots.
	EtOAc:AcOH:H_2O = 18:1:1	No spots.
	IPA:AcOH:H_2O = 7:1:2	No spots.
	BuOH:AcOH:H_2O = 5:1:4	No spots.
31-50	70% MeOH in Water	No spots.
	90% MeOH in Water	No spots.

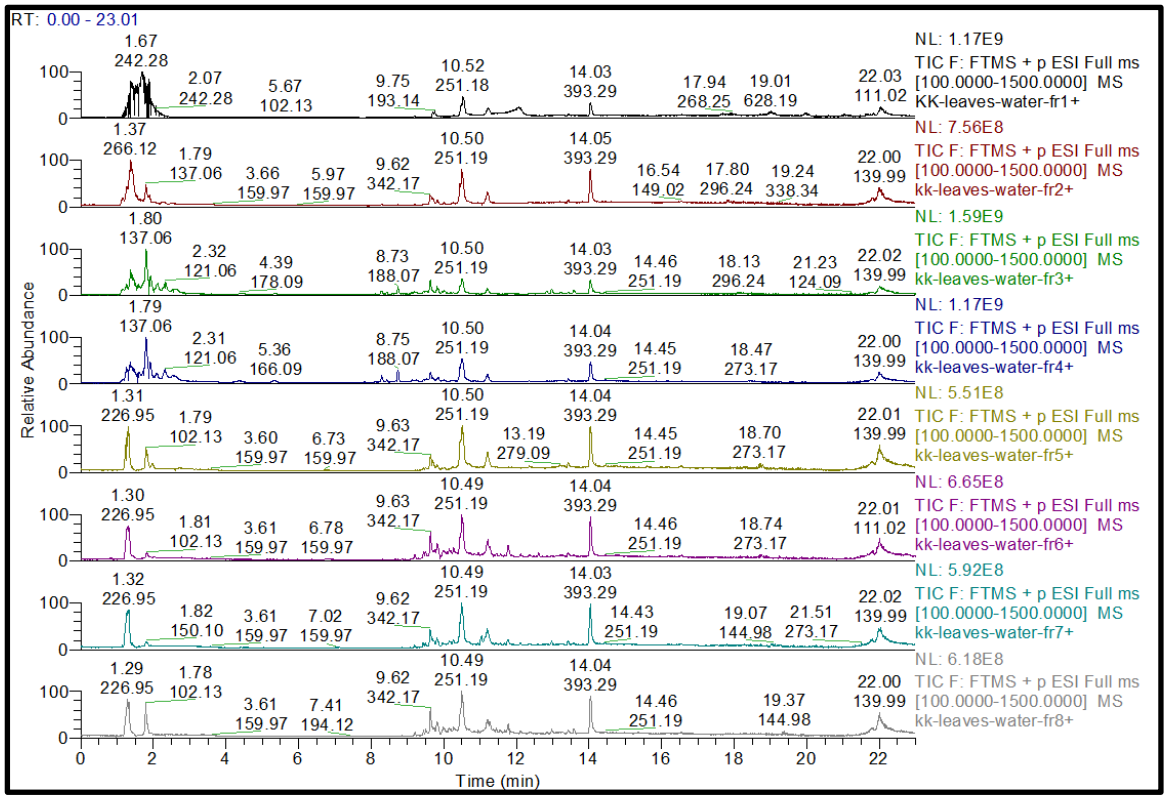
Table A.5. Result of the observation of RP-TLC study with the initial fractions obtained from the RP-FC separation of the KK-May20-leaf-Water-Fraction.

Initial fraction	*Yield in mg*
1	96
2	336
3	12
4	8
5	30
6	43
7	31
8	29
9	12
10	13

Table A.6. Yield of each initial combined fraction obtained from the RP-FC run of KK-May20-leaf-Water-Fraction.

Combination of fraction(s) having the same LC-MS result	*Final fraction*
1	KK-May20-leaf-Water Fr 1
2	KK-May20-leaf-Water Fr 2
3-4	KK-May20-leaf-Water Fr 3
5-7	KK-May20-leaf-Water Fr 4
8	KK-May20-leaf-Water Fr 5
9	KK-May20-leaf-Water Fr 6
10	KK-May20-leaf-Water Fr 7

Table A.7. Final fractions of the KK-May20-leaf-Water-Fraction through combination of initial fractions based on LC-MS analysis.



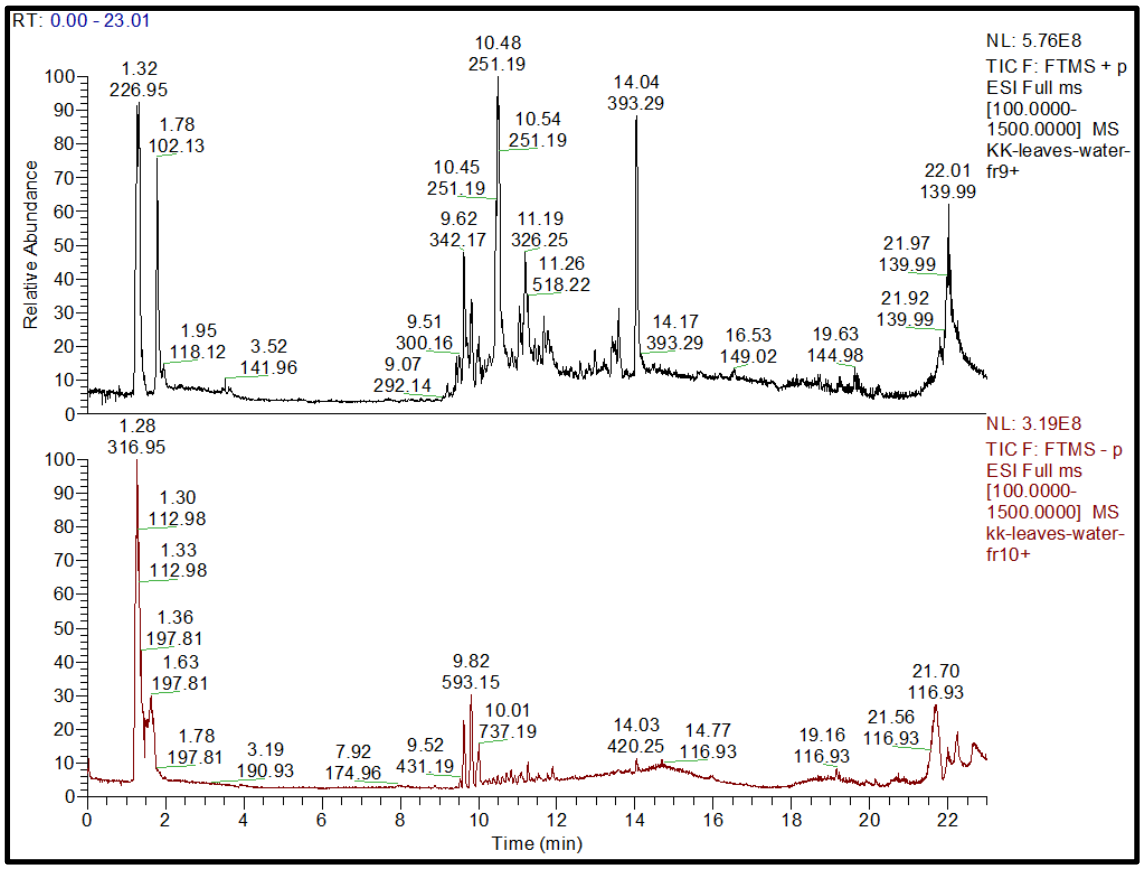


Figure A.18. LC-MS chromatograms of Initial Fractions 1-10 of the KK-May20-leaf-Water-Fraction with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.4. Separation of the KK-May20-leaf-MeOH-Fraction Using RP-FC

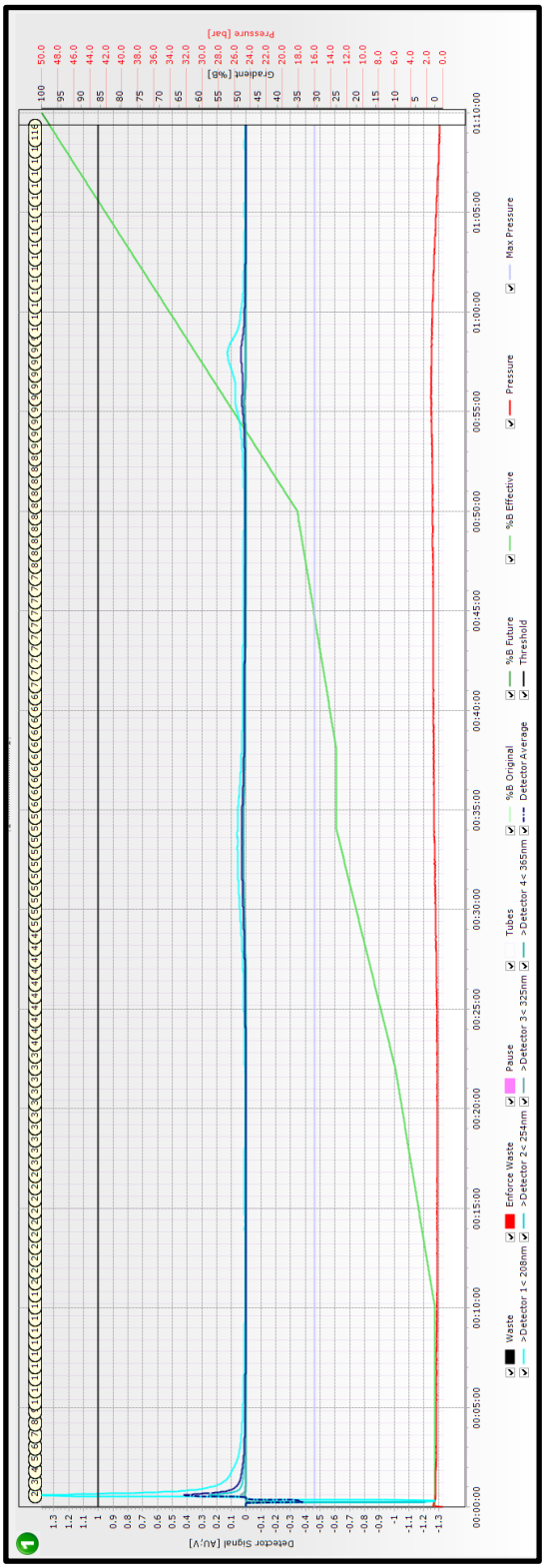


Figure A.19. Flash chromatogram of the RP-FC analysis of the KK-May20-leaf-MeOH-Fraction.

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***	***Yield in mg***
2	1st hypothesis	1	215
3-7	2nd hypothesis	2	41
8-18	2nd hypothesis	3	38
19-45	2nd hypothesis	4	51
46-66	3rd and 4th hypothesis	5	50
67-88	2nd hypothesis	6	22
89-92	3rd and 4th hypothesis	7	33
93-96	3rd and 4th hypothesis	8	33
97-101	3rd and 4th hypothesis	9	16
102-116	2nd hypothesis	10	28

Table A.8. Combination of the fractions obtained from RP-FC run of the KK-May20-leaf-MeOH-Fraction and dry matter yield of each.

Combination of fraction(s) having the same LC-MS result	***Final fraction***
1	KK-May20-leaf-MeOH Fr 1
2	KK-May20-leaf-MeOH Fr 2
3	KK-May20-leaf-MeOH Fr 3
4	KK-May20-leaf-MeOH Fr 4
5	KK-May20-leaf-MeOH Fr 5
6	KK-May20-leaf-MeOH Fr 6
7-8	KK-May20-leaf-MeOH Fr 7
9-10	KK-May20-leaf-MeOH Fr 8

Table A.9. Final fractions of the KK-May20-leaf-MeOH-Fraction through combination of initial fractions based on LC-MS analysis.

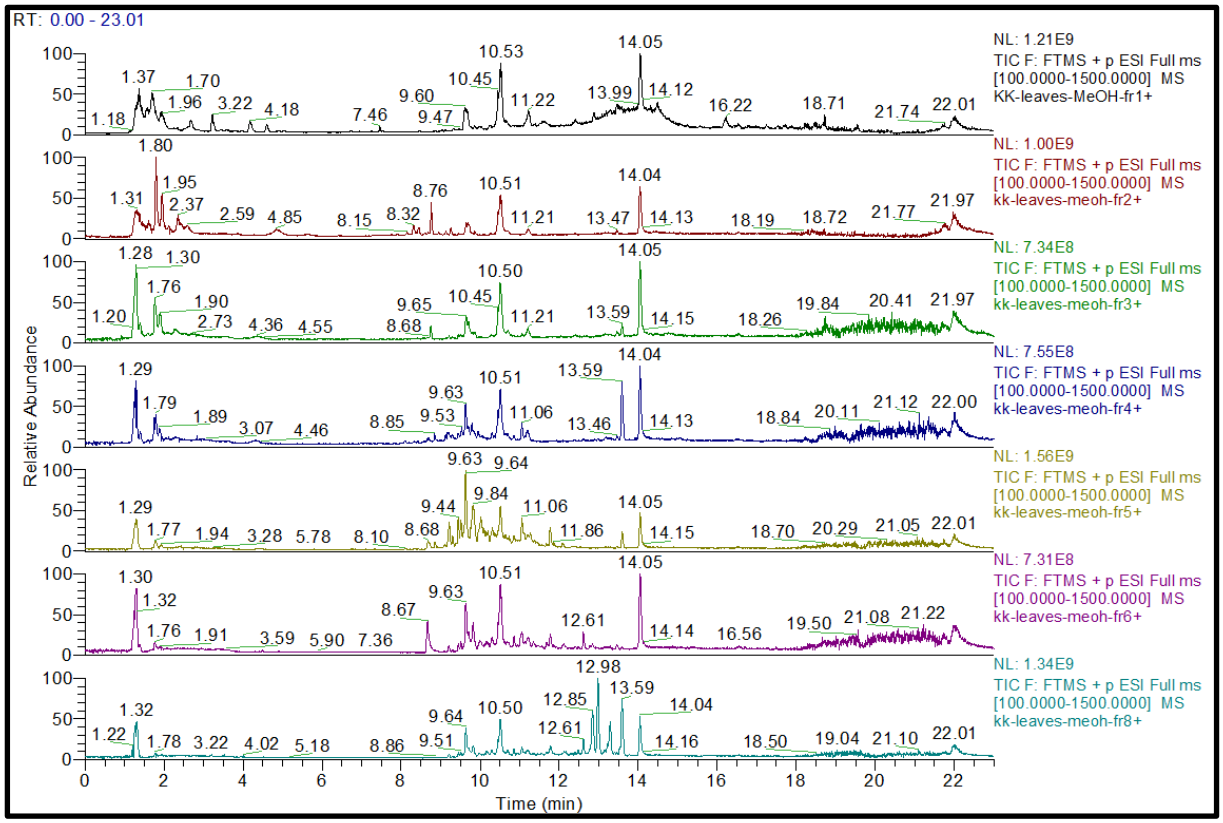


Figure A.20. LC-MS chromatograms of Initial Fractions 1-8 of the KK-May20-leaf-MeOH-Fraction with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.5. Separation of the KK-Oct20-leaf-Water-Fraction Using RP-FC

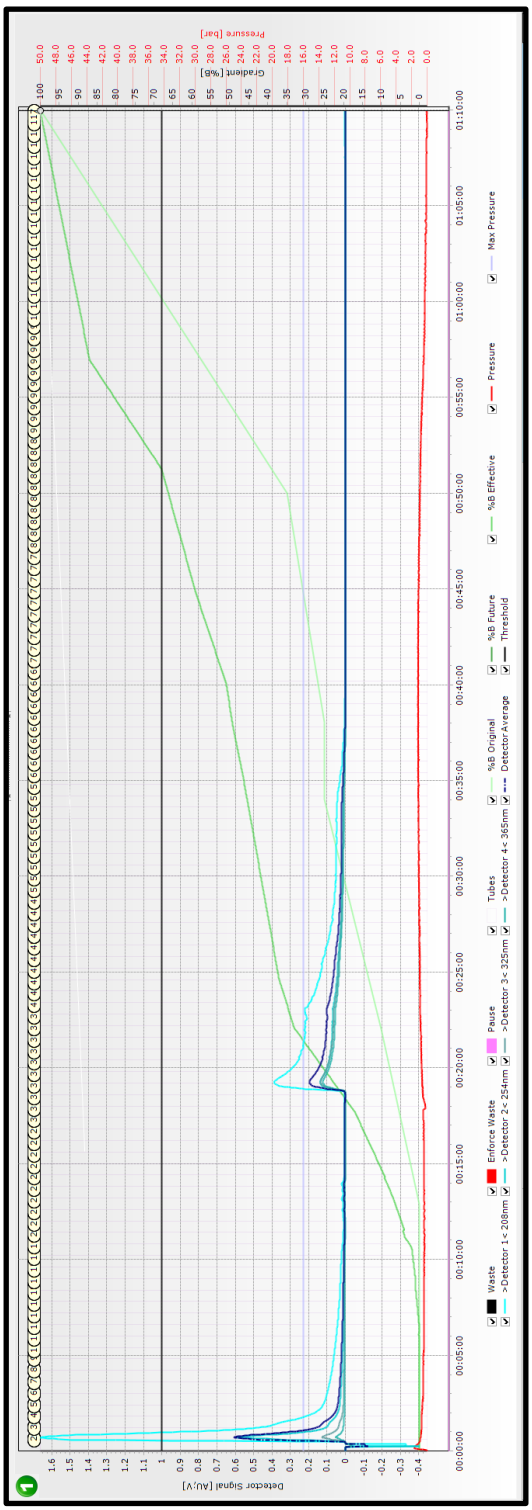


Figure A.21. Flash chromatogram of the RP-FC analysis of the KK-Oct20-leaf-Water-Fraction.

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1-2	1st hypothesis	1
3-4	3rd and 4th hypothesis	2
5-23	3rd and 4th hypothesis	3
24-26	3rd and 4th hypothesis	4
27-32	2nd hypothesis	5
33-34	1st hypothesis	6
35-39	3rd and 4th hypothesis	7
40-41	1st hypothesis	8
43-52	3rd and 4th hypothesis	9
53-62	3rd and 4th hypothesis	10
62-67	3rd and 4th hypothesis	11
67-117	2nd hypothesis	12

Table A.10. Combination of the fractions obtained from RP-FC run of the KK-Oct20-leaf-Water-Fraction.

Combination of initial fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1	KK-Oct20-leaf-Water-Fr 1	902
2-3	KK-Oct20-leaf-Water-Fr 2	67
4-5	KK-Oct20-leaf-Water-Fr 3	5
6-8	KK-Oct20-leaf-Water-Fr 4	52
9-10	KK-Oct20-leaf-Water-Fr 5	44
11-12	KK-Oct20-leaf-Water-Fr 6	3

Table A.11. Final fractions of the KK-Oct20-Water-Fraction through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

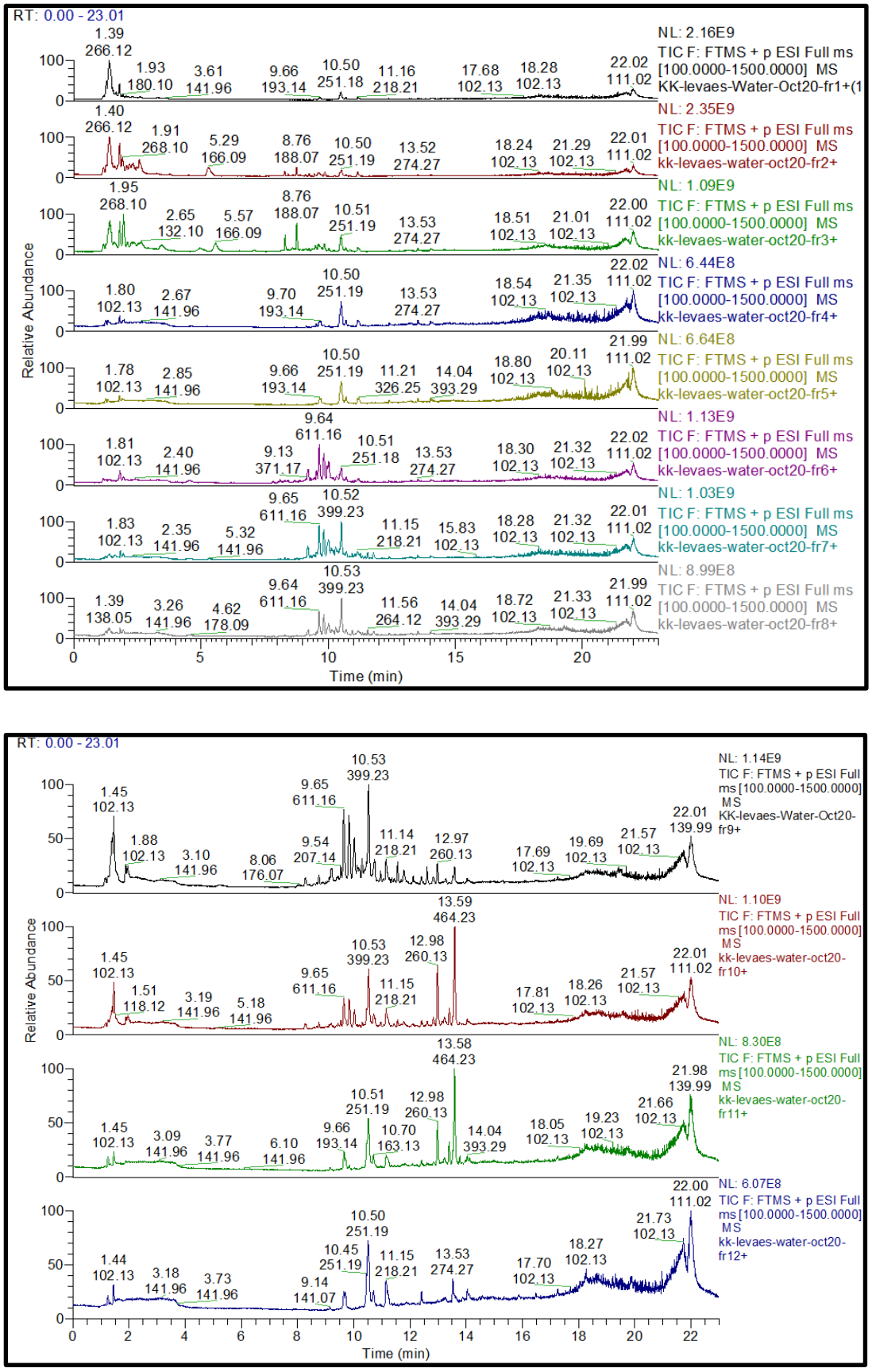


Figure A.22. LC-MS chromatograms of Initial Fractions 1-12 of the KK-Oct20-leaf-Water-Fraction with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.6. Separation of the KK-Oct20-leaf-MeOH-Fraction Using RP-FC

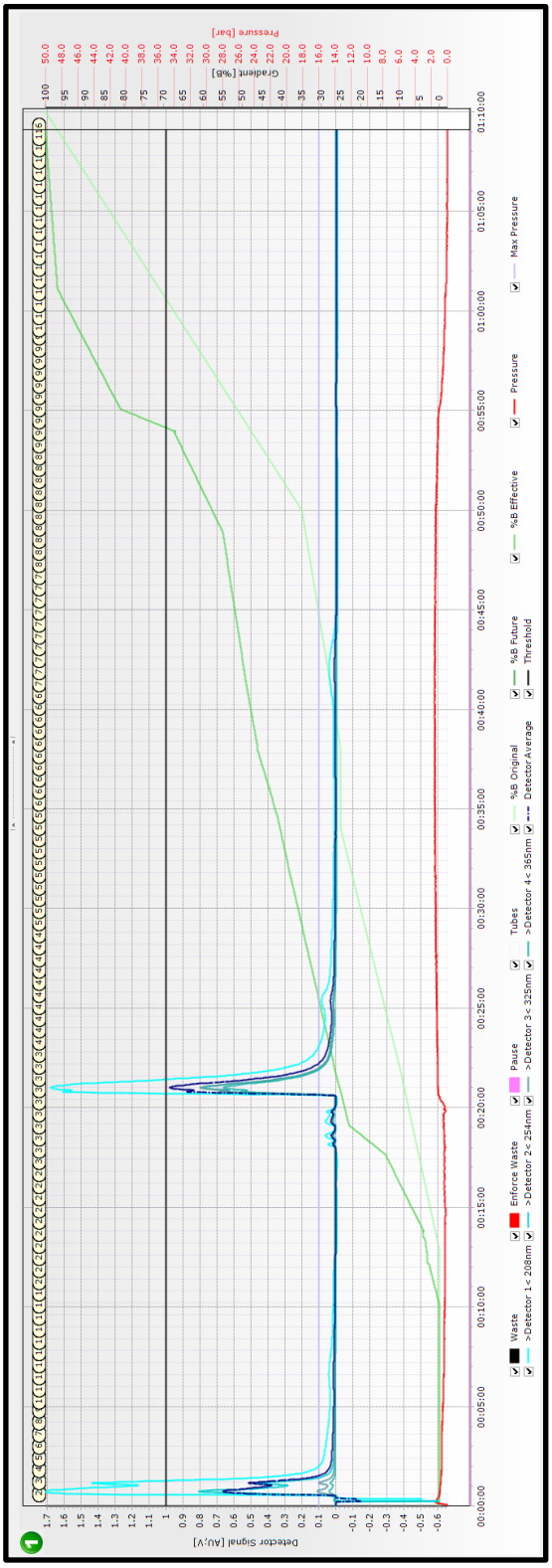


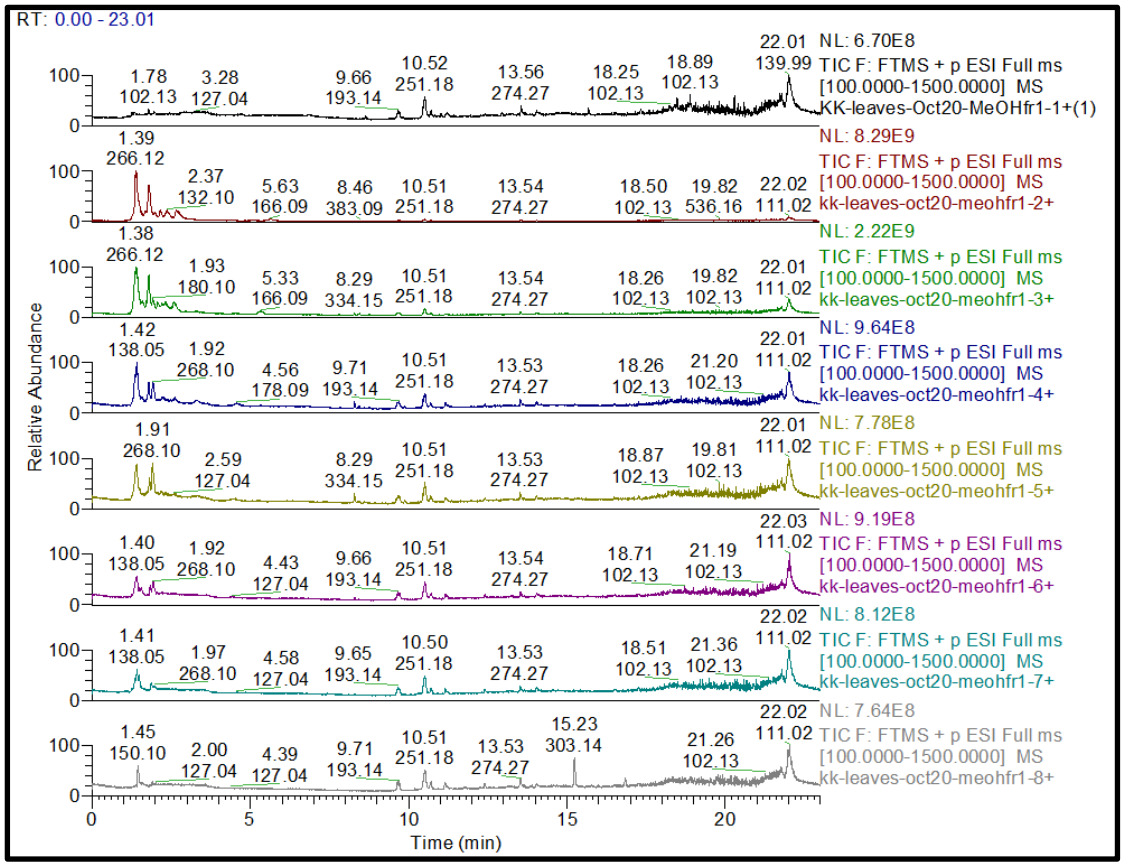
Figure A.23. Flash chromatogram of the RP-FC analysis of the KK-Oct20-leaf-MeOH-Fraction.

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
2	1st hypothesis	1
3	1st hypothesis	2
4-8	3rd and 4th hypothesis	3
9-30	3rd and 4th hypothesis	4
31-34	3rd and 4th hypothesis	5
35-36	1st hypothesis	6
37-38	1st hypothesis	7
39-43	3rd and 4th hypothesis	8
44-47	3rd and 4th hypothesis	9
49-67	2nd hypothesis	10
68-75	3rd and 4th hypothesis	11
76-116	2nd hypothesis	12

Table A.12. Combination of the fractions obtained from RP-FC run of the KK-Oct20-leaf-MeOH-Fraction.

Combination of initial fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1	KK-Oct20-leaf-MeOH-Fr 1	597
2-3	KK-Oct20-leaf-MeOH-Fr 2	78
4	KK-Oct20-leaf-MeOH-Fr 3	19
5-6	KK-Oct20-leaf-MeOH-Fr 4	51
7	KK-Oct20-leaf-MeOH-Fr 5	6
8-9	KK-Oct20-leaf-MeOH-Fr 6	11
10	KK-Oct20-leaf-MeOH-Fr 7	4
11-12	KK-Oct20-leaf-MeOH-Fr 8	3

Table A.13. Final fractions of the KK-Oct20-MeOH-Fraction through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.



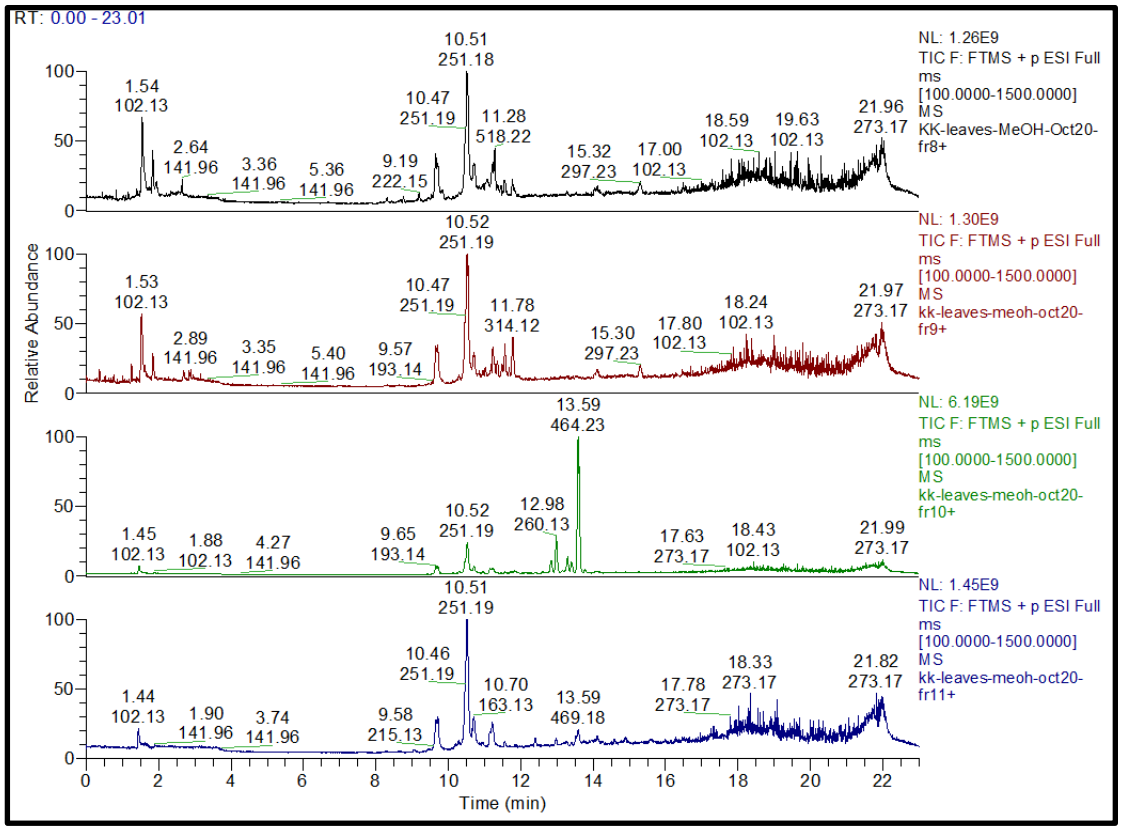


Figure A.24. LC-MS chromatograms of Initial Fractions 1-12 of the KK-Oct20-leaf-MeOH-Fraction with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.7. Separation of the KK-Oct20-leaf-MeOH-Fr-1 Using RP-FC

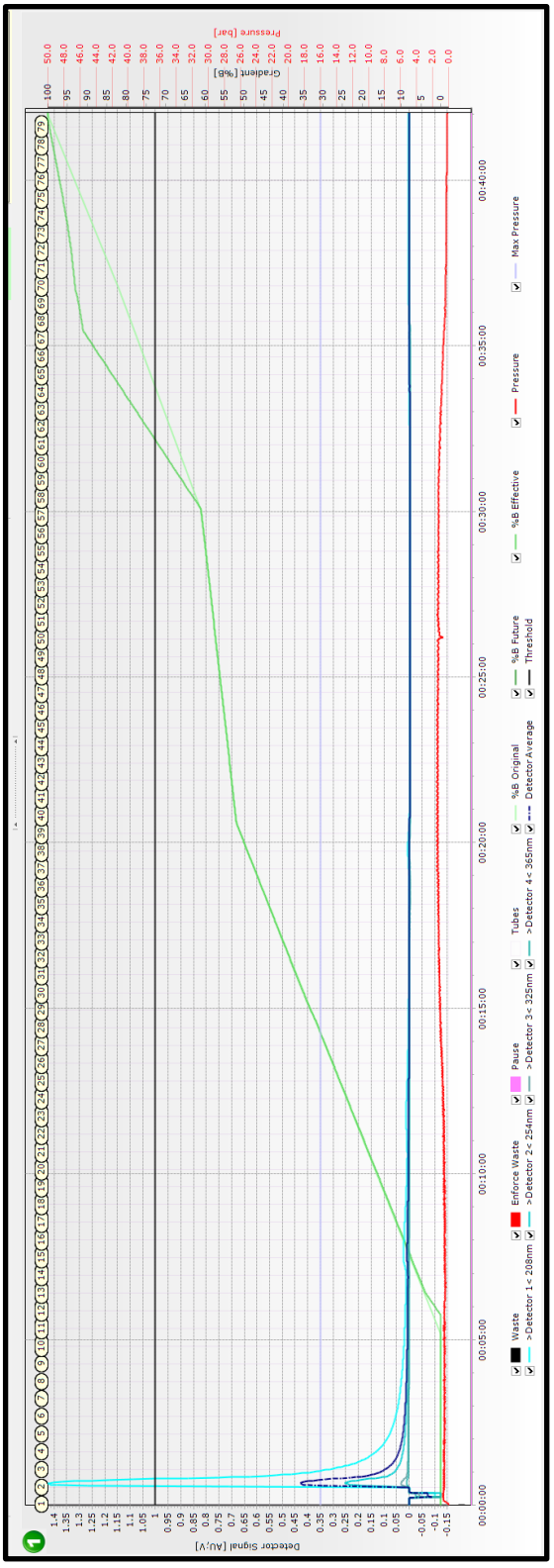


Figure A.25. Flash chromatogram of the RP-FC analysis of the KK-Oct20-leaf-MeOH-Fr-1.

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1	2nd hypothesis	1
2	1st hypothesis	2
3-10	3rd and 4th hypothesis	3
11-15	2nd hypothesis	4
16-19	3rd and 4th hypothesis	5
20-26	3rd and 4th hypothesis	6
27-37	2nd hypothesis	7
38-79	3rd and 4th hypothesis	8

Table A.14. Combination of the fractions obtained from RP-FC run of the KK-Oct20-leaf-MeOH-Fr-1.

Combination of fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1	KK-Oct20-leaf-MeOH-Fr 1-1	2
2	KK-Oct20-leaf-MeOH-Fr 1-2	170
3-4	KK-Oct20-leaf-MeOH-Fr 1-3	20
5-7	KK-Oct20-leaf-MeOH-Fr 1-4	5
8	KK-Oct20-leaf-MeOH-Fr 1-5	5

Table A.15. Final fractions of the KK-Oct20-leaf-MeOH-Fr-1 through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

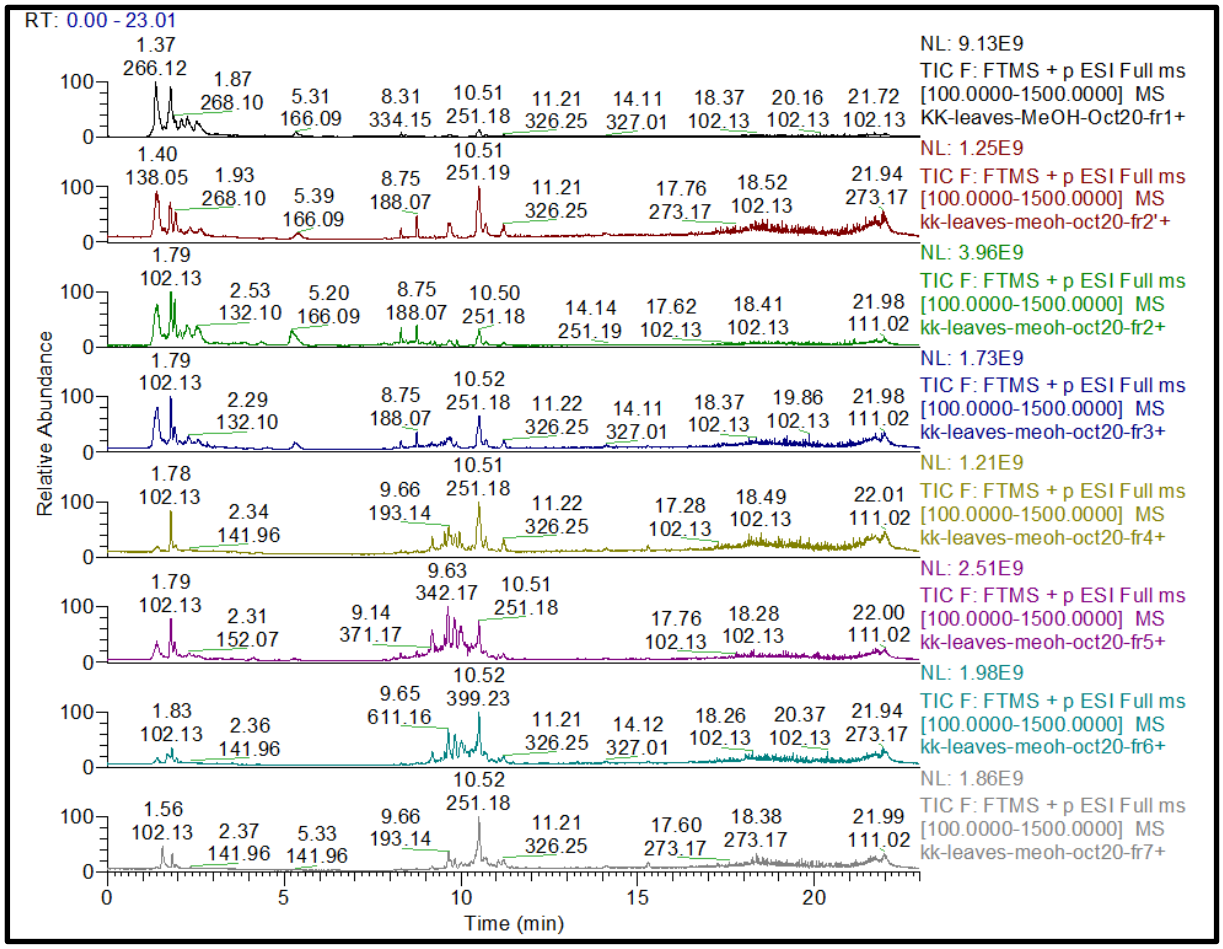


Figure A.26. LC-MS chromatograms of Initial Fractions 1-8 of the KK-Oct20-leaf-MeOH-Fr-1 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.8. Separation of the KK-Oct20-leaf-MeOH-Fr-1-2 Using RP-FC

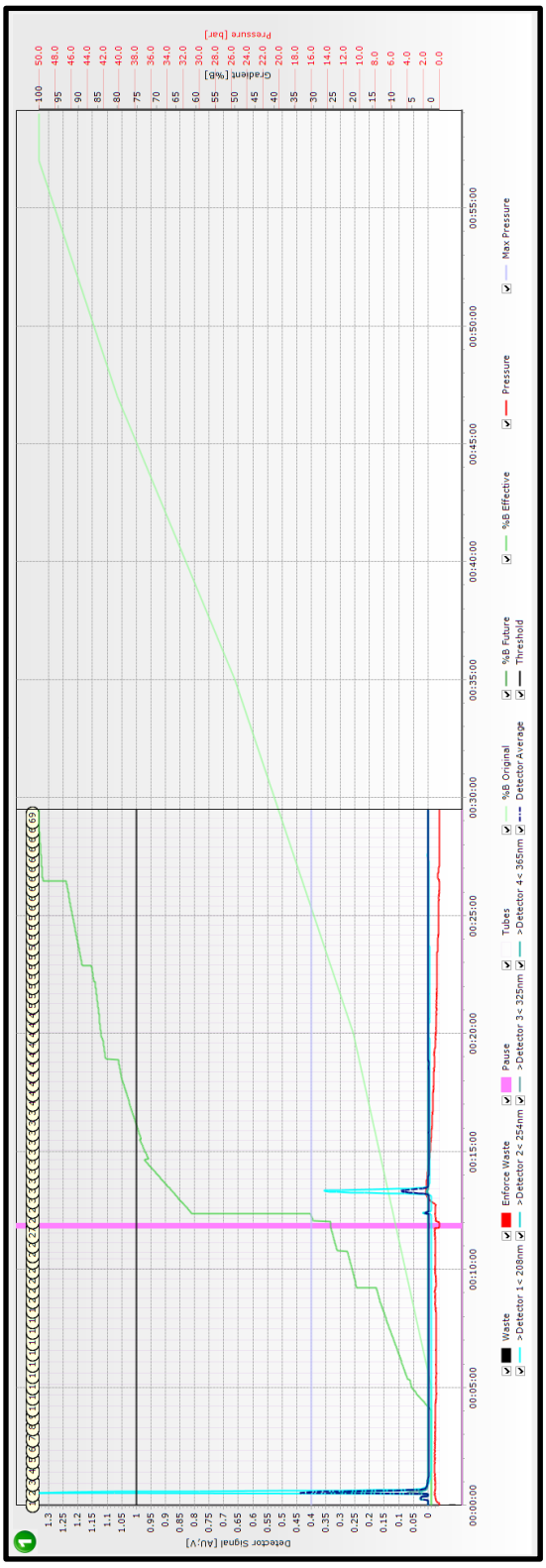


Figure A.27. Flash chromatogram of the RP-FC analysis of the KK-Oct20-leaf-MeOH-Fr-1-2.

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1	1st hypothesis	1
2-3	1st hypothesis	2
4-6	3rd and 4th hypothesis	3
7-28	2nd hypothesis	4
29	1st hypothesis	5
30-31	2nd hypothesis	6
32	1st hypothesis	7
33-34	3rd and 4th hypothesis	8
35-69	2nd hypothesis	9

Table A.16. Combination of the fractions obtained from RP-FC run of the KK-Oct20-leaf-MeOH-Fr-1-2.

Combination of fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1	KK-Oct20-leaf-MeOH-Fr-1-2-1	1
2	KK-Oct20-leaf-MeOH-Fr-1-2-2	68
3	KK-Oct20-leaf-MeOH-Fr-1-2-3	3
4	KK-Oct20-leaf-MeOH-Fr-1-2-4	4
5	KK-Oct20-leaf-MeOH-Fr-1-2-5	3
6-9	KK-Oct20-leaf-MeOH-Fr-1-2-6	12

Table A.17. Final fractions of the KK-Oct20-leaf-MeOH-Fr-1-2 through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

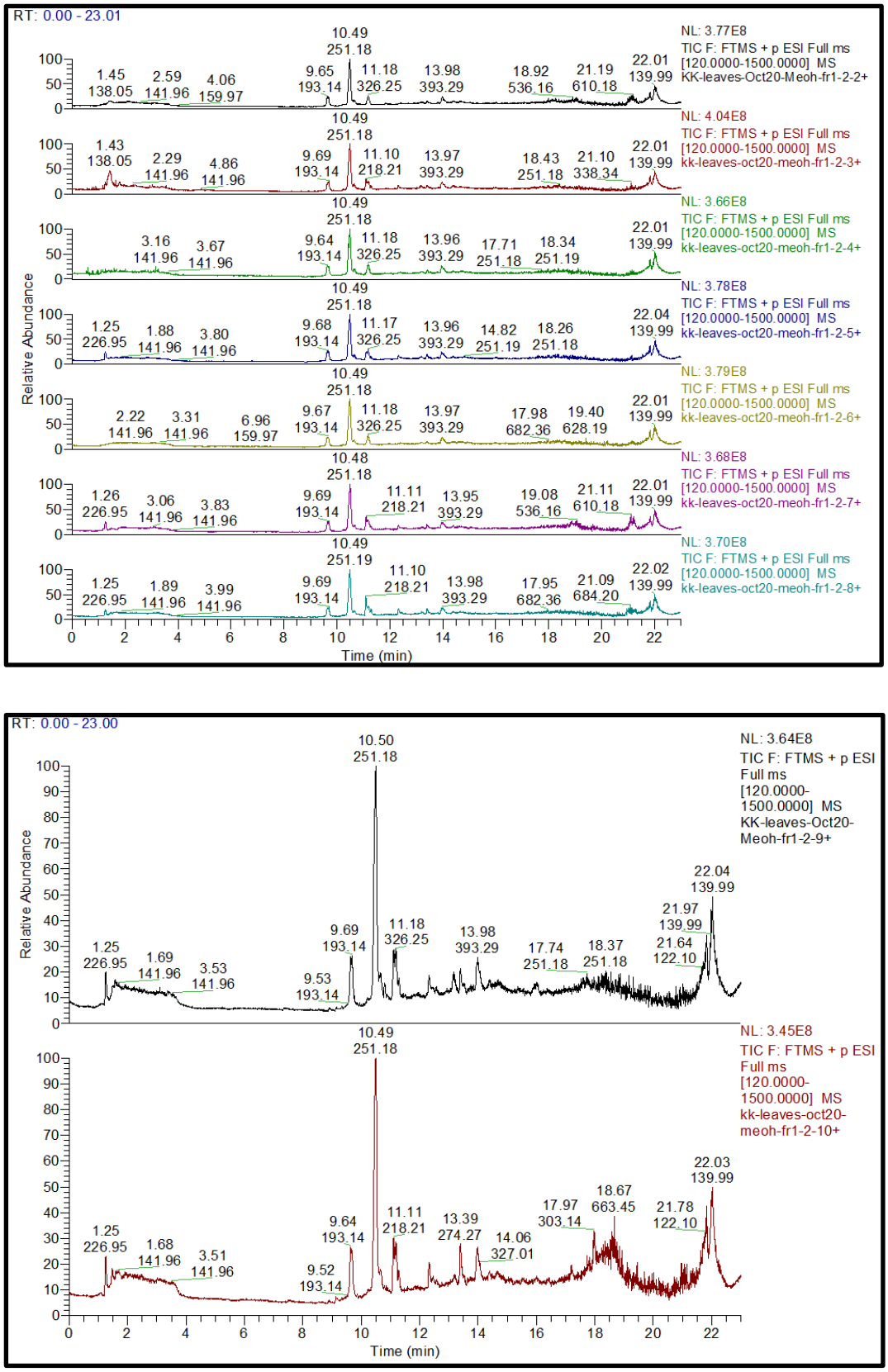


Figure A.28. LC-MS chromatograms of Initial Fractions 1-9 of the KK-Oct20-leaf-MeOH-Fr-1-2 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.9. Separation of the KK-Dec20-leaf-MeOH-Fraction Using RP-FC (RUN 1-3)

Run 1:

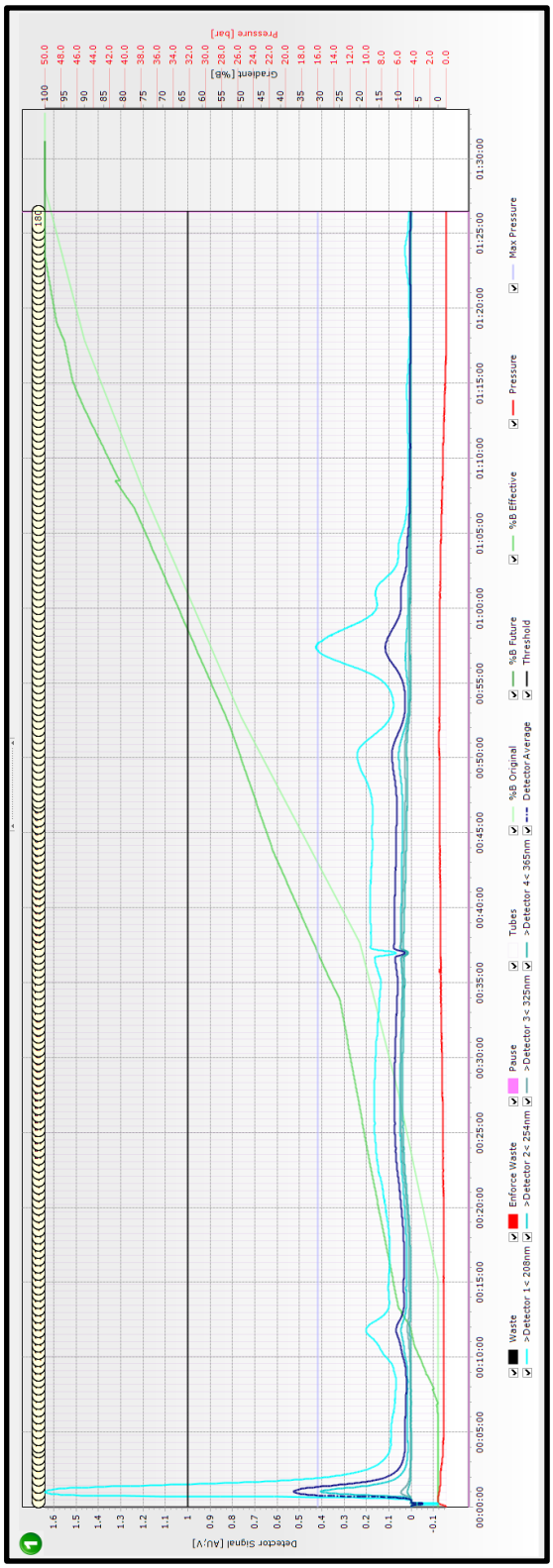


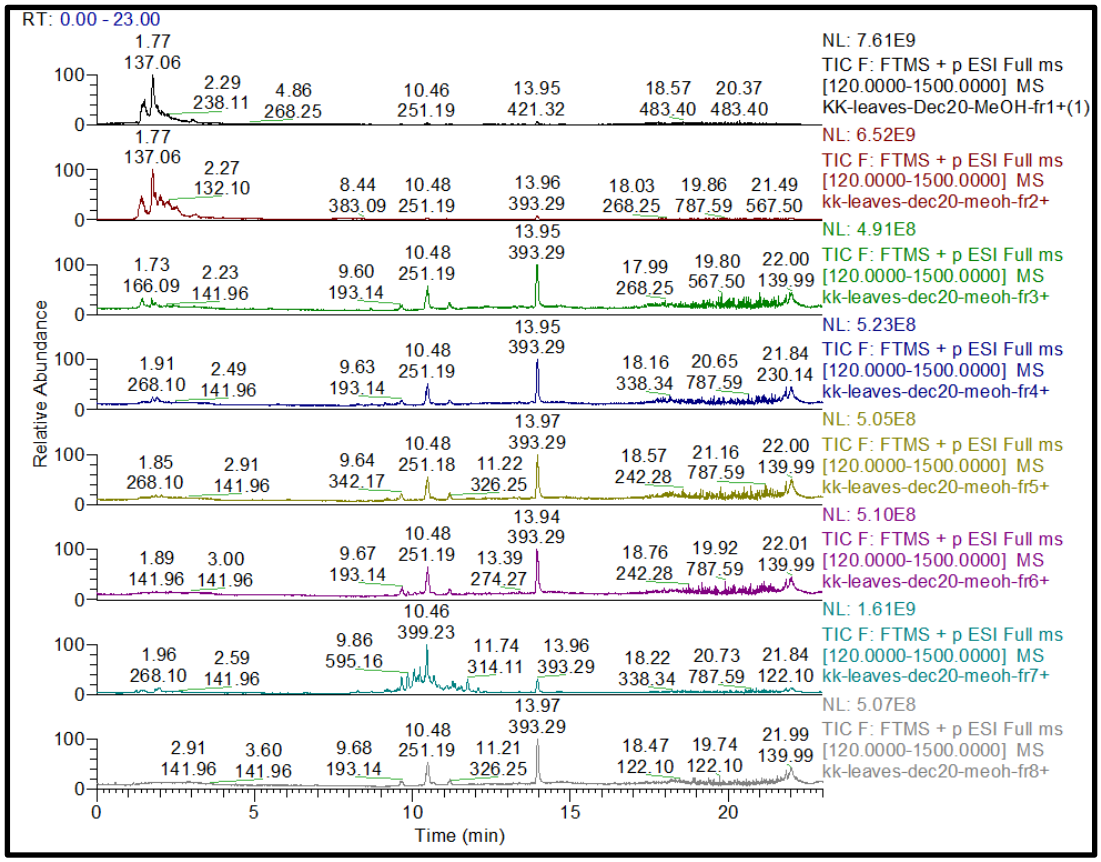
Figure A.29. Flash chromatogram of the RP-FC analysis of the KK-Dec20-leaf-MeOH-Fraction (RUN 1).

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
2-3	1st hypothesis	1
4-5	3rd and 4th hypothesis	2
6-20	3rd and 4th hypothesis	3
21-30	1st hypothesis	4
31-44	2nd hypothesis	5
45-78	3rd and 4th hypothesis	6
79-81	3rd and 4th hypothesis	7
82-101	2nd hypothesis	8
107-110	1st hypothesis	9
117-127	2nd hypothesis	10
128-133	1st hypothesis	11
134-140	1st hypothesis	12
141-169	3rd and 4th hypothesis	13
170-180	2nd hypothesis	14

Table A.18. Combination of the fractions obtained from RP-FC run of the KK-Dec20-leaf-MeOH-Fraction (RUN 1).

Combination of fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1-2	KK-Dec20-MeOH-R1-Fr 1	1478
3-6	KK-Dec20-MeOH-R1-Fr 2	265
7	KK-Dec20-MeOH-R1-Fr 3	15
8	KK-Dec20-MeOH-R1-Fr 4	122
9	KK-Dec20-MeOH-R1-Fr 5	45
10	KK-Dec20-MeOH-R1-Fr 6	20
11	KK-Dec20-MeOH-R1-Fr 7	29
12-13	KK-Dec20-MeOH-R1-Fr 8	18
14	KK-Dec20-MeOH-R1-Fr 9	4

Table A.19. Final fractions of the KK-Dec20-leaf-MeOH-Fraction (RUN 1) through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.



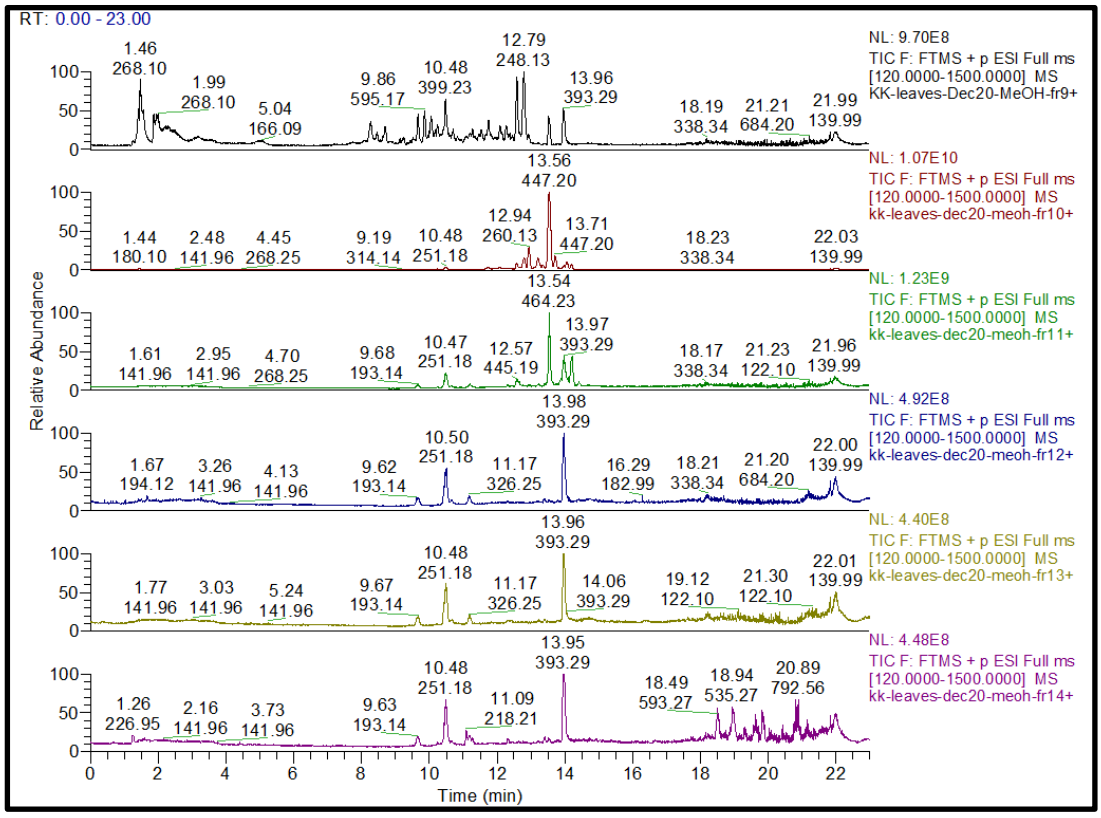


Figure A.30. LC-MS chromatograms of Initial Fractions 1-14 of the KK-Dec20-leaf-MeOH-Fraction (RUN 1) with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

RUN 2:

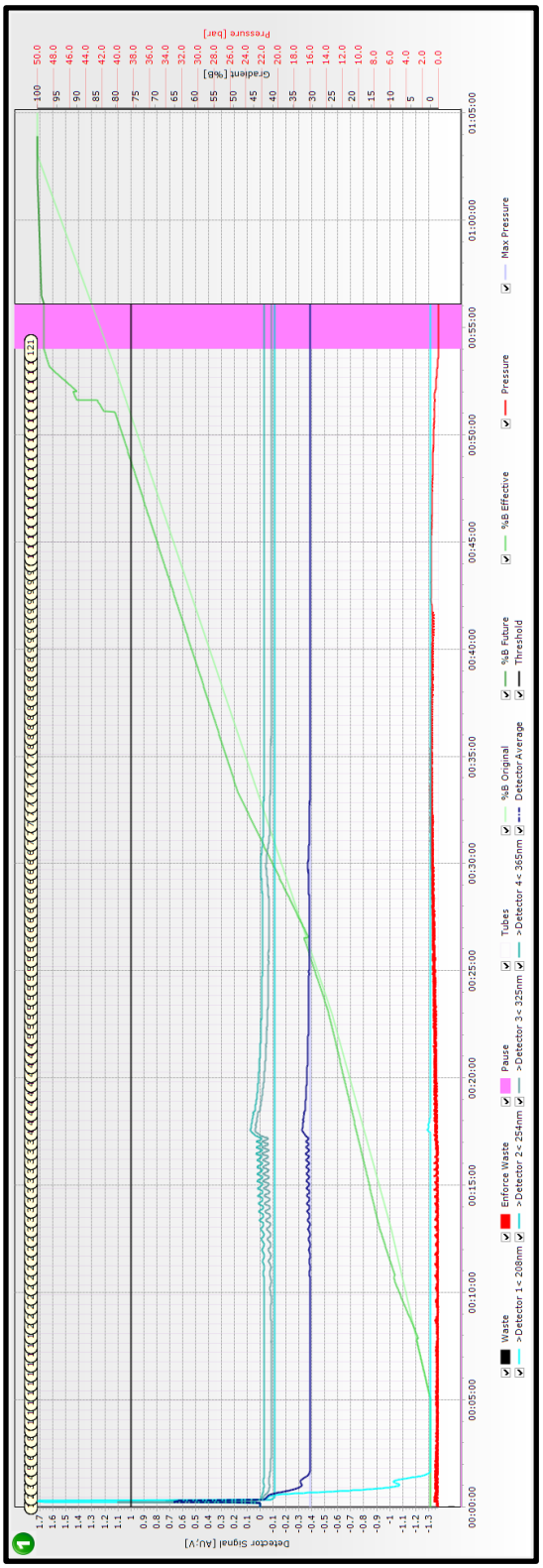


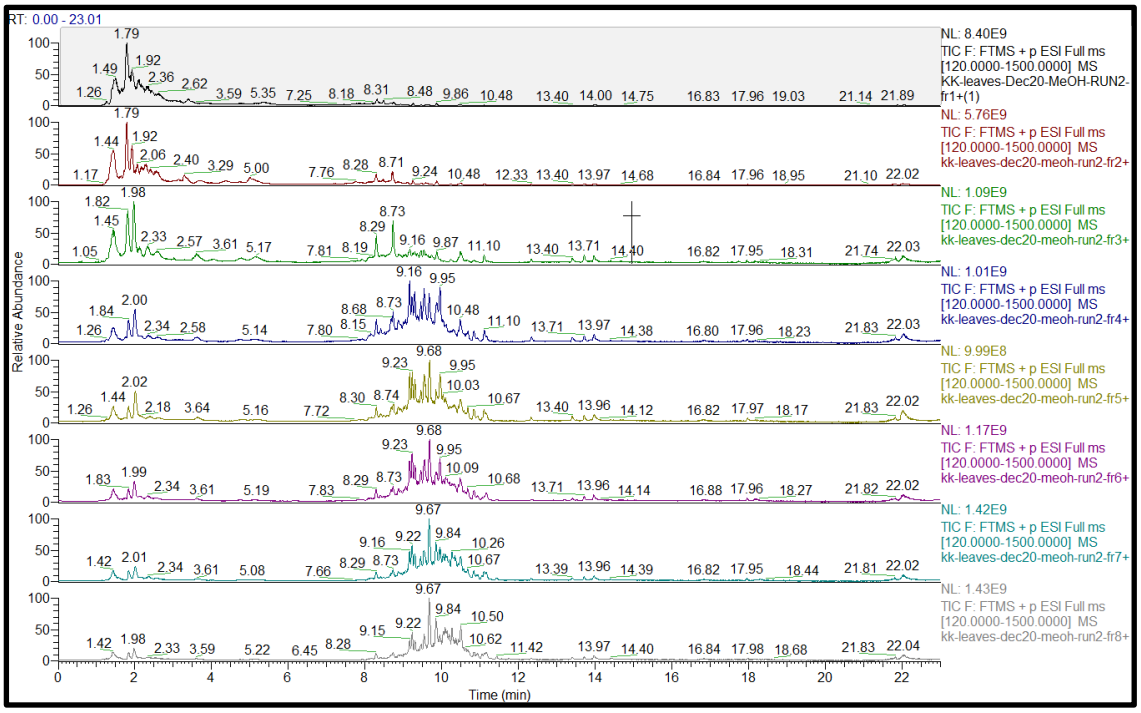
Figure A.31. Flash chromatogram of the RP-FC analysis of the KK-Dec20-leaf-MeOH-Fraction (RUN 2).

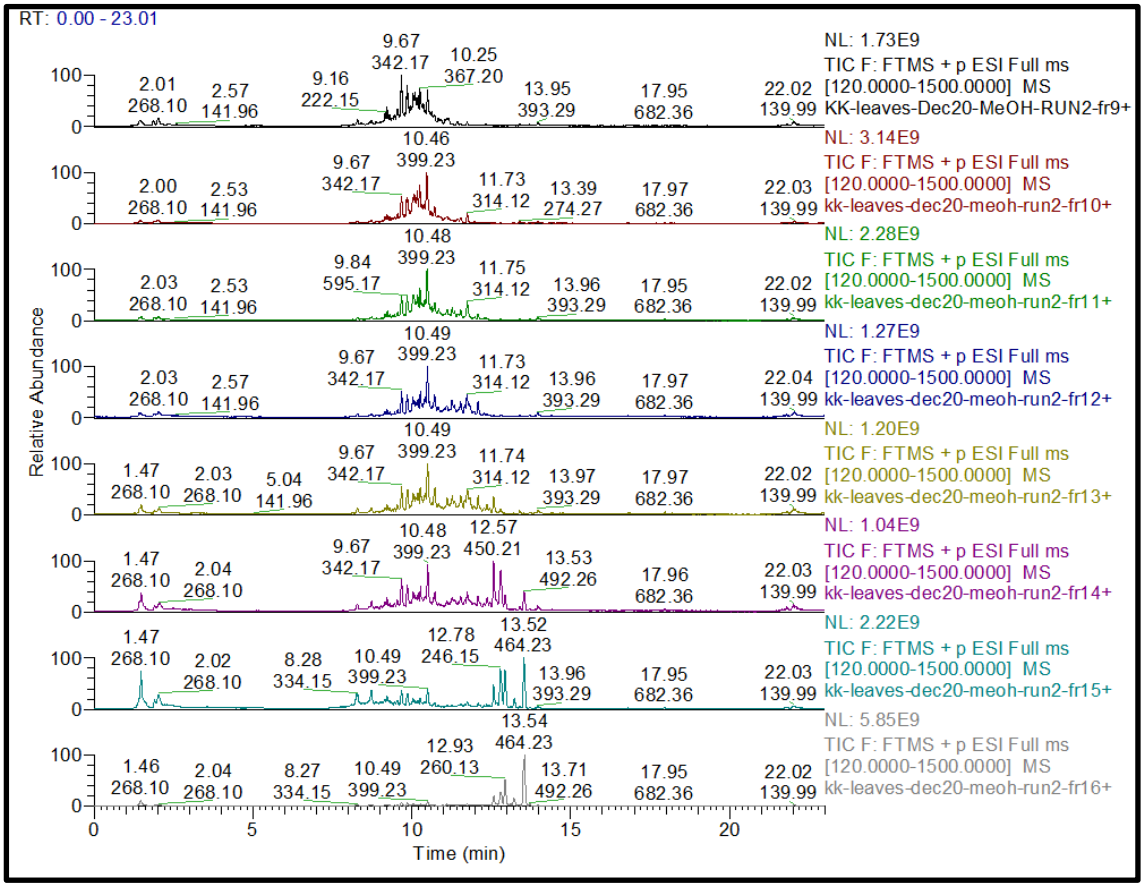
Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1-2	1st hypothesis	1
3-4	3rd and 4th hypothesis	2
5-21	2nd hypothesis	3
22-25	3rd and 4th hypothesis	4
26	1st hypothesis	5
27	1st hypothesis	6
28-32	3rd and 4th hypothesis	7
33	1st hypothesis	8
34-39	3rd and 4th hypothesis	9
40-42	1st hypothesis	10
43-51	3rd and 4th hypothesis	11
52-53	3rd and 4th hypothesis	12
54-58	3rd and 4th hypothesis	13
59-65	2nd hypothesis	14
66-69	3rd and 4th hypothesis	15
70-71	3rd and 4th hypothesis	16
72-76	3rd and 4th hypothesis	17
77-116	2nd hypothesis	18
117-121	2nd hypothesis	19

Table A.20. Combination of the fractions obtained from RP-FC run of the KK-Dec20-leaf-MeOH-Fraction (RUN 2).

Combination of fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1-2	KK-Dec20-MeOH-R2-Fr 1	648
3	KK-Dec20-MeOH-R2-Fr 2	45
4-9	KK-Dec20-MeOH-R2-Fr 3	27
10-13	KK-Dec20-MeOH-R2-Fr 4	57
14-15	KK-Dec20-MeOH-R2-Fr 5	32
16	KK-Dec20-MeOH-R2-Fr 6	5
17	KK-Dec20-MeOH-R2-Fr 7	10
18	KK-Dec20-MeOH-R2-Fr 8	12
19	KK-Dec20-MeOH-R2-Fr 9	10

Table A.21. Final fractions of the KK-Dec20-leaf-MeOH Fraction (RUN 2) through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.





(Contd. on the next page)

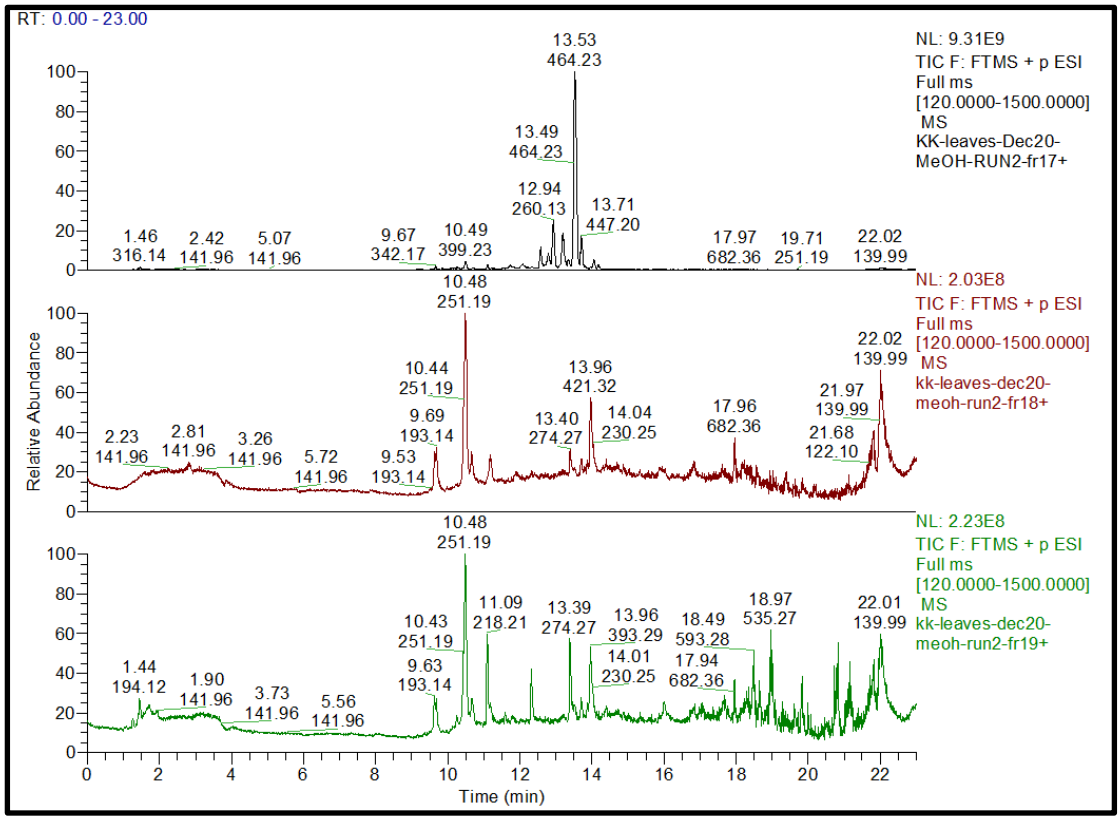


Figure A.32. LC-MS chromatograms of Initial Fractions 1-19 of the KK-Dec20-leaf-MeOH-Fraction (RUN 2) with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

RUN 3:

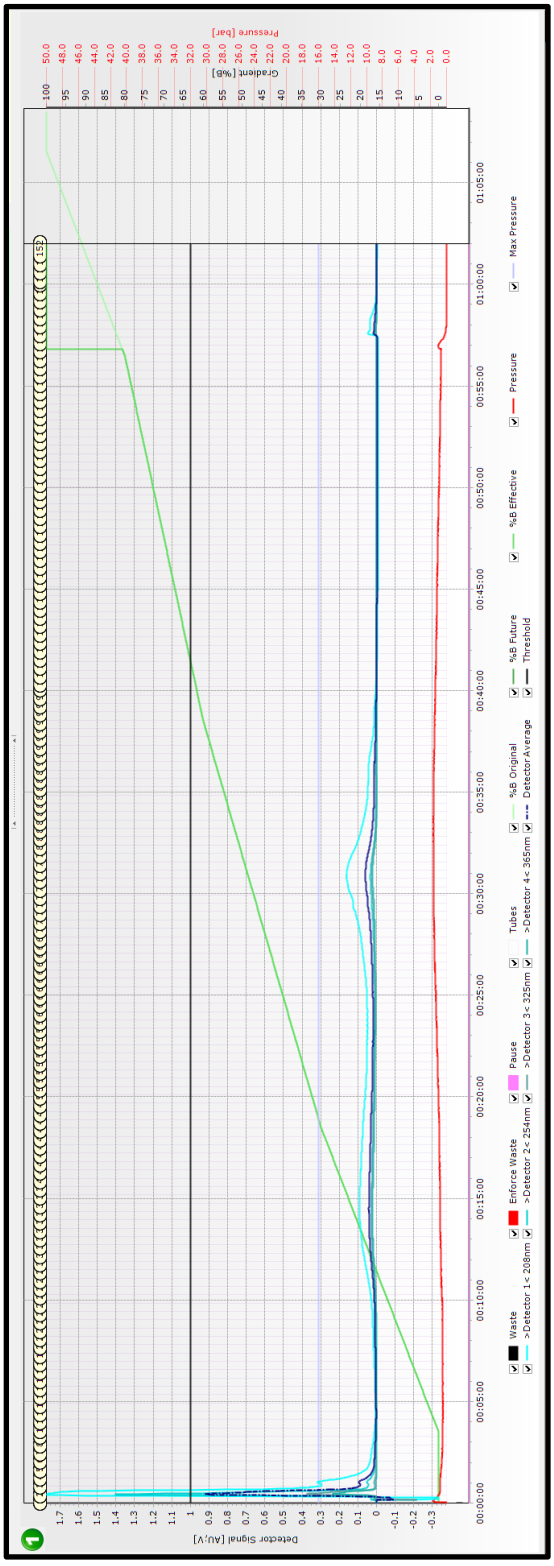


Figure A.33. Flash chromatogram of the RP-FC analysis of the KK-Dec20-leaf-MeOH-Fraction (RUN 3).

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1-2	1st hypothesis	1
3-4	1st hypothesis	2
5-6	3rd and 4th hypothesis	3
7-9	3rd and 4th hypothesis	4
10-11	3rd and 4th hypothesis	5
12-13	3rd and 4th hypothesis	6
14-19	3rd and 4th hypothesis	7
20-24	3rd and 4th hypothesis	8
25-52	3rd and 4th hypothesis	9
53-64	3rd and 4th hypothesis	10
65-85	3rd and 4th hypothesis	11
86-94	3rd and 4th hypothesis	12
95-140	2nd hypothesis	13
141-152	3rd and 4th hypothesis	14

Table A.22. Combination of the fractions obtained from RP-FC run of THE KK-Dec20-leaf-MeOH-Fraction (RUN 3).

Combination of fraction(s) having the same LC-MS/MS result	***Final fraction***	***Yield in mg***
1	KK-Dec20-MeOH-R3-Fr 1	602
2	KK-Dec20-MeOH-R3-Fr 2	30
3-6	KK-Dec20-MeOH-R3-Fr 3	9
7-8	KK-Dec20-MeOH-R3-Fr 4	9
9	KK-Dec20-MeOH-R3-Fr 5	56
10	KK-Dec20-MeOH-R3-Fr 6	30
11-12	KK-Dec20-MeOH-R3-Fr 7	12
13-14	KK-Dec20-MeOH-R3-Fr 8	11

Table A.23. Final fractions of the KK-Dec20-leaf-MeOH Fraction (RUN 3) through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

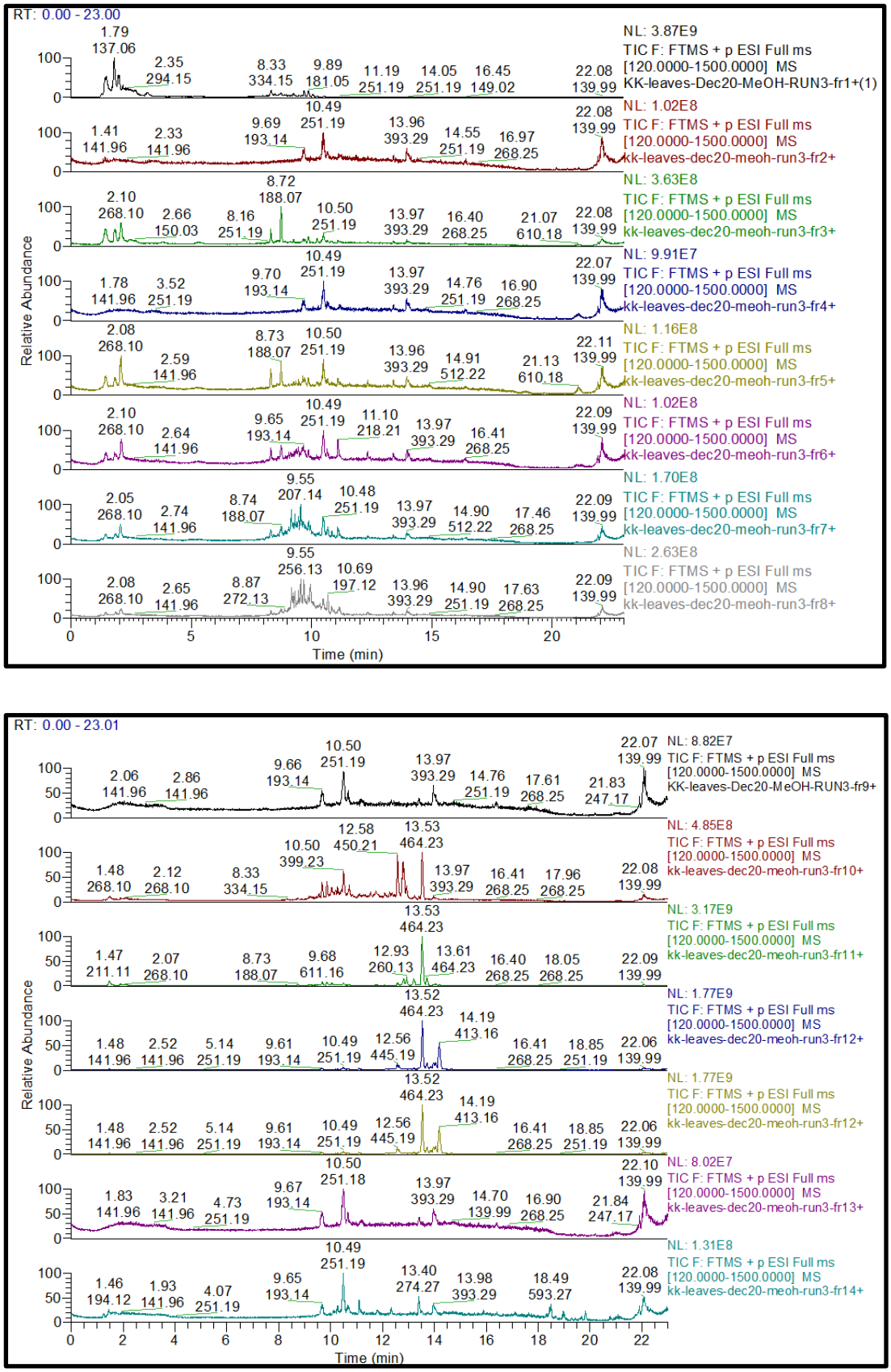


Figure A.34. LC-MS chromatograms of Initial Fractions 1-14 of the KK-Dec20-leaf-MeOH-Fraction (RUN 3) with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.10. Separation of the KK-Dec20-leaf-Water-Fraction Using RP-FC (RUN 1-2)

RUN 1:

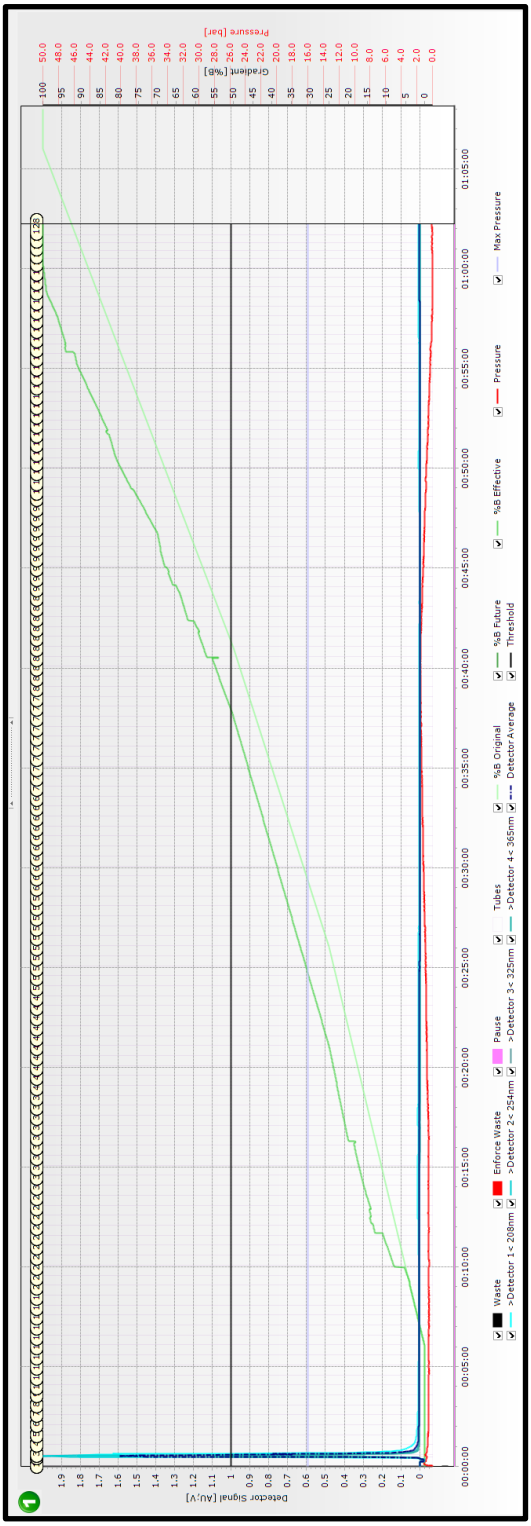


Figure A.35. Flash chromatogram of the RP-FC analysis of the KK-Dec20-leaf-Water-Fraction (RUN 1).

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
2-3	1st hypothesis	1
4-7	3rd and 4th hypothesis	2
8-34	2nd hypothesis	3
35-37	3rd and 4th hypothesis	4
38-39	3rd and 4th hypothesis	5
40-56	2nd hypothesis	6
57-86	2nd hypothesis	7
87-102	3rd and 4th hypothesis	8
103-106	3rd and 4th hypothesis	9
107-118	2nd hypothesis	10
119-128	3rd and 4th hypothesis	11

Table A.24. Combination of the fractions obtained from RP-FC run of the KK-Dec20-leaf-Water-Fraction (RUN 1).

Combination of fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1	KK-Dec20-Water-R1-Fr 1	596
2-4	KK-Dec20-Water-R1-Fr 2	8
5	KK-Dec20-Water-R1-Fr 3	8
6-7	KK-Dec20-Water-R1-Fr 4	3
8-10	KK-Dec20-Water-R1-Fr 5	3
11	KK-Dec20-Water-R1-Fr 6	4

Table A.25. Final fractions of the KK-Dec20-leaf-Water-Fraction (RUN 1) through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

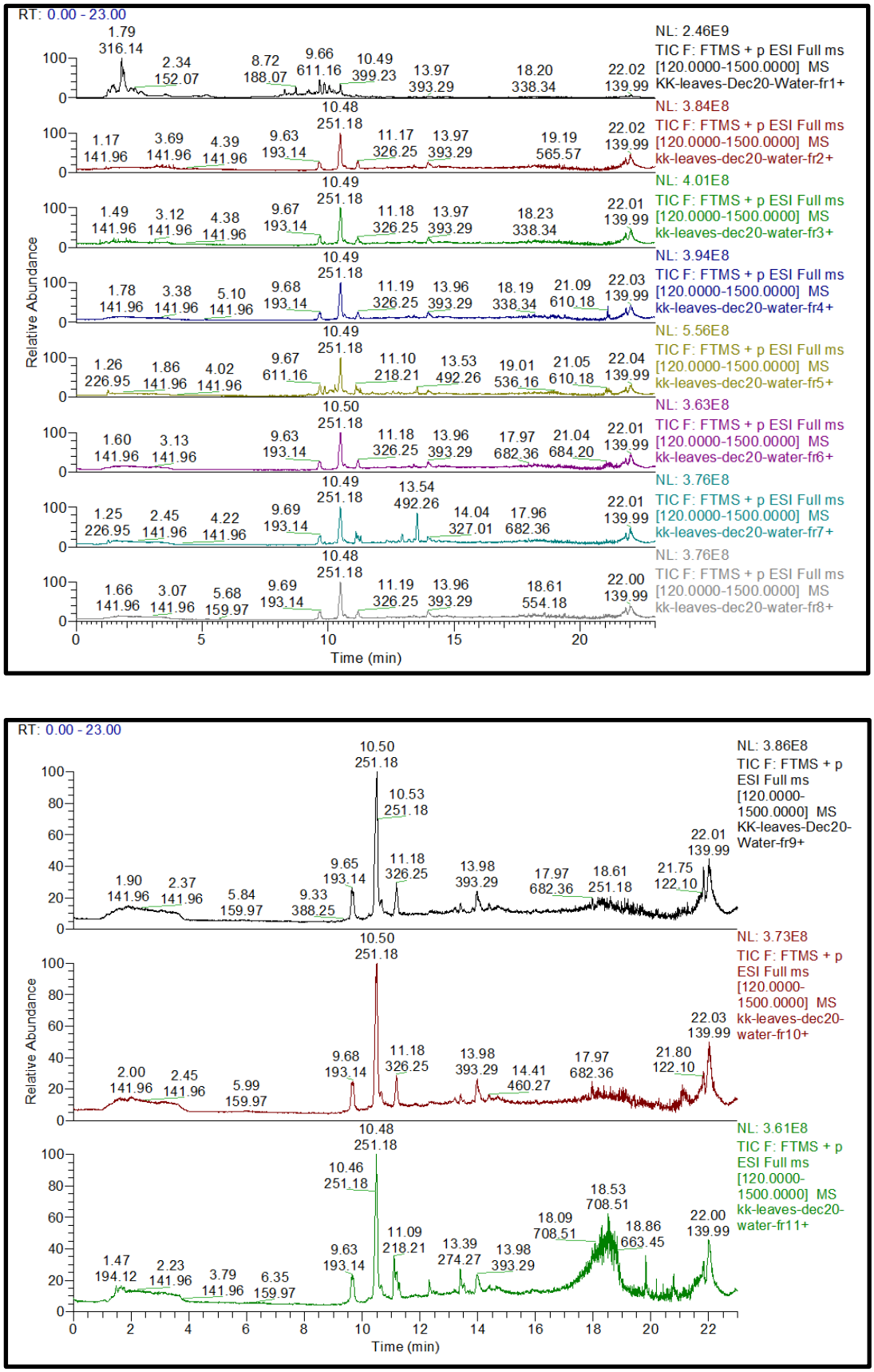


Figure A.36. LC-MS chromatograms of Initial Fractions 1-11 of the KK-Dec20-leaf-Water-Fraction (RUN 1) with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

RUN 2:

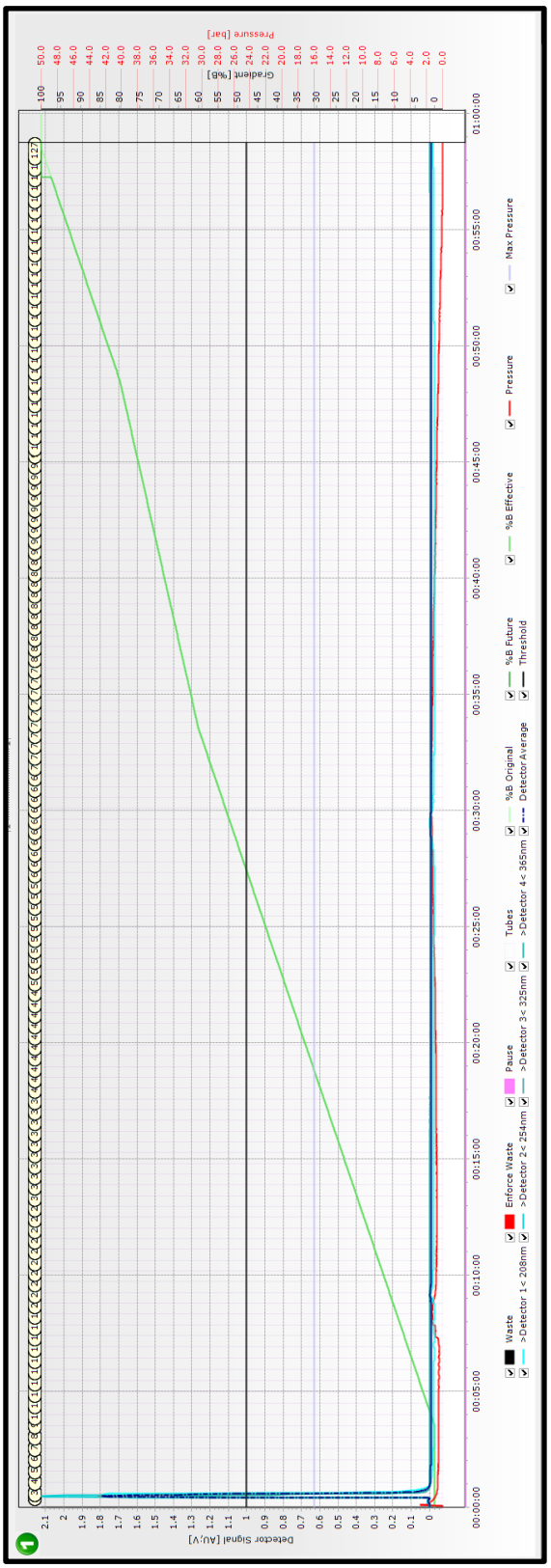


Figure A.37. Flash chromatogram of RP-FC analysis of the KK-Dec20-leaf-Water-Fraction (RUN 2).

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1-3	1st hypothesis	1
4-5	3rd and 4th hypothesis	2
6-20	2nd hypothesis	3
21-22	3rd and 4th hypothesis	4
23-62	2nd hypothesis	5
63-65	3rd and 4th hypothesis	6
66-98	2nd hypothesis	7
99-124	2nd hypothesis	8
125-127	3rd and 4th hypothesis	9

Table A.26. Combination of the fractions obtained from RP-FC run of the KK-Dec20-leaf-Water-Fraction (RUN 2).

Combination of fraction(s) having the same LC-MS result	***Final fraction***	***Yield in mg***
1	KK-Dec20-Water-R2-Fr 1	718
2	KK-Dec20-Water-R2-Fr 2	8
3-5	KK-Dec20-Water-R2-Fr 3	6
6	KK-Dec20-Water-R2-Fr 4	3
7-9	KK-Dec20-Water-R2-Fr 5	2

Table A.27. Final fractions of the KK-Dec20-leaf-Water-Fraction (RUN 2) through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

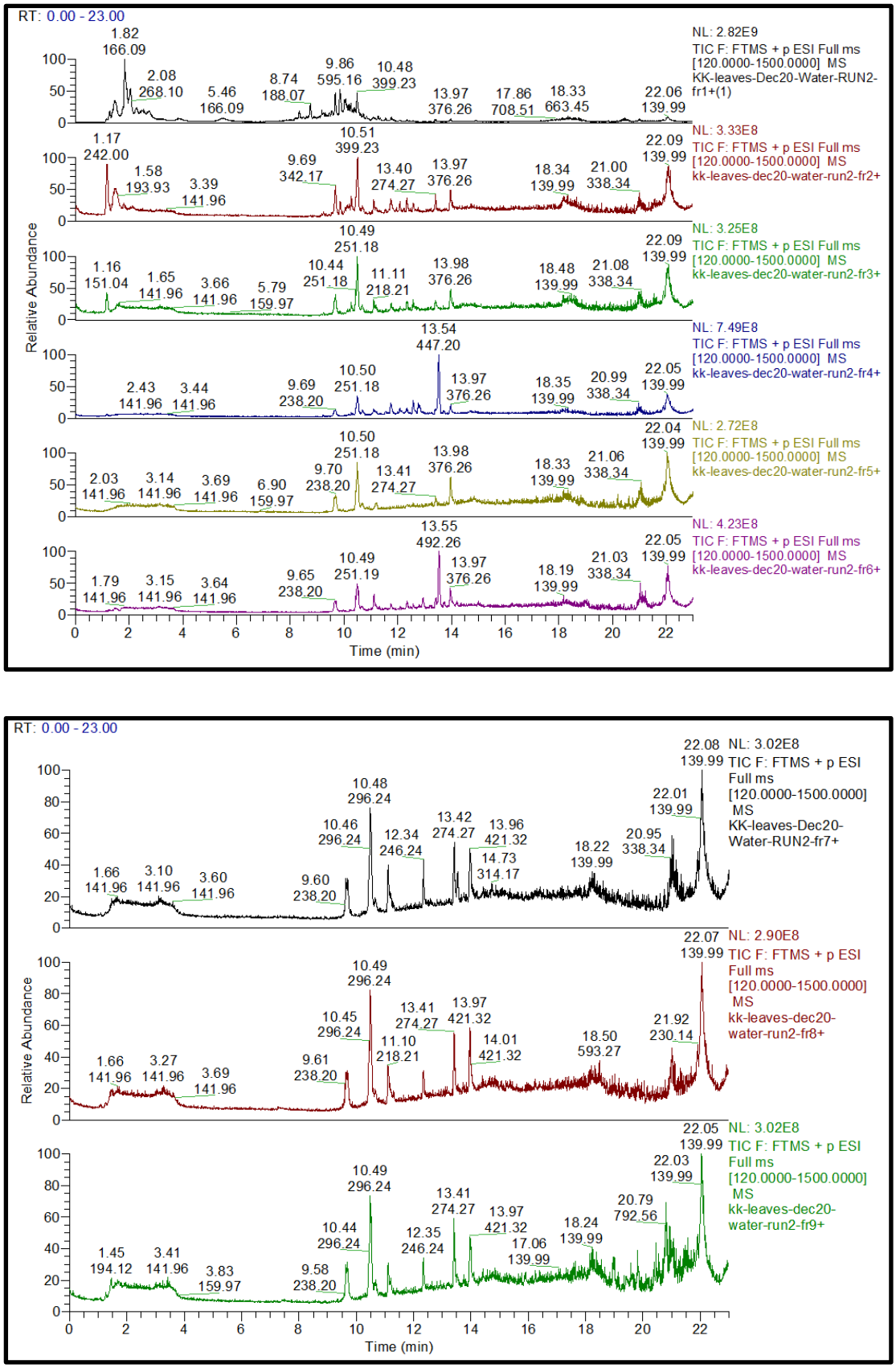


Figure A.38. LC-MS chromatograms of Initial Fractions 1-9 of the KK-Dec20-leaf-Water-Fraction (RUN 2) with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.11. Separation of the KK-Dec20-leaf-Water-Fr-1 Using RP-FC

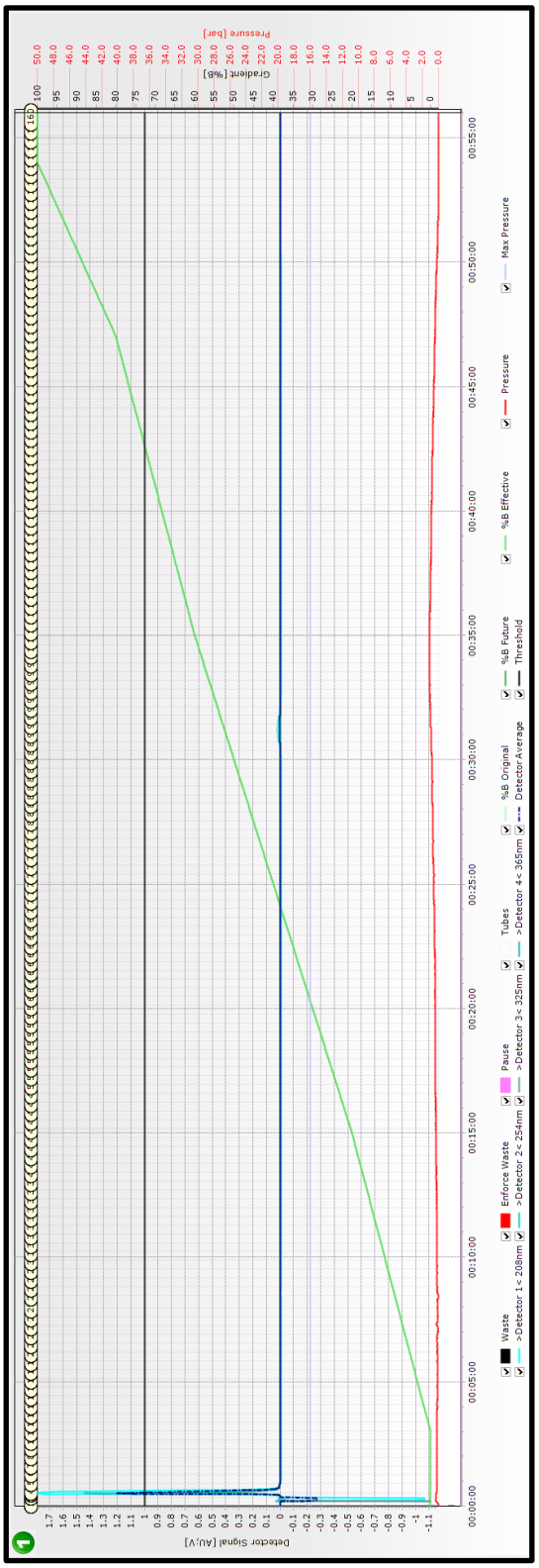


Figure A.39. Flash chromatogram of RP-FC analysis of the KK-Dec20-leaf-Water-Fr-1.

Combination of the fractions obtained from RP-FC run	***Sub-Fraction***	***Yield in mg***
1-4	Water fraction 1-1	324.9
88-93	Water fraction 1-2	1.2

Table A.28. Final fractions of the KK-Dec20-leaf-Water-Fr-1 and the dry matter yield of each fraction.

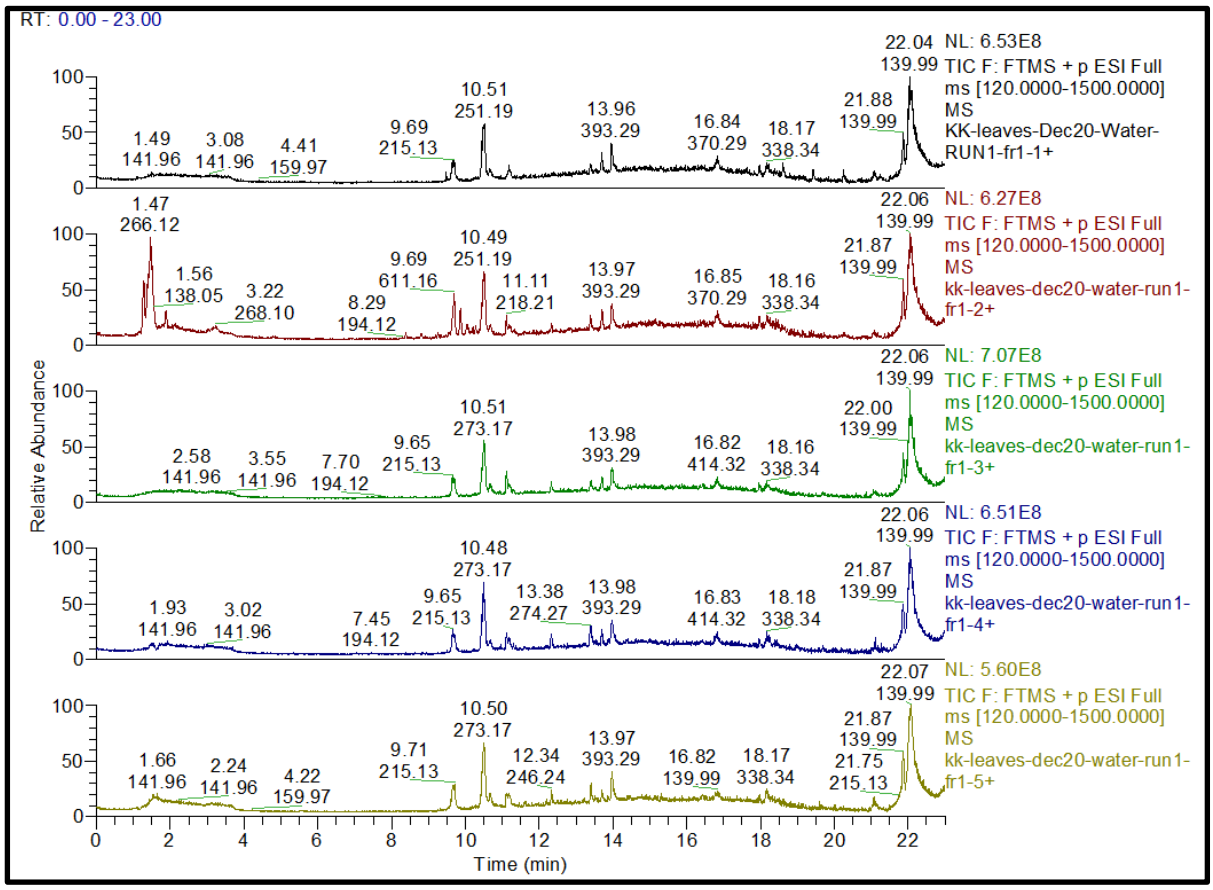


Figure A.40. LC-MS chromatograms of Initial Fractions 1-5 of the KK-Dec20-leaf-Water-Fr-1 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.1.12: Separation of the KK-Dec20-leaf-Water-Fr-1-1 Using RP-FC

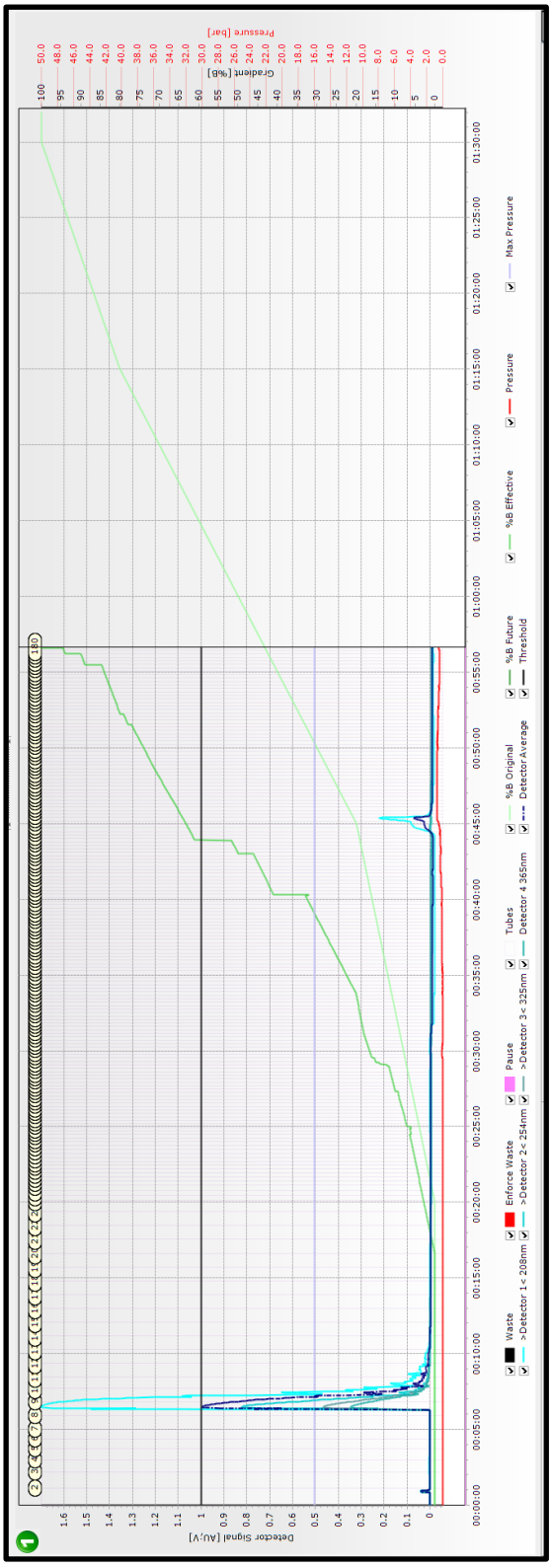


Figure A.41. Flash chromatogram of the RP-FC analysis of the KK-Dec20-leaf-Water-Fr-1-1.

Combination of the fractions obtained from RP-FC run	***Combination based on hypothesis presented in Section 2.3.9.3***	***Initial fraction***
1-2	1st hypothesis	1
8-9	1st hypothesis	2
10	3rd and 4th hypothesis	3
11	3rd and 4th hypothesis	4
12	3rd and 4th hypothesis	5
13-14	3rd and 4th hypothesis	6
15-74	2nd hypothesis	7
75-107	3rd and 4th hypothesis	8
108-127	2nd hypothesis	9
128-129	1st hypothesis	10
130-132	1st hypothesis	11

Table A.29. Combination of the fractions obtained from RP-FC run of the KK-Dec20-leaf-Water-Fr-1-1.

Combination of fraction(s) having the same LC-MS result	***Final fraction***
1	KK-Dec20-Water-Fr-1-1-1
2-6	KK-Dec20-Water-Fr-1-1-2
10-11	KK-Dec20-Water-Fr-1-1-3

Table A.30. Final fractions of the KK-Dec20-leaf-Water-Fr-1-1 through combination of initial fractions based on LC-MS analysis and dry matter yield of each fraction.

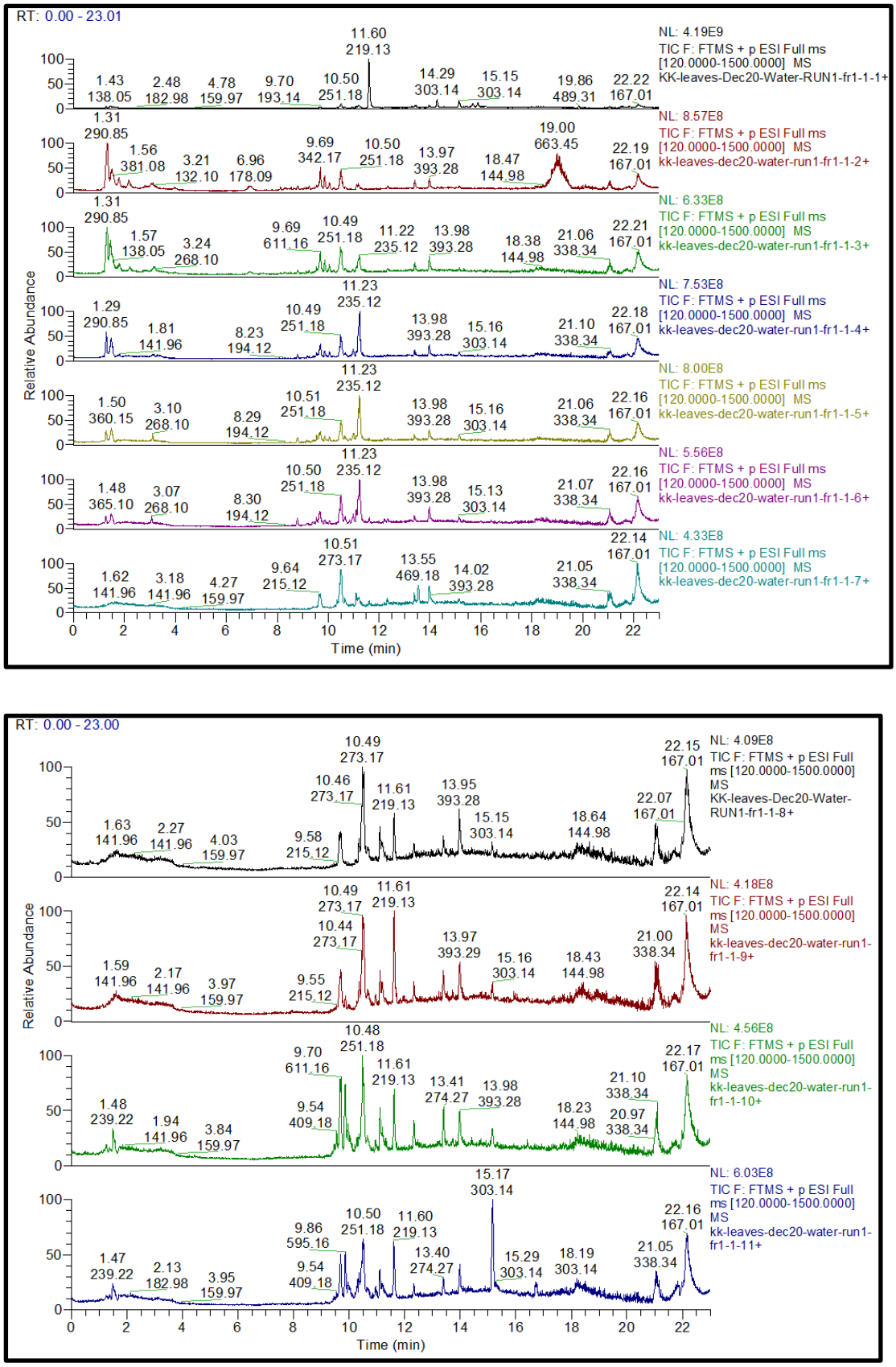


Figure A.42. LC-MS chromatograms of Initial Fractions 1-11 of the KK-Dec20-leaf-Water-Fr-1-1 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.2. Results of Nematocidal Experiments of KK Leaf Fractions

Appendix 7.2.1. Result of Experiment 7.1

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Dec19-leaf-Hexane	8	10	9	9.0 ± 0.5^{g}
KK-Dec19-leaf-EtOAc	15	14	14	14.3 ± 0.3^{f}
KK-Dec19-leaf-Water-MeOH	34	36	39	36.3 ± 1.4^{b}
KK-Dec19-leaf-Water	10	9	10	9.7 ± 0.3^{g}
KK-Dec19-leaf-MeOH	18	18	17	17.7 ± 0.3^{e}
BZ	21	23	22	22.0 ± 0.5^{d}
AB	26	27	26	26.3 ± 0.3^{c}
IVM	88	86	88	87.3 ± 0.6^{a}
PBS	0	0	0	0

Table A.31. Result of Experiment 7.1: Mean of % mortality of the KK-Dec19 leaf crude solvent fractions (n=3; T1-3) against Batch 6 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.2. Result of Experiment 7.2

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Dec19-leaf-Hexane	11	12	11	11.3 ± 0.3^{g}
KK-Dec19-leaf-EtOAc	17	18	16	17.0 ± 0.5^{f}
KK-Dec19-leaf-Water-MeOH	59	60	59	59.3 ± 0.3^{c}
KK-Dec19-leaf-Water	81	82	82	81.7 ± 0.3^{b}
KK-Dec19-leaf-MeOH	40	41	42	41.0 ± 0.5^{d}
BZ	36	37	36	36.3 ± 0.3^{e}
AB	80	80	81	80.3 ± 0.3^{b}
IVM	88	88	88	88.0 ± 0^{a}
PBS	0	0	0	0

Table A.32. Result of Experiment 7.2: Mean of % mortality of the KK-Dec19 leaf crude solvent fractions (n=3; T1-3) against Batch 7 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.3. Result of Experiment 7.3

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Dec19-leaf-Water-MeOH	69	69	70	$69.3 \pm 0.3^{a,b}$
KK-Dec19-leaf-MeOH	66	68	69	67.7 ± 0.8^{b}
KK-Dec19-leaf-Water	63	66	63	64.0 ± 1.0^{b}
IVM	79	72	-	75.5 ± 3.5^{a}
AB	53	55	54	54.0 ± 0.5^{c}
BZ	36	47	40	41.0 ± 3.2^{d}
PBS	0	0	-	0

Table A.33. Result of Experiment 7.3: Mean of % mortality of the KK-Dec19 leaf crude solvent fractions (n=3; T1-3) against Batch 8 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.4. Result of Experiment 7.4

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Me+IVM	80	75	67	74.0 ± 3.7^{b}
KK-Me+AB	30	31	37	$32.7 \pm 2.1^{d,e}$
KK-Me+BZ	15	17	18	16.7 ± 0.8^{f}
KK-W+IVM	82	82	-	82.0 ± 0^{a}
KK-W+AB	61	60	57	59.3 ± 1.2^{c}
KK-W+BZ	39	41	38	39.3 ± 0.8^{d}
BZ+AB	33	38	40	37.0 ± 2.0^{d}
IVM	88	88	-	88.0 ± 0^{a}
AB	25	27	25	$25.7 \pm 0.7^{e,f}$
BZ	22	18	25	21.7 ± 2.8^{f}
PBS	0	0	0	0

Table A.34. Result of Experiment 7.4: Mean of % mortality of combination formulations (n=3; T1-3) against Batch 6 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.5. Result of Experiment 7.5

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Me+IVM	80	75	76	$77.0 \pm 1.5^{b,c}$
KK-Me+AB	70	75	71	72.0 ± 1.5^{c}
KK-Me+BZ	23	24	26	24.3 ± 0.8^{f}
KK-W+IVM	81	89	85	$85.0 \pm 2.3^{a,b}$
KK-W+AB	81	80	79	80.0 ± 0.5^{b}
KK-W+BZ	57	58	57	57.3 ± 0.3^{d}
BZ+AB	83	81	85	$83.0 \pm 1.1^{a,b}$
IVM	88	88	-	88.0 ± 0^{a}
AB	80	81	80	80.3 ± 0.3^{b}
BZ	33	38	39	36.7 ± 1.8^{e}
PBS	0	0	0	0

Table A.35. Result of Experiment 7.5: Mean of % mortality of combination formulations (n=3; T1-3) against Batch 7 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.6. Result of Experiment 7.6

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Me+IVM	88	82	-	85.0 ± 3.0^{a}
KK-Me+BZ	68	59	65	$64.0 \pm 2.6^{b,c}$
KK-W+IVM	90	95	91	92.0 ± 1.5^{a}
KK-W+BZ	42	44	-	$43.0 \pm 1.0^{d,e}$
IVM	79	72	-	75.5 ± 3.5^{b}
AB	53	55	54	$54.0 \pm 0.5^{c,d}$
BZ	36	47	40	41.0 ± 3.2^{e}
PBS	0	0	0	0

Table A.36. Result of Experiment 7.6: Mean of % mortality of combination formulations (n=3; T1-3) against Batch 8 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.7. Result of Experiment 7.7

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec19-leaf-Water + QWF	25	26	25	25.3 ± 0.3^{b}
KK-Dec19-leaf-MeOH +QWF	29	28	29	28.7 ± 0.3^{a}
KK-Dec19-leaf-Water	10	9	10	9.7 ± 0.3^{d}
KK-Dec19-leaf-MeOH	18	18	17	17.7 ± 0.3^{c}
QWF	10	11	11	10.7 ± 0.3^{d}
PBS	0	0	0	0

Table A.37. Result of Experiment 7.7: Mean of % mortality of combination formulations made with the KK and QWF samples (n=3; T1-3) against Batch 6 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.8. Result of Experiment 7.8

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec19-leaf-Water + QWF	63	63	63	63.0 ± 0^{b}
KK-Dec19-leaf-MeOH +QWF	49	49	50	49.3 ± 0.3^{c}
KK-Dec19-leaf-Water	81	82	82	81.7 ± 0.3^{a}
KK-Dec19-leaf-MeOH	40	41	42	41.0 ± 0.5^{d}
QWF	34	35	36	35.0 ± 0.5^{e}
PBS	0	0	0	0

Table A.38. Result of Experiment 7.8: Mean of % mortality of combination formulations made with the KK and QWF samples (n=3; T1-3) against Batch 7 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.9. Result of Experiment 7.9

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec19-leaf-Water + QWF	67	66	68	67.0 ± 0.6[a]
KK-Dec19-leaf-MeOH +QWF	59	59	60	59.3 ± 0.3[b]
KK-Dec19-leaf-Water	63	66	63	64.0 ± 1.0[a]
KK-Dec19-leaf-MeOH	66	68	69	67.7 ± 0.8[a]
QWF	45	47	43	45.0 ± 1.1[c]
PBS	0	0	0	0

Table A.39. Results of Experiment 7.9: Mean of % mortality of combination formulations made with the KK and QWF samples (n=3; T1-3) against Batch 8 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.10. Result of Experiment 7.10

Sample	***% Larval Mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-May20-leaf-Water	80	76	77	77.6 ± 2.0[a]
KK-May20-leaf-MeOH	74	72	71	72.3 ± 1.5[b]
KK-May20-leaf-Water-MeOH	32	33	32	32.3 ± 0.5[d]
KK-May20-leaf-EtOAc	0	3	4	2.3 ± 2.0[e]
KK-May20-leaf-Hexane	0	2	1	1.0 ± 1.0[e]
IVM	56	55	58	56.3 ± 1.5[c]
AB	75	78	80	77.6 ± 2.5[a]
BZ	0	5	4	3.0 ± 2.6[e]
PBS	0	0	0	0

Table A.40. Result of Experiment 7.10: Mean of % mortality of the KK-May20 leaf crude solvent fractions (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of samples (%)***			
	Water-MeOH (*dead/total for the species*)	**Water** (*dead/total for the species*)	**MeOH** (*dead/total for the species*)	**AB** (*dead/total for the species*)
H. contortus (56%)	11% (*6/56*)	64% (*36/56*)	50% (*28/56*)	59% (*33/56*)
T. circumcincta (4%)	-	100% (*4/4*)	100% (*4/4*)	100% (*4/4*)
Trichostrongylus spp. *(7%)*	57% (*4/7*)	100% (*7/7*)	86% (*6/7*)	86% (*6/7)*
LT (*33%*)	59% (*22/33*)	100% (*33/33*)	100% (*33/33*)	97% (*32/33*)
Total Dead count (%) (dead/total)	32% (*32/100*)	80% (*80/100*)	74% (*74/100*)	76% (*76/100*)

Table A.41. Dead larval identification of the species of Batch 9 larvae against the fractions of the KK leaf and the standard AB.

Appendix 7.2.11. Result of Experiment 7.11

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-May20-leaf-Water 16 mg/mL	43	60	56	53.0 ± 5.1^{a}
KK-May20-leaf-Water 12 mg/mL	62	58	61	60.3 ± 1.2^{a}
KK-May20-leaf-Water 8 mg/mL	60	60	65	61.7 ± 1.7^{a}
KK-May20-leaf-Water 6 mg/mL	53	55	53	53.7 ± 0.7^{a}
KK-May20-leaf-Water 4 mg/mL	36	38	37	37.0 ± 0.5^{b}
KK-May20-leaf-Water 2 mg/mL	10	11	14	11.7 ± 1.2^{c}
PBS	0	0	-	0

Table A.42. Result of Experiment 7.11: Mean of % mortality of different concentrations of the KK-May20-leaf-Water-Fraction (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.12. Result of Experiment 7.12

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-May20-leaf-MeOH 16 mg/mL	61	56	53	56.7 ± 2.3^{b}
KK-May20-leaf-MeOH 12 mg/mL	53	55	53	53.7 ± 0.7^{b}
KK-May20-leaf-MeOH 8 mg/mL	66	64	62	64.0 ± 1.1^{a}
KK-May20-leaf-MeOH 6 mg/mL	55	51	50	52.0 ± 1.5^{b}
KK-May20-leaf-MeOH 4 mg/mL	46	44	43	44.3 ± 0.8^{c}
KK-May20-leaf-MeOH 2 mg/mL	22	20	19	20.3 ± 0.8^{d}
PBS	0	0	-	0

Table A.43. Result of Experiment 7.12: Mean of % mortality of different concentrations of KK-May20-leaf-MeOH Fraction (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.13. Result of Experiment 7.13

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-May20-leaf-Hex-fr10	2	1	1	1.3 ± 0.3^{c}
KK-May20-leaf-Hex-fr9	0	0	2	0.7 ± 0.6^{c}
KK-May20-leaf-Hex-fr8	10	11	8	9.7 ± 0.8^{b}
KK-May20-leaf-Hex-fr7	0	6	2	2.7 ± 1.7^{c}
KK-May20-leaf-Hex-fr6	20	21	16	19.0 ± 1.5^{a}
KK-May20-leaf-Hex-fr5	13	12	13	$12.7 \pm 0.3^{a,b}$
KK-May20-leaf-Hex-fr4	0	0	0	0
KK-May20-leaf-Hex-fr3	0	0	0	0
KK-May20-leaf-Hex-fr2	0	0	0	0
KK-May20-leaf-Hex-fr1	0	0	0	0
KK-May20-leaf-Hexane crude	0	1	2	1.0 ± 0.5^{c}
BZ	15	5	8	9.3 ± 2.9^{b}
PBS	0	0	-	0

Table A.44. Result of Experiment 7.13: Mean of % mortality of the separated fractions of KK-May20-leaf-Hexane-Fraction (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.14. Result of Experiment 7.14

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-May20-leaf-Et-fr10	10	12	14	12.0 ± 1.1^{c}
KK-May20-leaf-Et-fr9	5	2	9	5.3 ± 2.0^{d}
KK-May20-leaf-Et-fr8	8	11	12	$10.3 \pm 1.2^{c,d}$
KK-May20-leaf-Et-fr7	9	12	14	$11.7 \pm 1.4^{c,d}$
KK-May20-leaf-Et-fr6	16	18	16	$16.7 \pm 0.6^{a,b}$
KK-May20-leaf-Et-fr5	14	12	15	$13.7 \pm 0.8^{b,c}$
KK-May20-leaf-Et-fr4	20	18	23	20.3 ± 1.4^{a}
KK-May20-leaf-Et-fr3	3	5	8	5.3 ± 1.4^{d}
KK-May20-leaf-Et-fr2	3	2	5	3.3 ± 0.8^{d}
KK-May20-leaf-Et-fr1	1	0	0	0.3 ± 0.3^{e}
KK-May20-leaf-EtoAC crude	2	3	5	3.3 ± 0.8^{d}
BZ	12	8	8	$9.7 \pm 1.2^{c,d}$
PBS	0	0	-	0

Table A.45. Result of Experiment 7.14: Mean of % mortality of the separated fractions of KK-May20-leaf-EtOAc-Fraction (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.15. Result of Experiment 7.15

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-May20-leaf-Water-fr 7	1	2	0	1.0 ± 0.5^{g}
KK-May20-leaf-Water-fr 6	5	3	2	3.3 ± 0.8^{g}
KK-May20-leaf-Water-fr 5	31	30	-	30.5 ± 0.5^{e}
KK-May20-leaf-Water-fr 4	20	17	-	18.5 ± 1.5^{f}
KK-May20-leaf-Water-fr 3	65	70	69	68.0 ± 1.5^{b}
KK-May20-leaf-Water-fr 2	47	45	42	44.7 ± 1.4^{d}
KK-May20-leaf-Water-fr 1	31	30	27	29.3 ± 1.2^{e}
KK-May20-leaf-Water crude	80	76	77	77.6 ± 2.0^{a}
IVM	57	61	61	60.0 ± 1.5^{c}
AB	48	49	51	49.3 ± 0.8^{d}
PBS	0	0	-	0

Table A.46. Result of Experiment 7.15: Mean of % mortality of the separated fractions of KK-May20-leaf-Water-Fraction (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of KK-May20-leaf-Water-Fr 3 (%)*** (*immotile/motile for that species*)
H. contortus (56%)	53% (30/56)
T. circumcincta (4%)	50% (2/4)
Trichostrongylus spp. *(7%)*	71% (5/7)
LT *(33%)*	97% (32/33)
Total dead count (%) (dead/total)	69% (69/100)

Table A.47. Dead larval identification of the species of Batch 9 larvae against the KK-May20-leaf-Water-Fraction 3.

Appendix 7.2.16. Result of Experiment 7.16

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-May20-leaf-Water-Fr3 8 mg/mL	66	70	71	69.0 ± 1.5^{b}
KK-May20-leaf-Water-Fr3 6 mg/mL	85	92	90	89.0 ± 2.0^{a}
KK-May20-leaf-Water-Fr3 4 mg/mL	92	93	89	91.3 ± 1.2^{a}
KK-May20-leaf-Water-Fr3 2 mg/mL	72	76	75	74.3 ± 1.2^{b}
KK-May20-leaf-Water-Fr3 1 mg/mL	10	8	5	7.7 ± 1.4^{c}

Table A.48. Result of Experiment 7.16: Mean of % mortality of different concentrations of KK-May20-leaf-Water-Fraction-3 (n=3; T1-3) against Batch 9 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of KK-May20-leaf-Water-Fr 3 (%)*** (*immotile/motile for that species*)
H. contortus (56%)	86% (*43/50*)
T. circumcincta (4%)	100% (*3/3*)
Trichostrongylus spp. *(7%)*	100% (*6/6*)
LT *(33%)*	100% (*31/31*)
Total dead count (%) (dead/total)	92% (*83/90*)

Table A.49. Dead larval identification of the species of Batch 9 larvae against the KK-May20-leaf-Water-Fraction-3.

Appendix 7.2.17. Result of Experiment 7.17

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-May20-leaf-MeOH-fr 8	0	1	0	0.3 ± 0.3^{g}
KK-May20-leaf-MeOH-fr 7	9	8	8	8.3 ± 0.3^{f}
KK-May20-leaf-MeOH-fr 6	20	18	16	18.0 ± 1.1^{e}
KK-May20-leaf-MeOH-fr 5	21	25	23	23.0 ± 1.1^{e}
KK-May20-leaf-MeOH-fr 4	80	75	72	75.7 ± 2.3^{b}
KK-May20-leaf-MeOH-fr 3	62	61	59	60.7 ± 0.8^{c}
KK-May20-leaf-MeOH-fr 2	88	92	94	91.3 ± 1.7^{a}
KK-May20-leaf-MeOH-fr 1	78	75	78	77.0 ± 1.0^{b}
KK-May20-leaf-Water fraction	36	35	32	34.3 ± 1.2^{d}
KK-May20-leaf-MeOH fraction	32	34	31	32.3 ± 0.8^{d}
AB	72	69	74	71.7 ± 1.4^{b}
BZ	27	20	15	20.7 ± 3.4^{e}
PBS	0	0	0	0

Table A.50. Result of Experiment 7.17: Mean of % mortality of the separated fractions of KK-May20-leaf-MeOH-Fraction (n=3; T1-3) against Batch 10 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of KK-May20-leaf-MeOH-Fr 2 (%)*** (*immotile/motile for that species*)
H. contortus (44%)	81% (*34/42*)
T. circumcincta (2%)	100% (*2/2*)
Trichostrongylus spp. *(5%)*	100% (*5/5*)
Cooperia spp. *(1%)*	-
LT *(48%)*	100% (*48/48*)
Total dead count (%) (dead/total)	92% (*89/97*)

Table A.51. Dead larval identification of the species of Batch 10 larvae against the KK-May20-leaf-MeOH-Fraction-2.

Appendix 7.2.18. Result of Experiment 7.18

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Oct20-leaf-Water	33	37	31	33.7 ± 1.7^{b}
KK-Oct20-leaf-MeOH	43	38	40	40.3 ± 1.4^{a}
KK-Oct20-leaf-Water-MeOH	20	16	18	18.0 ± 1.1^{c}
KK-Oct20-leaf-EtOAC	2	5	3	3.3 ± 0.8^{e}
KK-Oct20-leaf-Hexane	1	0	1	0.7 ± 0.3^{e}
IVM	10	14	11	11.7 ± 1.2^{d}
BZ	0	0	0	0
PBS	0	0	-	0

Table A.52. Result of Experiment 7.18: Mean of % mortality of KK-Oct20 leaf crude solvent fractions (n=3; T1-3) against Batch 11 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of samples (%)***			
	Water-MeOH (*dead/total for the species*)	**Water** (*dead/total for the species*)	**MeOH** (*dead/total for the species*)	**IVM** (*dead/total for the species*)
H. contortus (72%)	0% (*0/72*)	12% (*9/72*)	25% (*18/72*)	0% (*0/72*)
T. circumcincta (4%)	50% (*2/4)*	100% (*4/4*)	100% (*4/4*)	50% (*2/4*)
Trichostrongylus spp. *(4%)*	25% (*1/4*)	50% (*2/4*)	25% (*1/4*)	0% (*0/4*)
LT (*20%)*	85% (*17/20*)	90% (*18/20*)	100% (*20/20*)	45% (*9/20*)
Total Dead count (%) (dead/total)	20% (*20/100*)	33% (*33/100*)	43% (*43/100*)	11% (11*/100*)

Table A.53. Dead larval identification of the species of Batch 11 larvae against the KK-Oct20-Water-MeOH, KK-Oct20-Water, KK-Oct20-MeOH and IVM.

Appendix 7.2.19. Result of Experiment 7.19

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Oct20-leaf-MeOH-Fr 8	30	34	40	34.7 ± 2.9^{d}
KK-Oct20-leaf-MeOH-Fr 7	19	15	17	17.0 ± 1.1^{f}
KK-Oct20-leaf-MeOH-Fr 6	20	26	21	$22.3 \pm 1.8^{e,f}$
KK-Oct20-leaf-MeOH-Fr 5	47	44	40	43.7 ± 2.0^{c}
KK-Oct20-leaf-MeOH-Fr 4	80	75	71	75.3 ± 2.6^{a}
KK-Oct20-leaf-MeOH-Fr 3	67	78	70	71.7 ± 3.2^{a}
KK-Oct20-leaf-MeOH-Fr 2	42	47	42	43.7 ± 1.7^{c}
KK-Oct20-leaf-MeOH-Fr 1	52	58	56	55.3 ± 1.7^{b}
PBS	0	0	0	0

Table A.54. Result of Experiment 7.19: Mean of % mortality of the separated fractions of KK-Oct20-leaf-MeOH Fraction (n=3; T1-3) against Batch 11 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of KK-Oct20-leaf-MeOH-Fr 4 (%)*** (*immotile/motile for that species*)
H. contortus (72%)	67% (*48/72*)
T. circumcincta (4%)	100% (*4/4*)
Trichostrongylus spp*. (4%)*	75% (*3/4*)
LT *(20%)*	100% (*20/20*)
Total dead count (%) (dead/total)	75% (*75/100*)

Table A.55. Dead larval identification of the species of Batch 9 larvae against the KK-May20-leaf-MeOH-Fraction-4.

Appendix 7.2.20. Result of Experiment 7.20

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Oct20-leaf-Water-Fr 6	10	11	-	10.5 ± 0.5^{d}
KK-Oct20-leaf-Water-Fr 5	10	-	-	10.0^{d}
KK-Oct20-leaf-Water-Fr 4	20	17	-	$18.5 \pm 1.5^{c,d}$
KK-Oct20-leaf-Water-Fr 3	38	41	-	$39.5 \pm 1.5^{b,c}$
KK-Oct20-leaf-Water-Fr 2	46	55	-	50.5 ± 4.5^{b}
KK-Oct20-leaf-Water-Fr 1	86	72	68	75.3 ± 5.4^{a}
PBS	0	0	-	0

Table A.56. Result of Experiment 7.20: Mean of % mortality of the separated fractions of KK-Oct20-leaf-MeOH Fraction (n=3 for Fr 1, T1-3; n=2 for Fr 2-4, 6, T=1-2; n=1 for Fr 5, T=1) against Batch 11 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Dead Larval Identifications:

It was observed with the 3 tests that were performed with Fraction-1 with Batch 11 larvae, the efficacies were 86% (T1), 72% (T2) and 68% (T3). It was found that with T1, a total of 70 larvae were present in the 100 µL aliquot taken from the bottle that contained the Batch 11 larvae. Of these 70 larvae, there were predominant presence of more LT than *H. contortus*. The Fraction-1 was able to

kill all of them and there were less *H. contortus* present in that aliquot, the total mortality count was 60 out 70 larvae (86%). This was an unusual finding, as most times the percentage of the composition had been found to be identical with each aliquot pipetted out from a bottle containing the batch of larvae. T2 and T3 had the usual larval % composition of Batch 11. Nonetheless, this result again indicated that the efficacy of a fraction was largely dependent on the species of the larvae present.

Appendix 7.2.21. Result of Experiment 7.21

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec20-leaf-Water	87	87	82	85.3 ± 1.6^{a}
KK-Dec20-leaf-MeOH	45	42	48	45.0 ± 1.7^{b}
KK-Dec20-leaf-Water-MeOH	13	12	13	12.7 ± 0.3^{d}
KK-Dec20-leaf-EtOAC	5	5	3	$4.3 \pm 0.6^{d,e}$
KK-Dec20-leaf-Hexane	2	3	2	2.3 ± 0.3^{e}
IVM	33	34	34	33.7 ± 0.3^{c}
AB	26	28	26	26.7 ± 0.6^{c}
BZ	38	22	25	28.3 ± 4.9^{c}
PBS	0	0	0	0

Table A.57. Result of Experiment 7.21: Mean of % mortality of the KK-Dec20 leaf crude solvent fractions (n=3; T1-3) against Batch 12 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Larval species	***Mortality count of samples (%)***		
	Water-MeOH (*dead/total for the species*)	**Water** (*dead/total for the species*)	**IVM** (*dead/total for the species*)
H. contortus (33%)	9% (*3/33*)	89% (*29/33*)	9% (*3/33*)
T. circumcincta (11%)	9% (*1/11*)	91% (*9/11*)	9% (*1/11*)
Trichostrongylus spp*. (11%)*	0% (*0/11*)	91% (*9/11*)	27% (*3/11*)
Cooperia spp*. (44%)*	18% (*8/44*)	93% (*35/44*)	61% (*27/44*)
LT (1%)	100% (*1/1*)	100% (*1/1*)	-
Total Dead count (%) (dead/total)	13% (*13/100*)	82% (*82/100*)	34% (*34/100*)

Table A.58. Dead larval identification of the species of Batch 11 larvae against the KK-Dec20-Water-MeOH, KK-Oct20-Water, and IVM.

Appendix 7.2.22. Result of Experiment 7.22

Sample	***% Larval mortality***			***Mean of % mortality ± SEM***
	T1	***T2***	***T3***	
KK-Dec20-leaf-MeOH-R1-fr 9	5	4	9	6.0 ± 1.5^{d}
KK-Dec20-leaf-MeOH-R1-fr 8	10	8	5	7.7 ± 1.4^{d}
KK-Dec20-leaf-MeOH-R1-fr 7	8	6	5	6.3 ± 0.8d
KK-Dec20-leaf-MeOH-R1-fr 6	10	15	14	$13.0 \pm 1.5^{c,d}$
KK-Dec20-leaf-MeOH-R1-fr 5	29	25	23	25.7 ± 1.7^{b}
KK-Dec20-leaf-MeOH-R1-fr 4	37	45	48	43.3 ± 3.2^{a}
KK-Dec20-leaf-MeOH-R1-fr 3	16	20	21	$19.0 \pm 1.5^{b,c}$
KK-Dec20-leaf-MeOH-R1-fr 2	10	12	10	10.7 ± 0.7^{d}
KK-Dec20-leaf-MeOH-R1-fr 1	10	9	12	10.3 ± 0.8^{d}
KK-Dec20-leaf-MeOH Fraction	23	28	25	25.3 ± 1.4^{b}
IVM	21	25	27	24.3 ± 1.7^{b}
BZ	8	6	6	6.7 ± 0.7^{d}
PBS	0	0	0	0

Table A.59. Result of Experiment 7.22: Mean of % mortality of the separated fractions of KK-Dec20-MeOH Fraction (n=3; T1-3) against Batch 13 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.2.23. Result of Experiment 7.23

Sample	*% Larval mortality*			*Mean of % mortality ± SEM*
	T1	*T2*	*T3*	
KK-Dec20-leaf-Water-R1-fr 6	1	3	-	2.0 ± 1.0^{d}
KK-Dec20-leaf-Water-R1-fr 5	5	5	4	4.7 ± 0.3^{d}
KK-Dec20-leaf-Water-R1-fr 4	5	4	1	3.3 ± 1.2^{d}
KK-Dec20-leaf-Water-R1-fr 3	4	5	3	4.0 ± 0.5^{d}
KK-Dec20-leaf-Water-R1-fr 2	30	42	46	39.3 ± 4.8^{b}
KK-Dec20-leaf-Water-R1-fr 1	17	19	22	19.3 ± 1.4^{c}
KK-Dec20-leaf-Water fraction	46	49	38	44.3 ± 3.2^{a}
IVM	21	25	27	25.3 ± 1.4^{c}
BZ	8	6	6	6.7 ± 0.7^{d}
PBS	0	0	0	0

Table A.60. Result of Experiment 7.23: Mean of % mortality of the separated fractions of KK-Dec20-Water Fraction (n=3; T1-3) against Batch 13 larvae with SEM=Standard Error of Mean. Superscript (a, b, c) represents statistical significance difference from Tukey's test (P<0.05).

Appendix 7.3. Chromatograms of the LC-MS Analysis of the Various KK leaf Fractions

Appendix 7.3.1. Chromatograms of the LC-MS Analysis of the Crude Water Fractions

(+) Ionisation Mode:

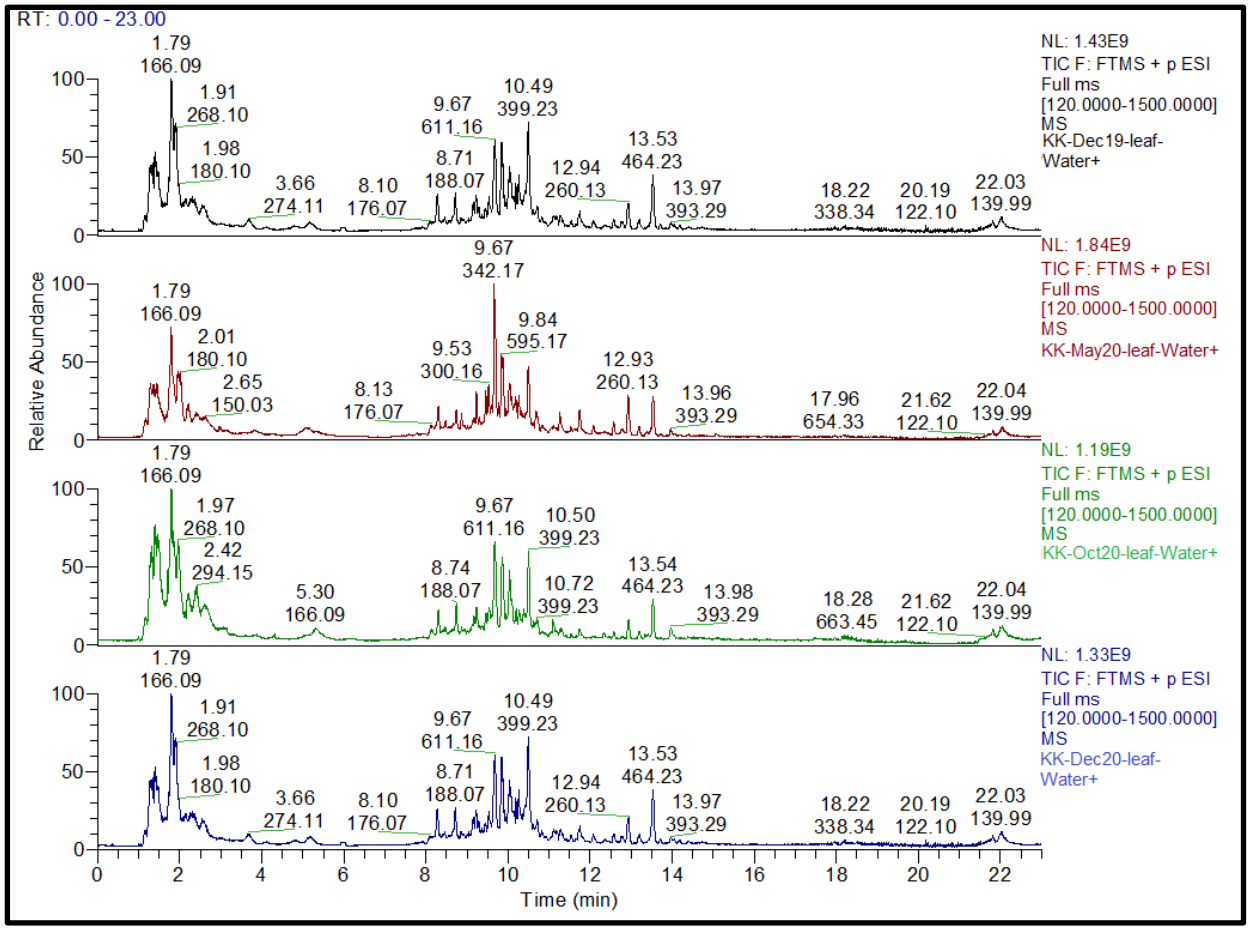


Figure A.43. LC-MS chromatograms of the Water Fractions of the KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(-) Ionisation Mode:

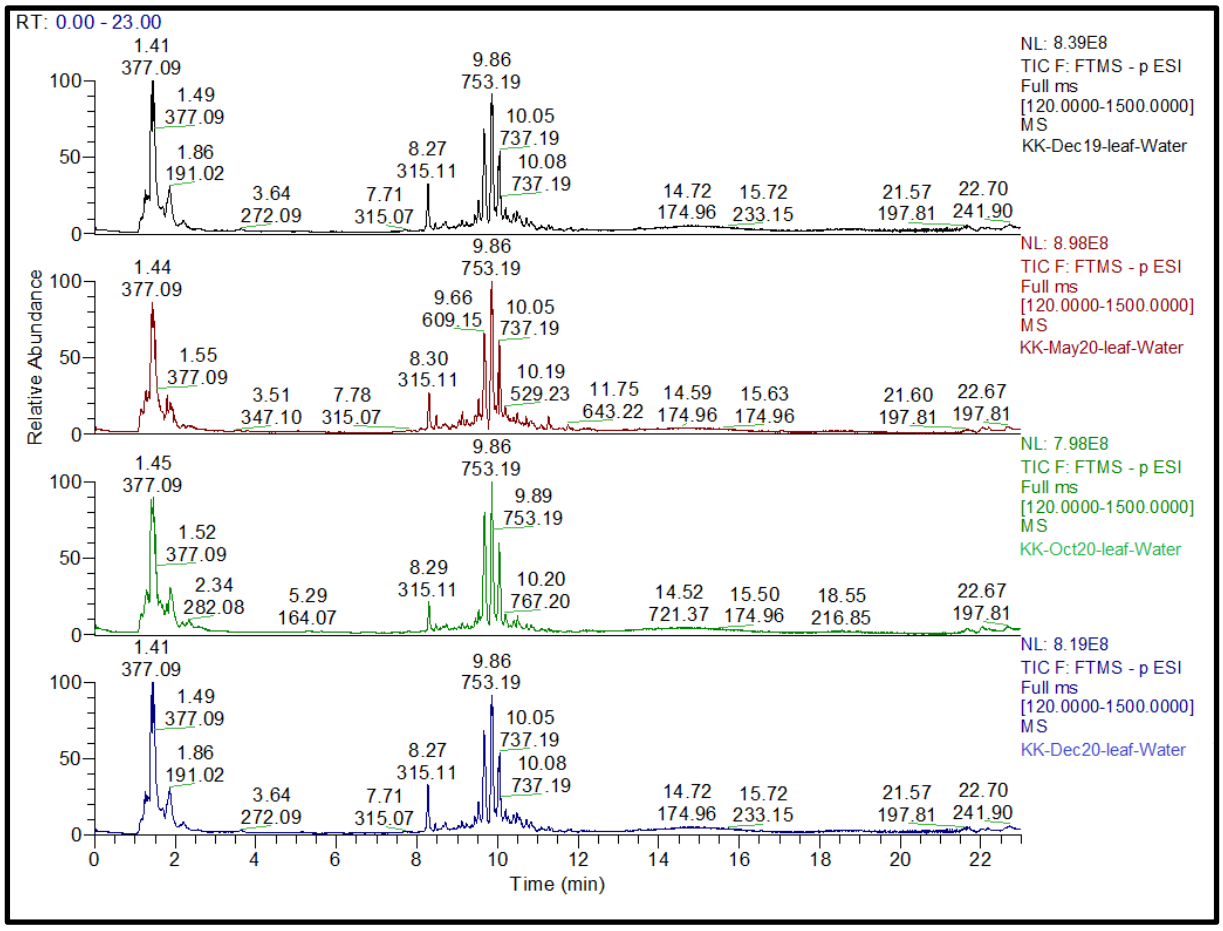


Figure A.44. LC-MS chromatograms of the Water Fractions of the KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 with (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.3.2. Chromatograms of the LC-MS Analysis of the Crude MeOH Fractions

(+) Ionisation Mode:

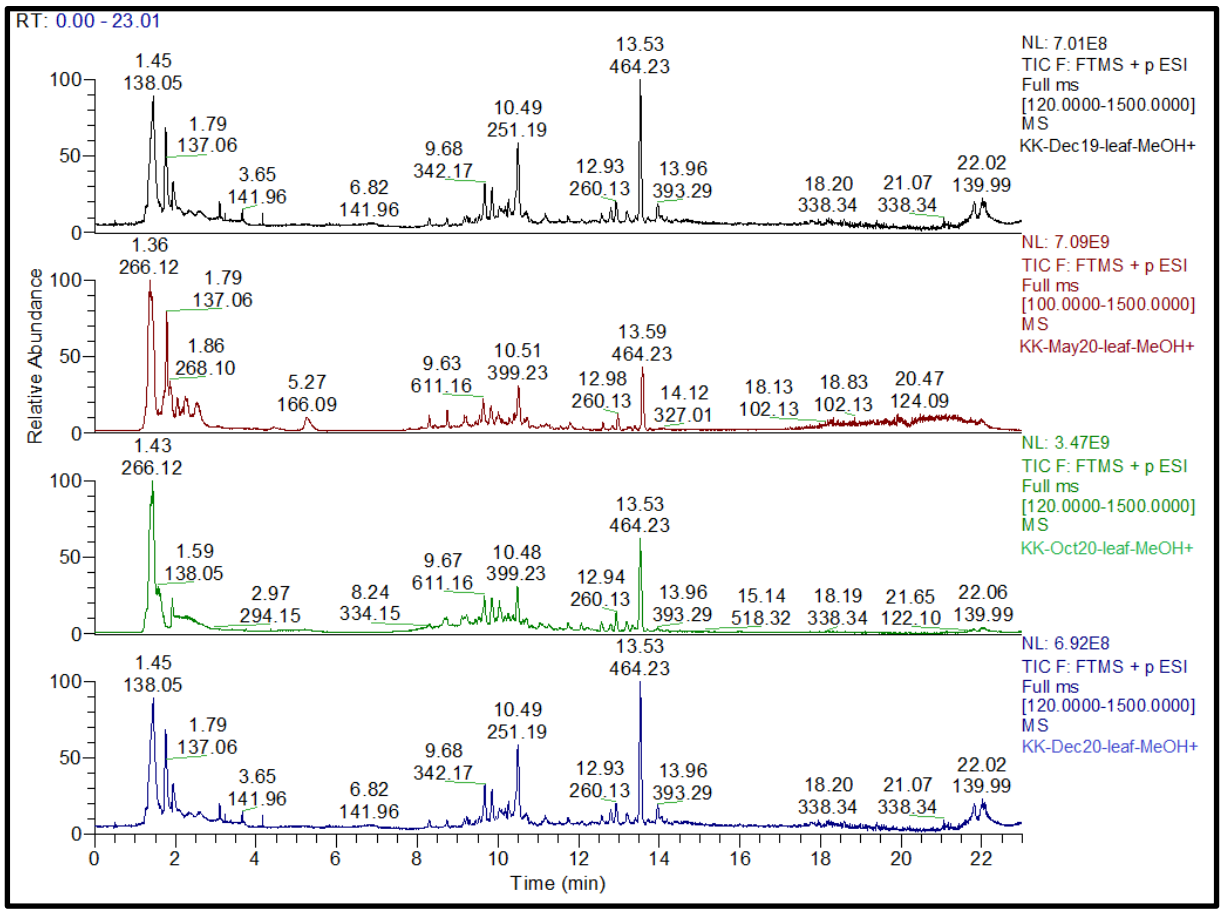


Figure A.45. LC-MS chromatograms of the MeOH Fractions of the KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(-) Ionisation Mode:

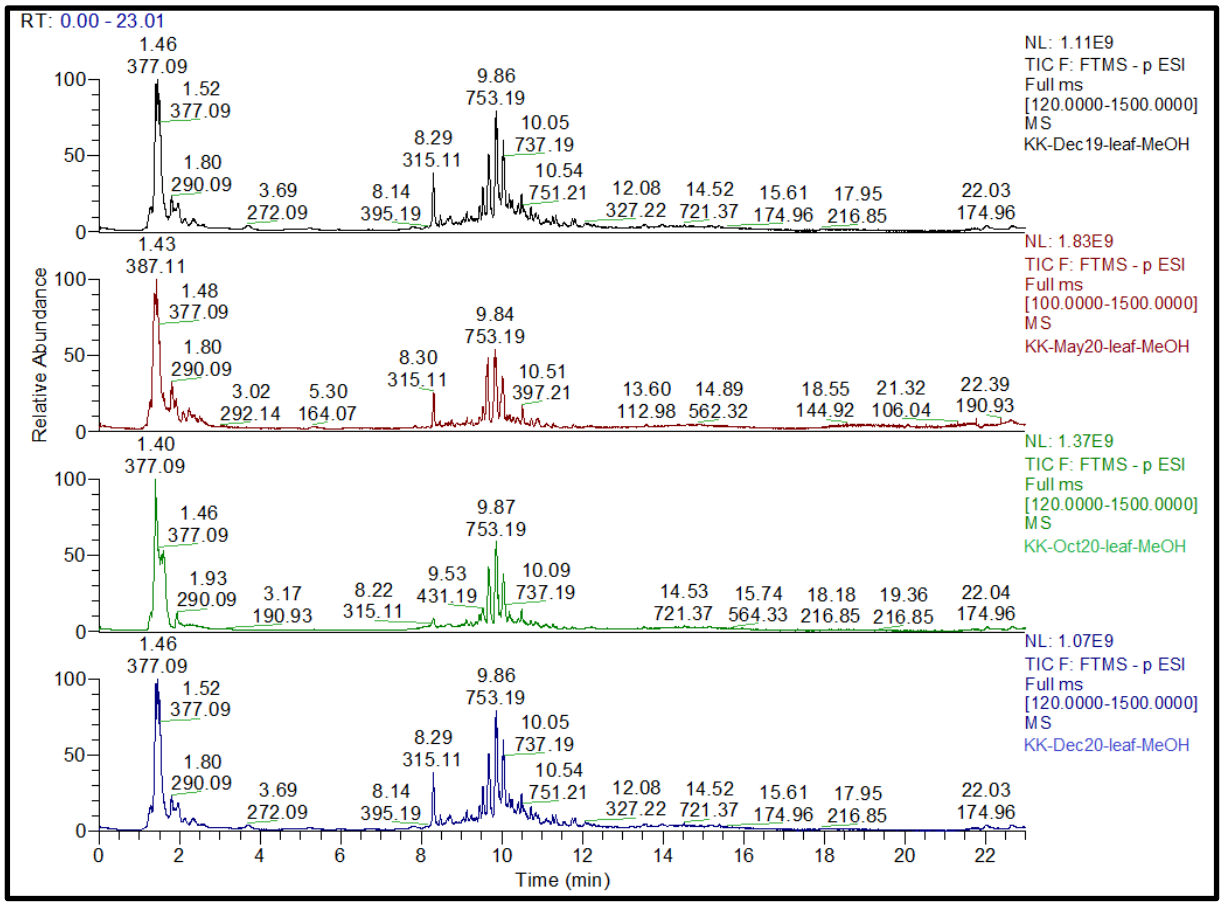


Figure A.46. LC-MS chromatograms of the MeOH Fractions of the KK-Dec19, KK-May20, KK-Oct20 and KK-Dec20 with (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.3.3. Chromatograms of the LC-MS Analysis of the Most Effective Separated MeOH Fractions

(+) Ionisation Mode:

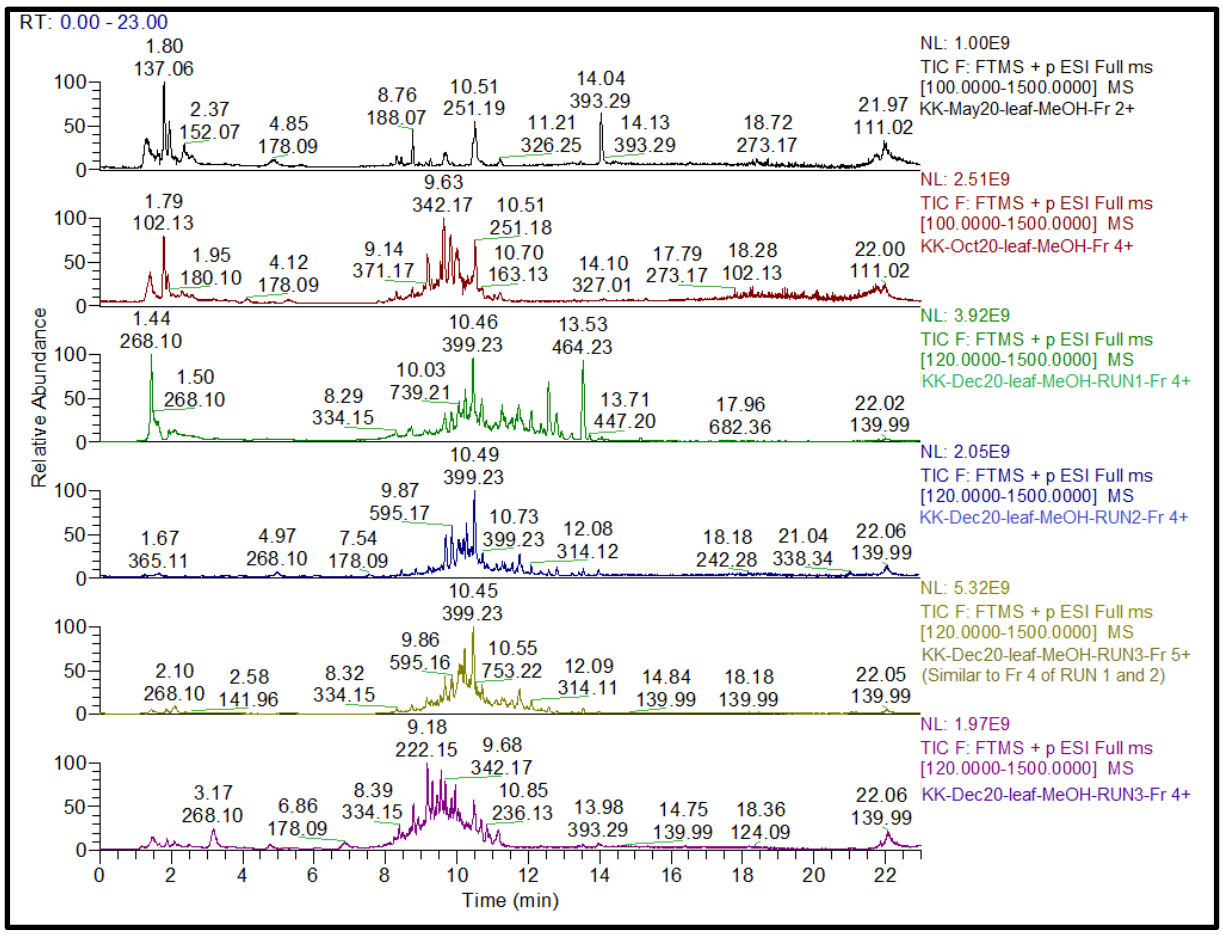


Figure A.47. LC-MS chromatograms of the most effective separated MeOH Fractions from the parent MeOH-Fraction of the KK-May20, KK-Oct20 and KK-Dec20 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(-) Ionisation Mode:

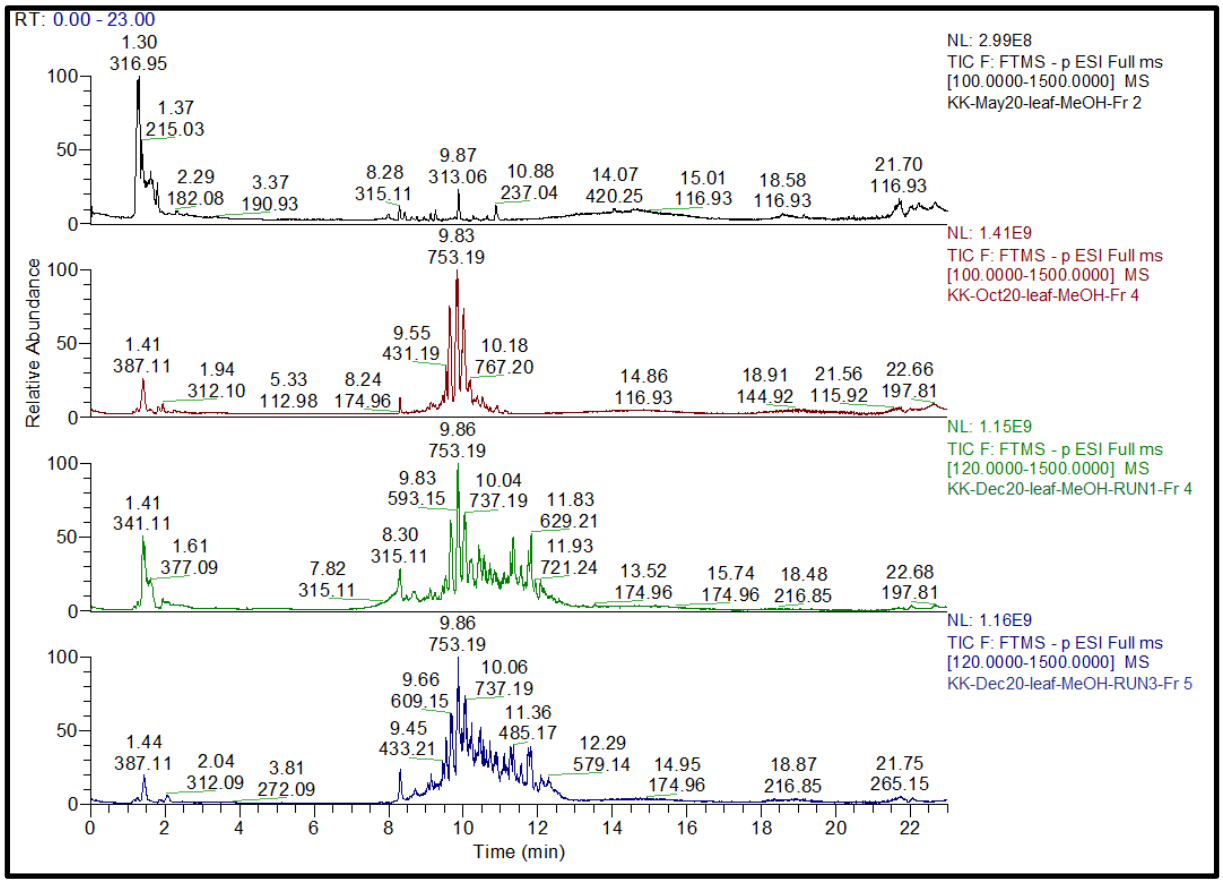


Figure A.48. LC-MS chromatograms of the most effective separated MeOH Fractions from the parent MeOH-Fraction of the KK-May20, KK-Oct20 and KK-Dec20 with (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.3.4. Chromatograms of the LC-MS Analysis of the Most Effective Separated Water Fractions

(+) Ionisation Mode:

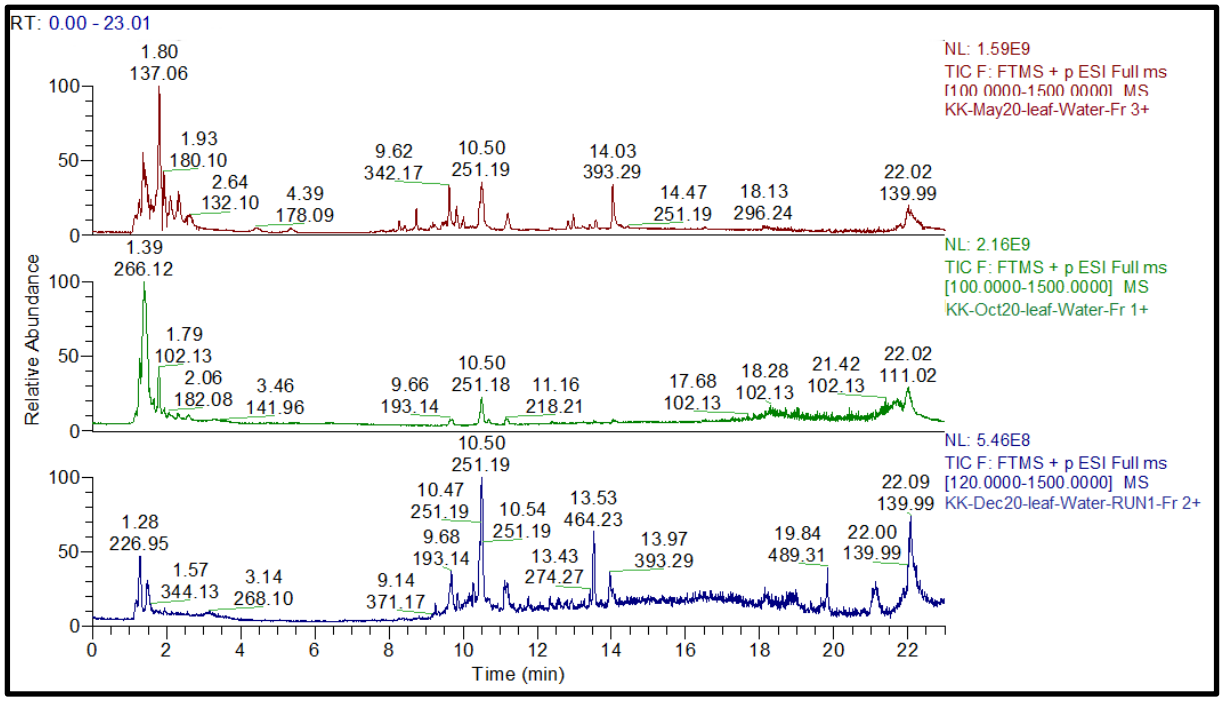


Figure A.49. LC-MS chromatograms of the most effective separated Water Fractions from the parent Water-Fraction of the KK-May20, KK-Oct20 and KK-Dec20 with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(-) Ionisation Mode:

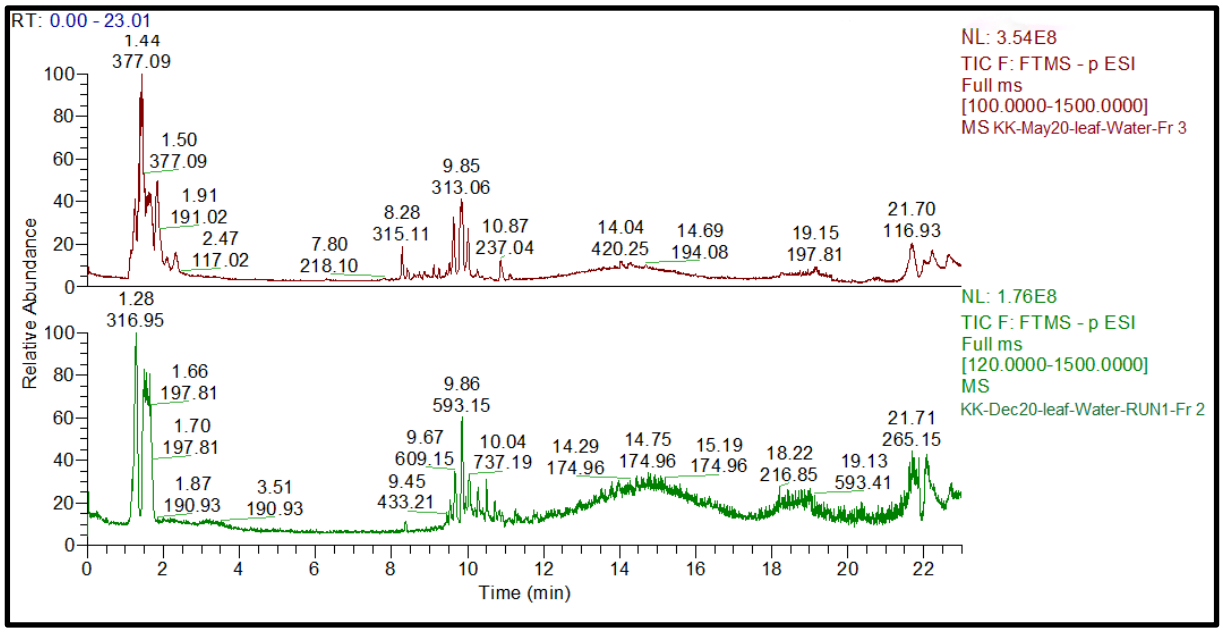


Figure A.50. LC-MS chromatograms of the most effective separated Water Fractions from the parent Water-Fraction of the KK-May20, and KK-Dec20 with (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.3.5. Chromatograms of the LC-MS Analysis of the Most Effective Separated Water Fractions Using HILIC Column

(+) Ionisation Mode:

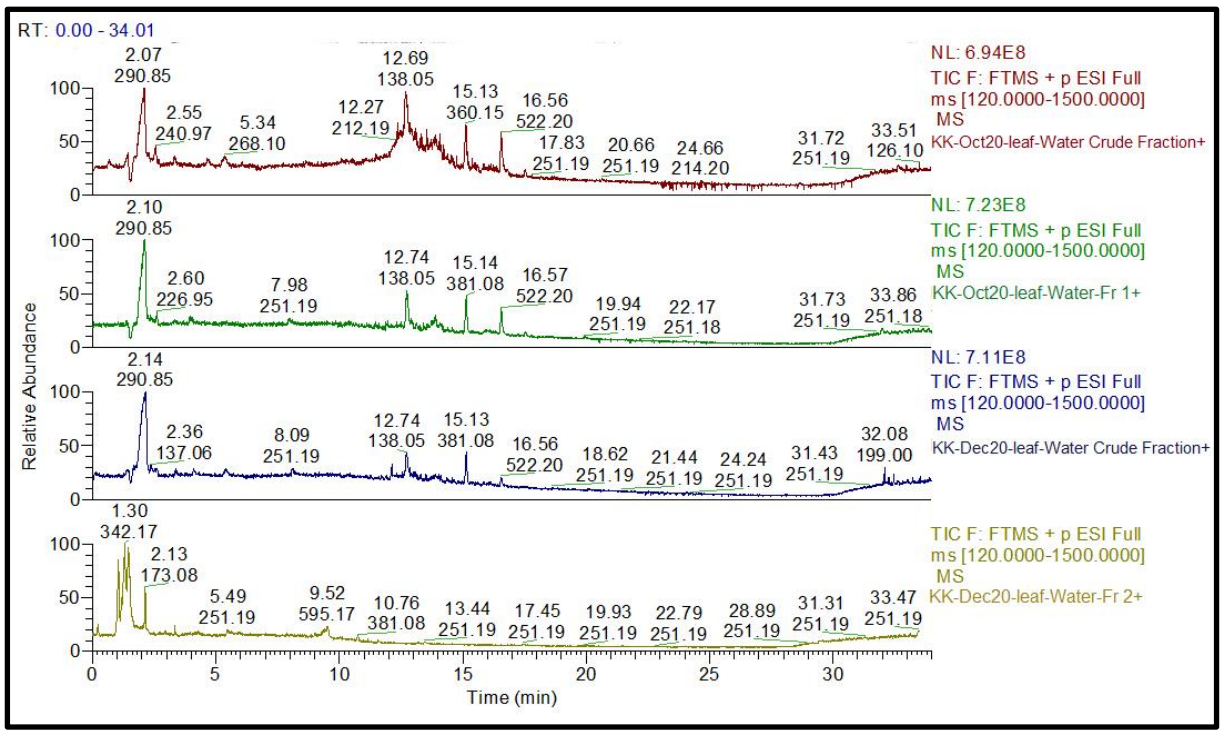


Figure A.51. LC-MS chromatograms of the most effective separated Water Fractions from the parent Water-Fraction of the KK-May20, and KK-Dec20 using HILIC column with (+) ionisation mode. Labels above peak represent the RT and m/z of base peak.

(-) Ionisation Mode:

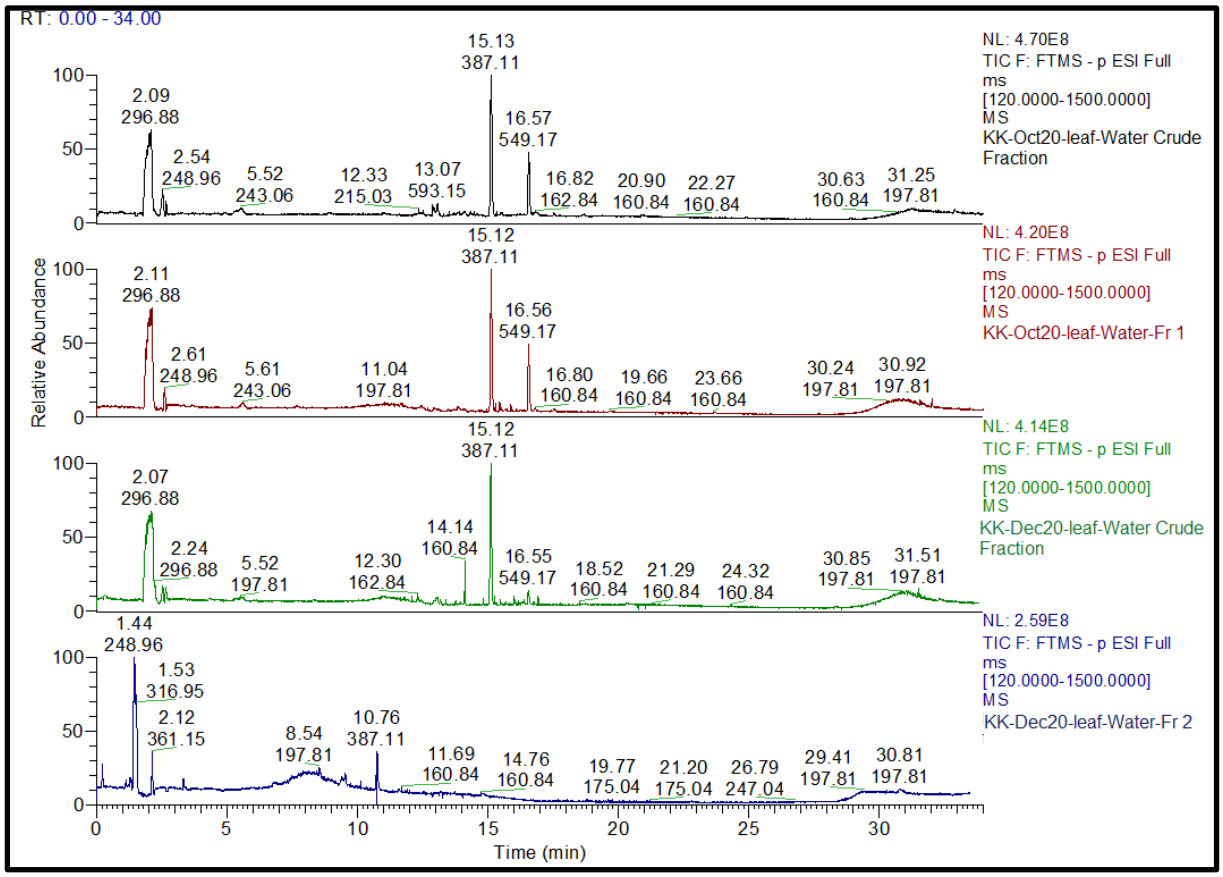


Figure A.52. LC-MS chromatograms of the most effective separated Water Fractions from the parent Water-Fraction of the KK-May20, and KK-Dec20 using HILIC column with (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 7.3.6. Chromatograms of the LC-MS Analysis of a Few Separated Fractions of the KK-Oct20-leaf-MeOH-Fraction Using HILIC Column

(+) Ionisation Mode:

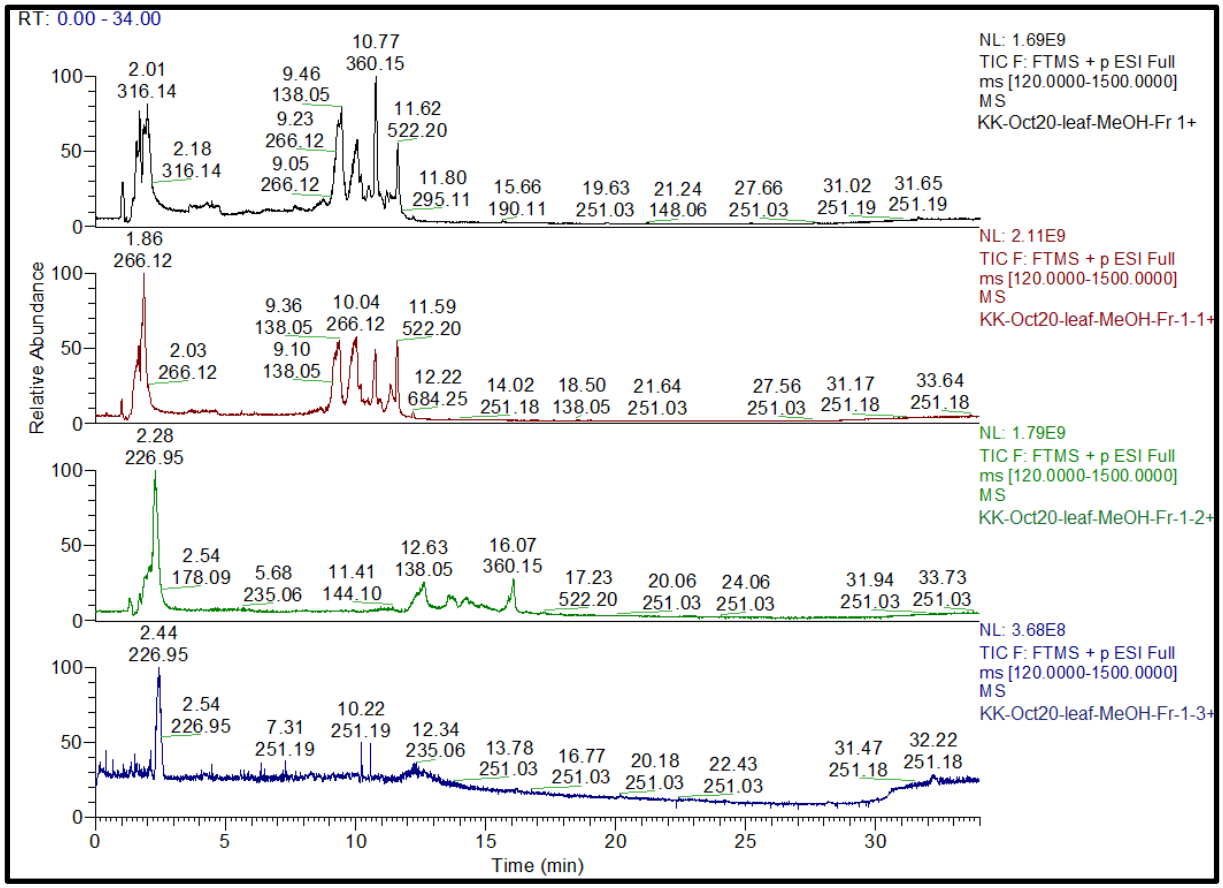


Figure A.53. LC-MS chromatograms of a few separated fractions of the KK-Oct20-leaf-MeOH-Fraction using HILIC column with (-) ionisation mode. Labels above peak represent the RT and m/z of base peak.

Appendix 8. Chapter 8 Appendix

Appendix 8.1. LC-MS Chromatograms of the Fractions Present in the Layers of the HCCCS Solvent Systems

Appendix 8.1.1. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-1 Solvent System

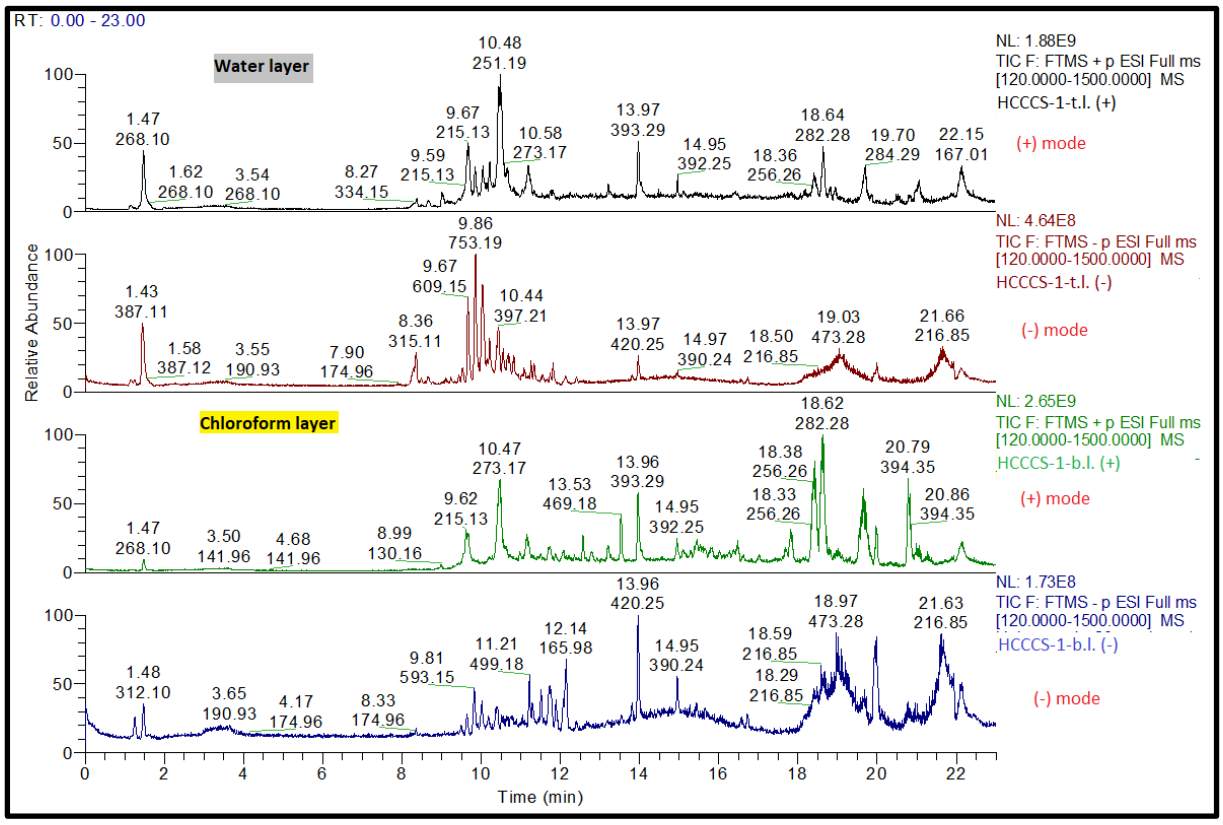


Figure A.54. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-1 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.2. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-2 Solvent System

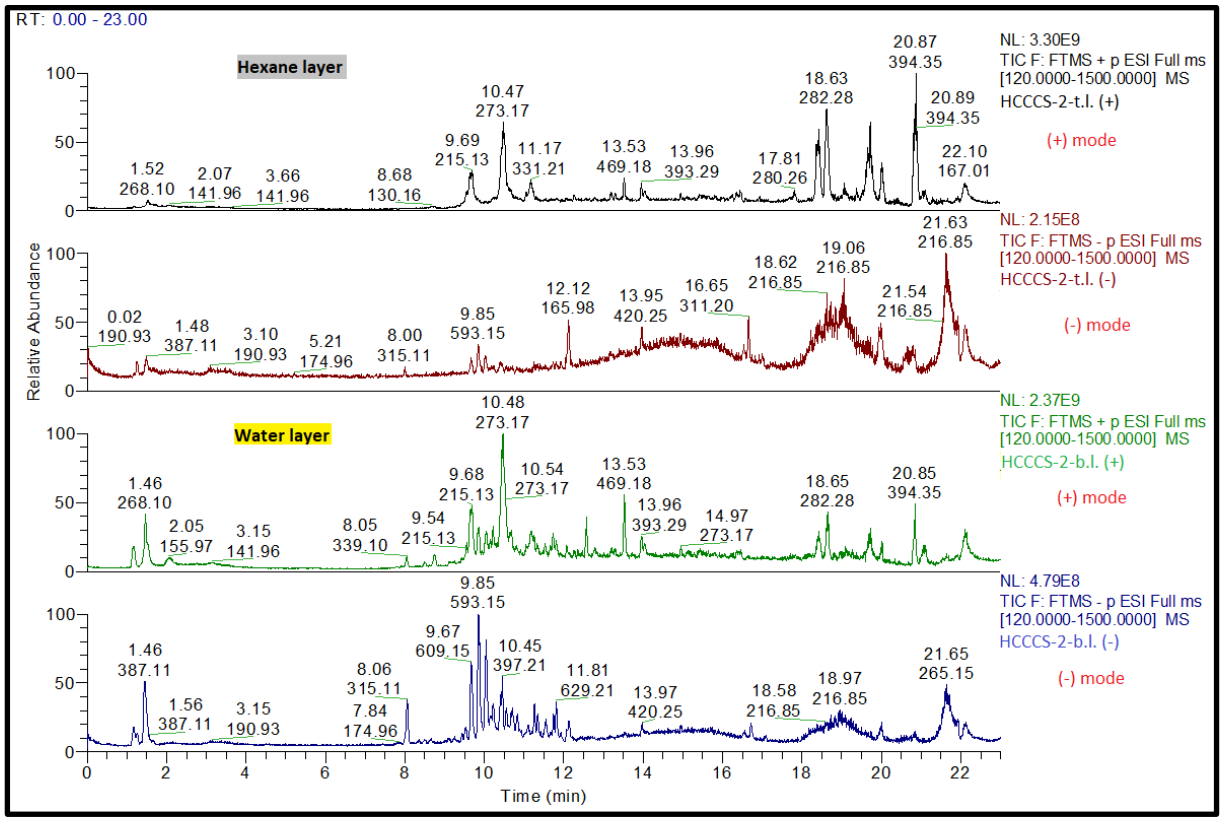


Figure A.55. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-2 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.3. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-3 Solvent System

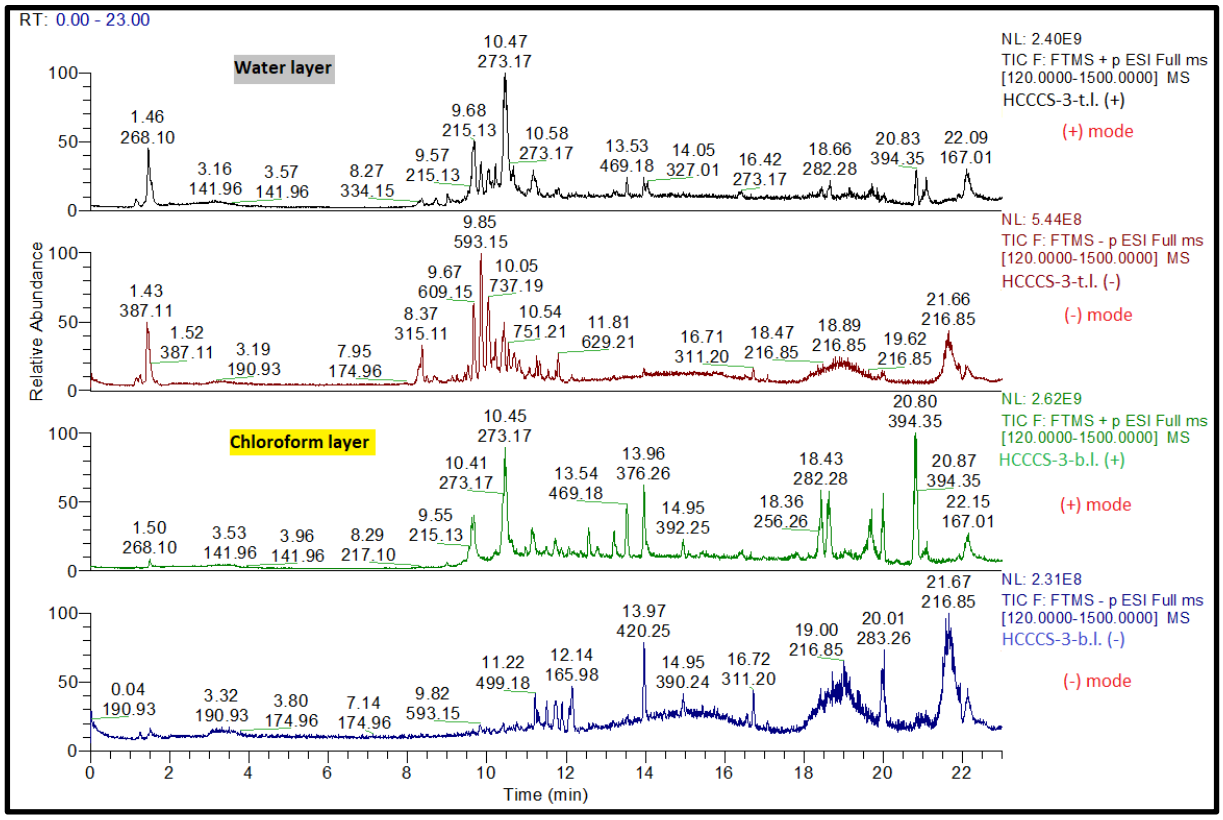


Figure A.56. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-3 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.4. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-4 Solvent System

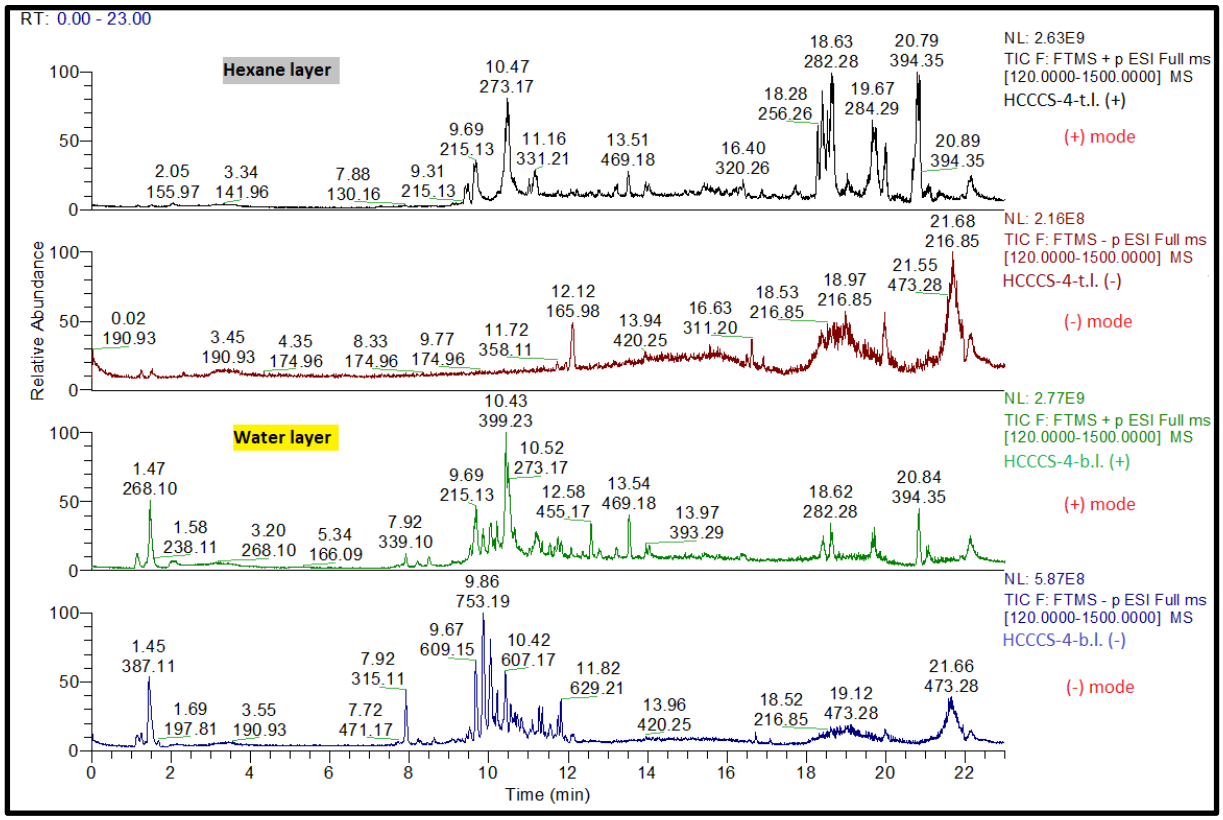


Figure A.57. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-4 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.5. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-5 Solvent System

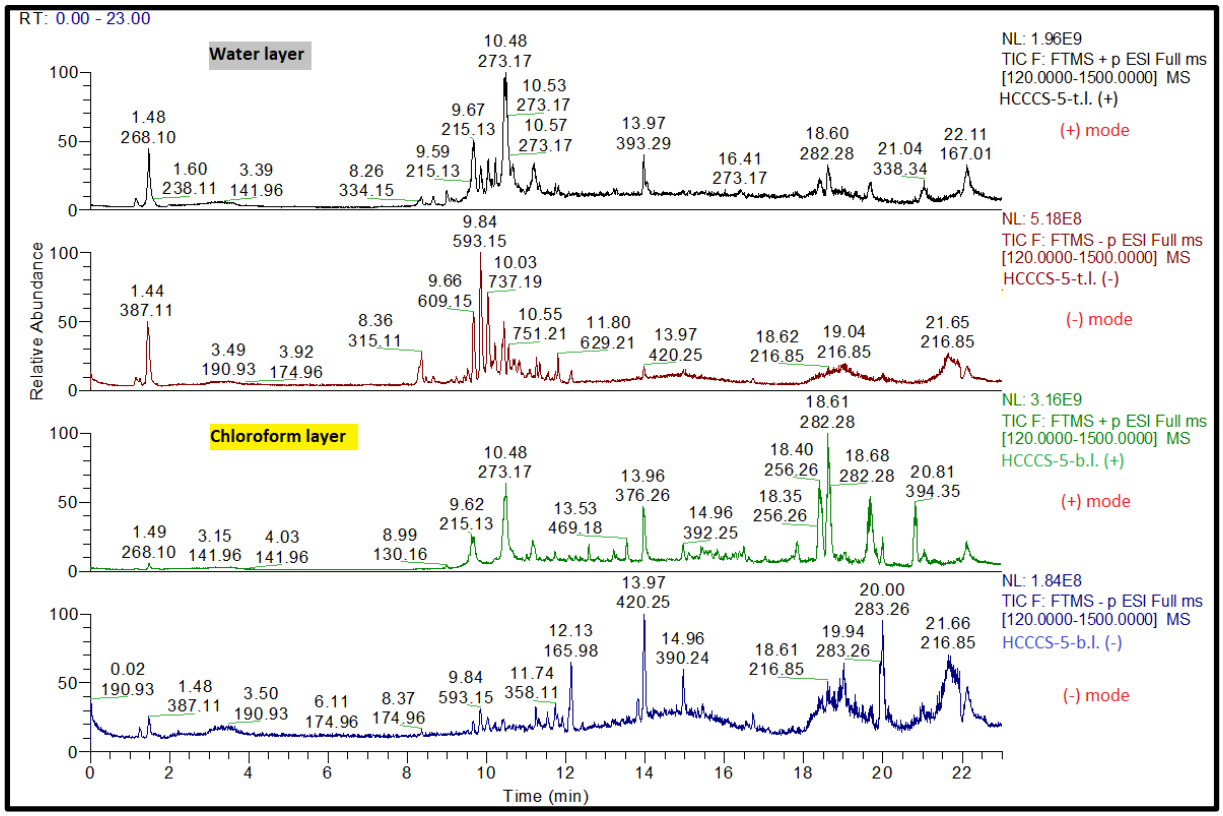


Figure A.58. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-5 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.6. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-6 Solvent System

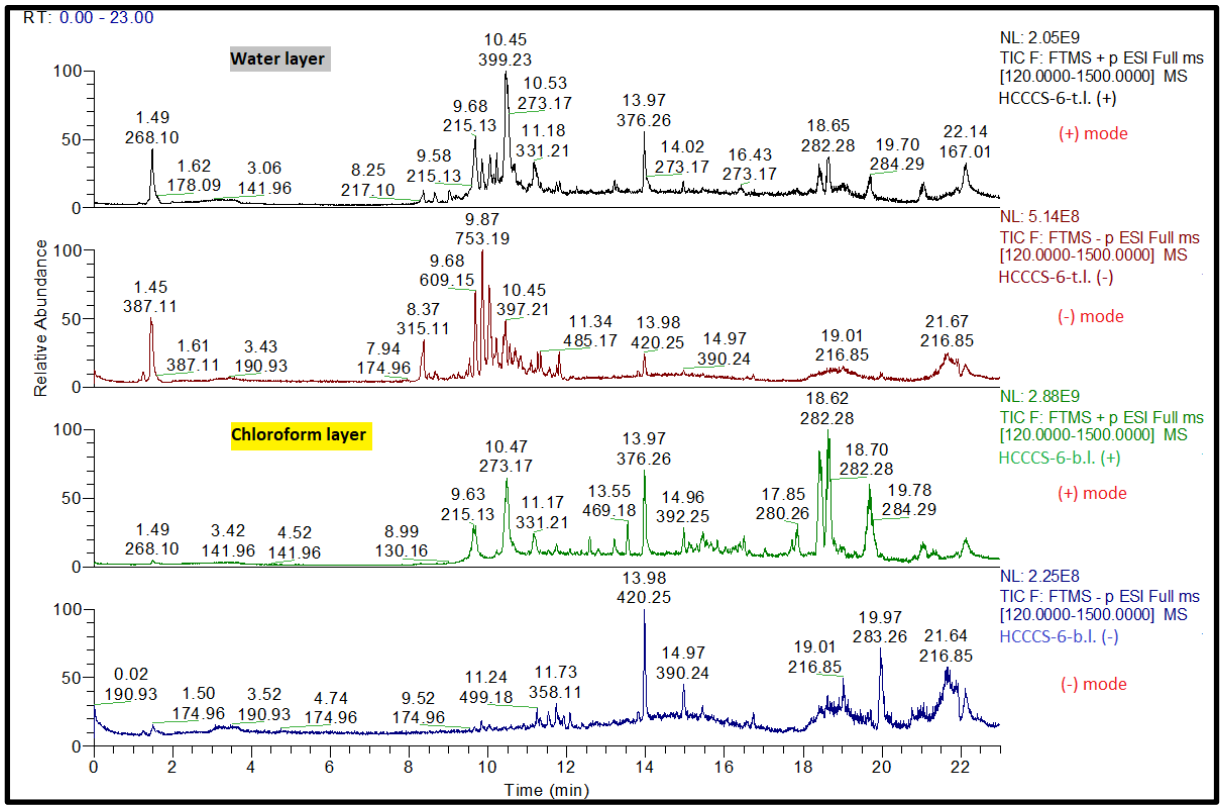


Figure A.59. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-6 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.7. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-7 Solvent System

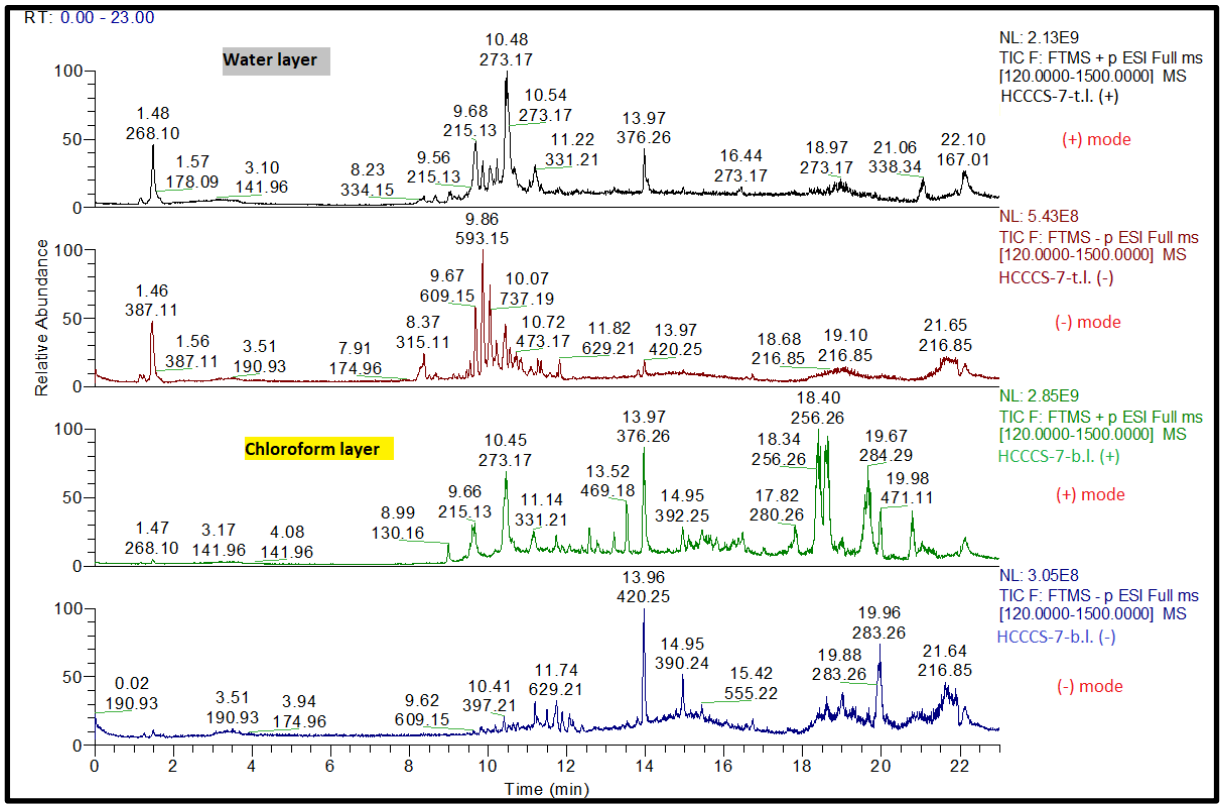


Figure A.60. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-7 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.8. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-8 Solvent System

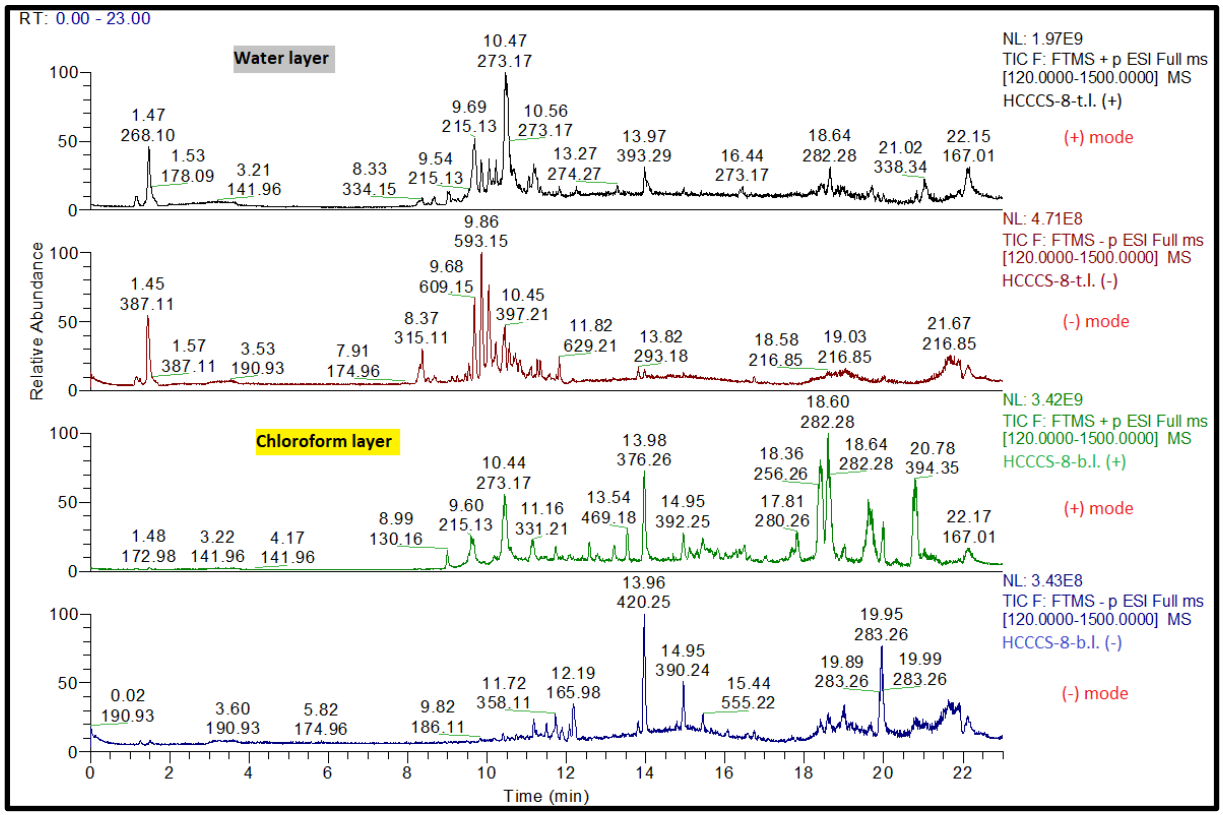


Figure A.61. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-8 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.9. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-9 Solvent System

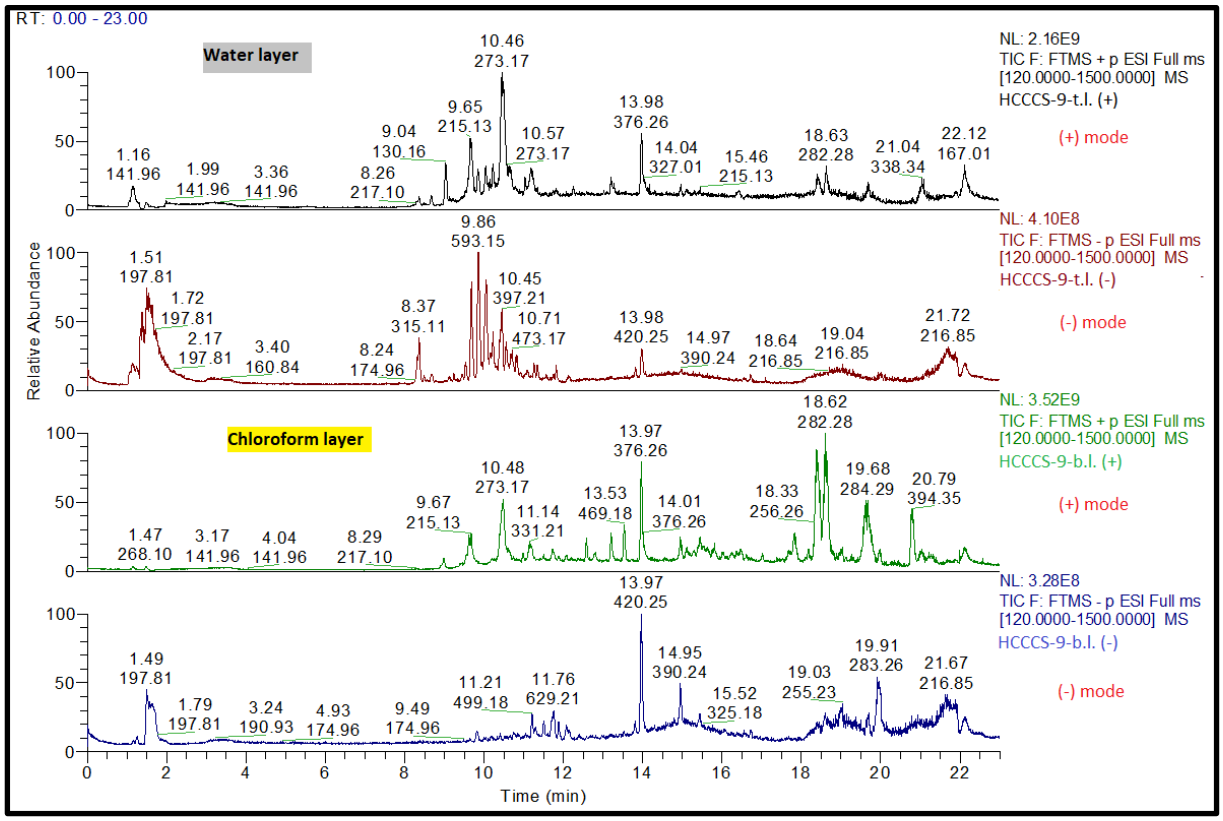


Figure A.62. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-9 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.10. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-10 Solvent System

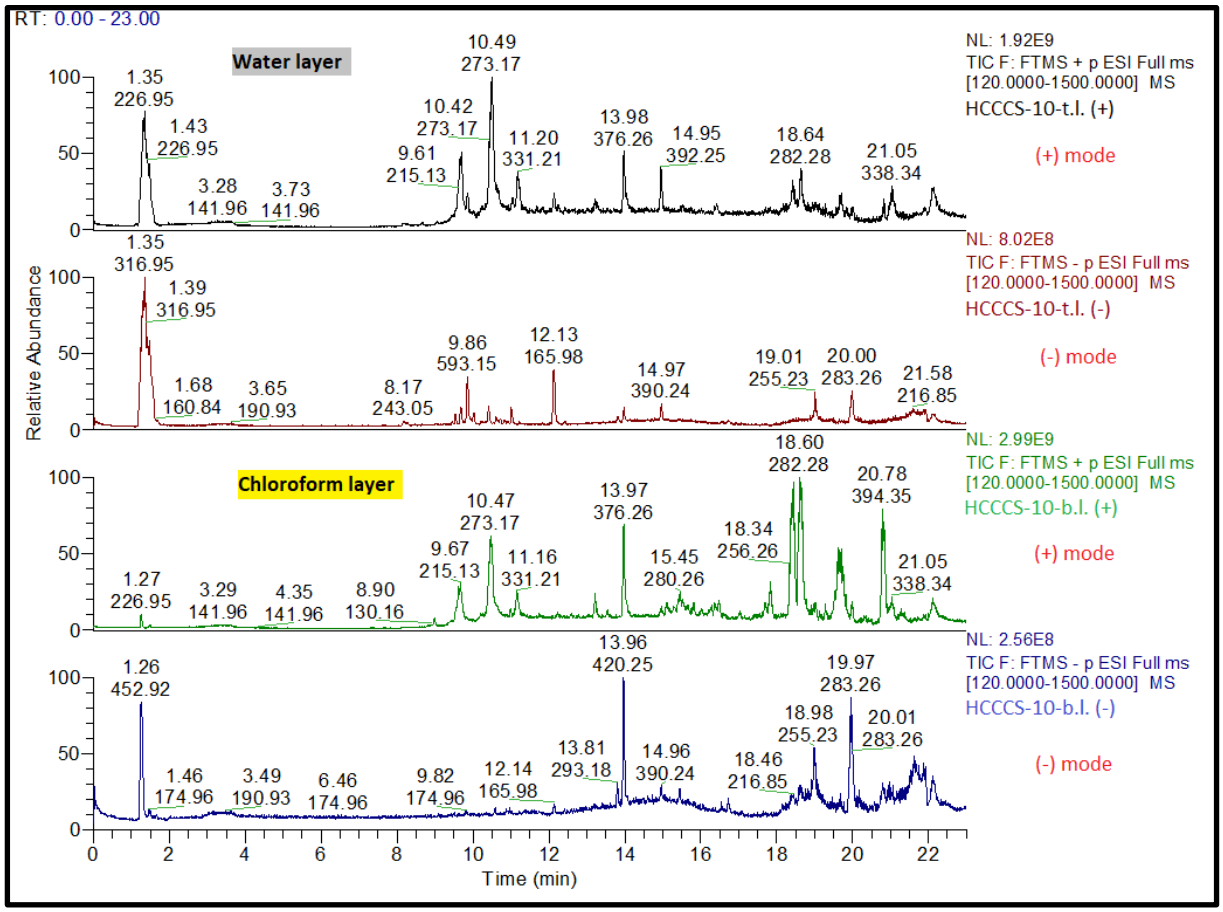


Figure A.63. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-10 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.11. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-11 Solvent System

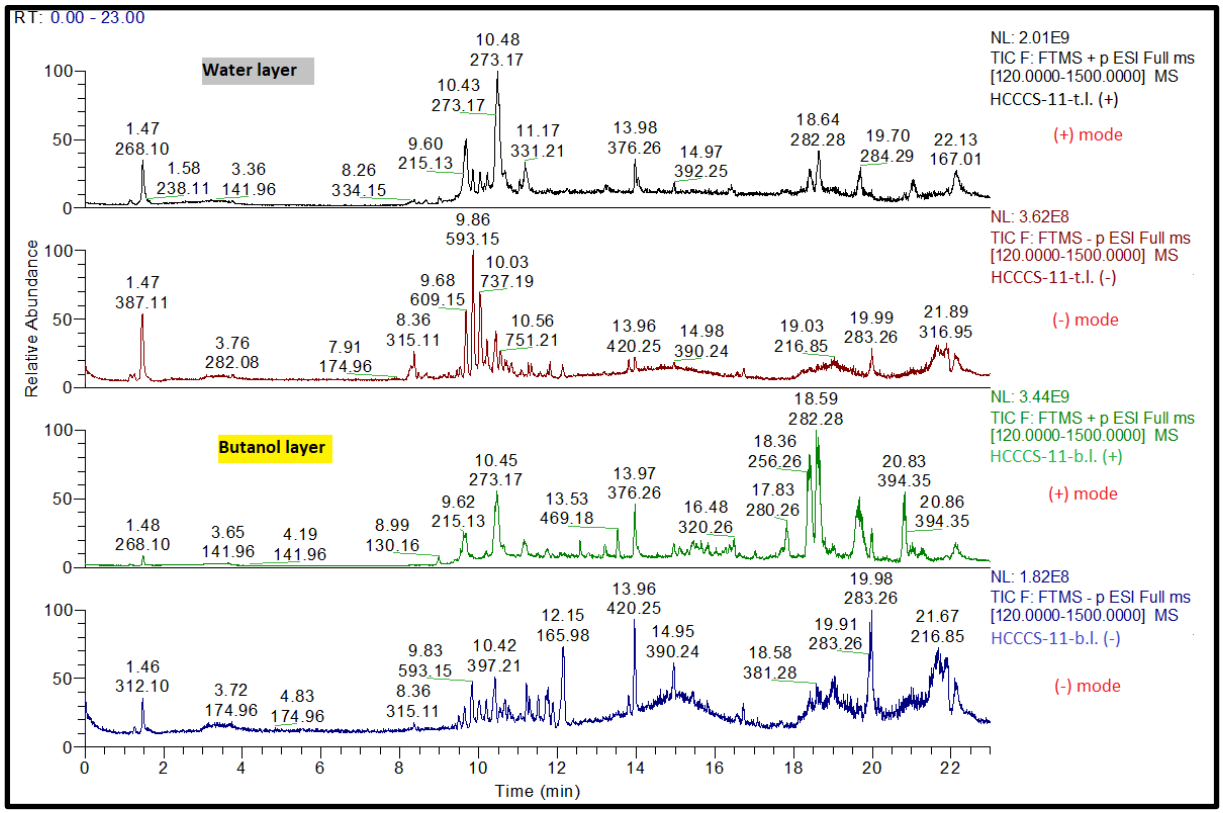


Figure A.64. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-11 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.12. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-12 Solvent System

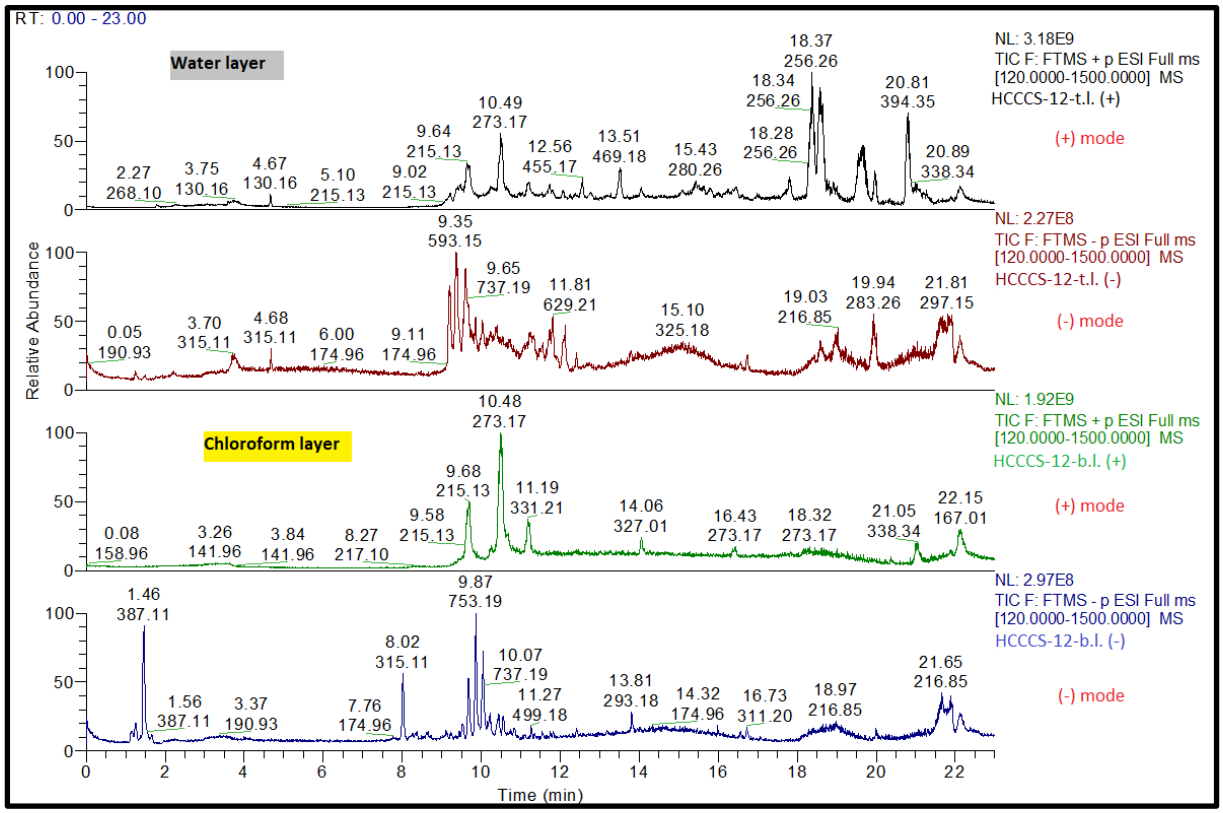


Figure A.65. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-12 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.13. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-13 Solvent System

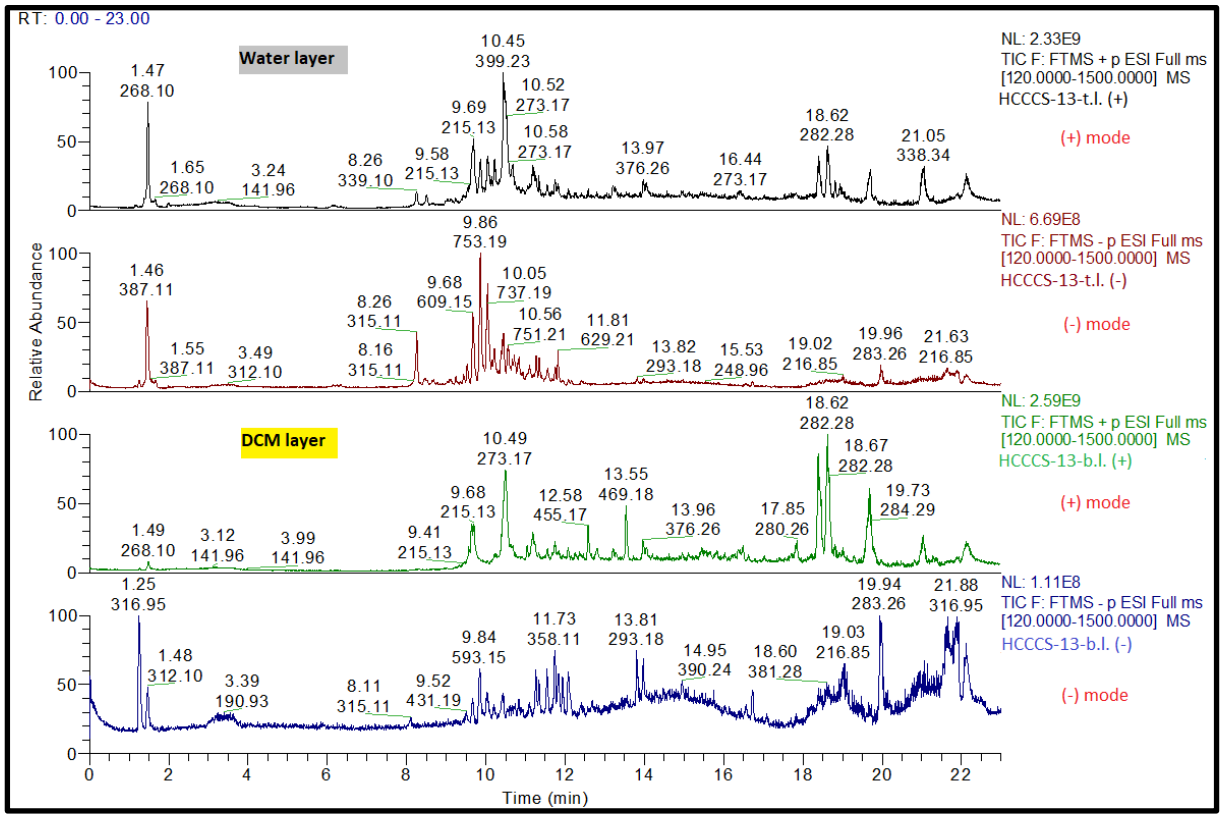


Figure A.66. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-13 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.14. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-14 Solvent System

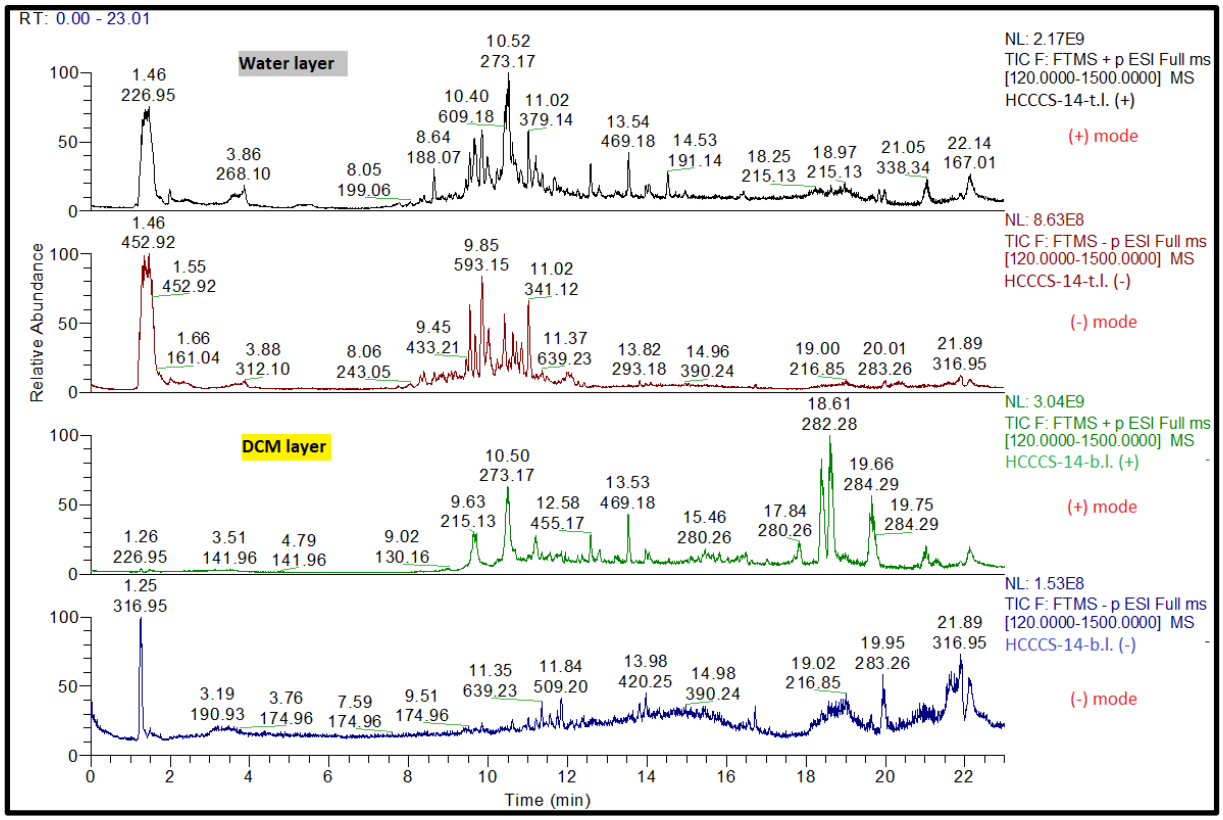


Figure A.67. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-14 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 8.1.15. LC-MS Chromatograms of the Fractions present in the Layers of the HCCCS-14 Solvent System

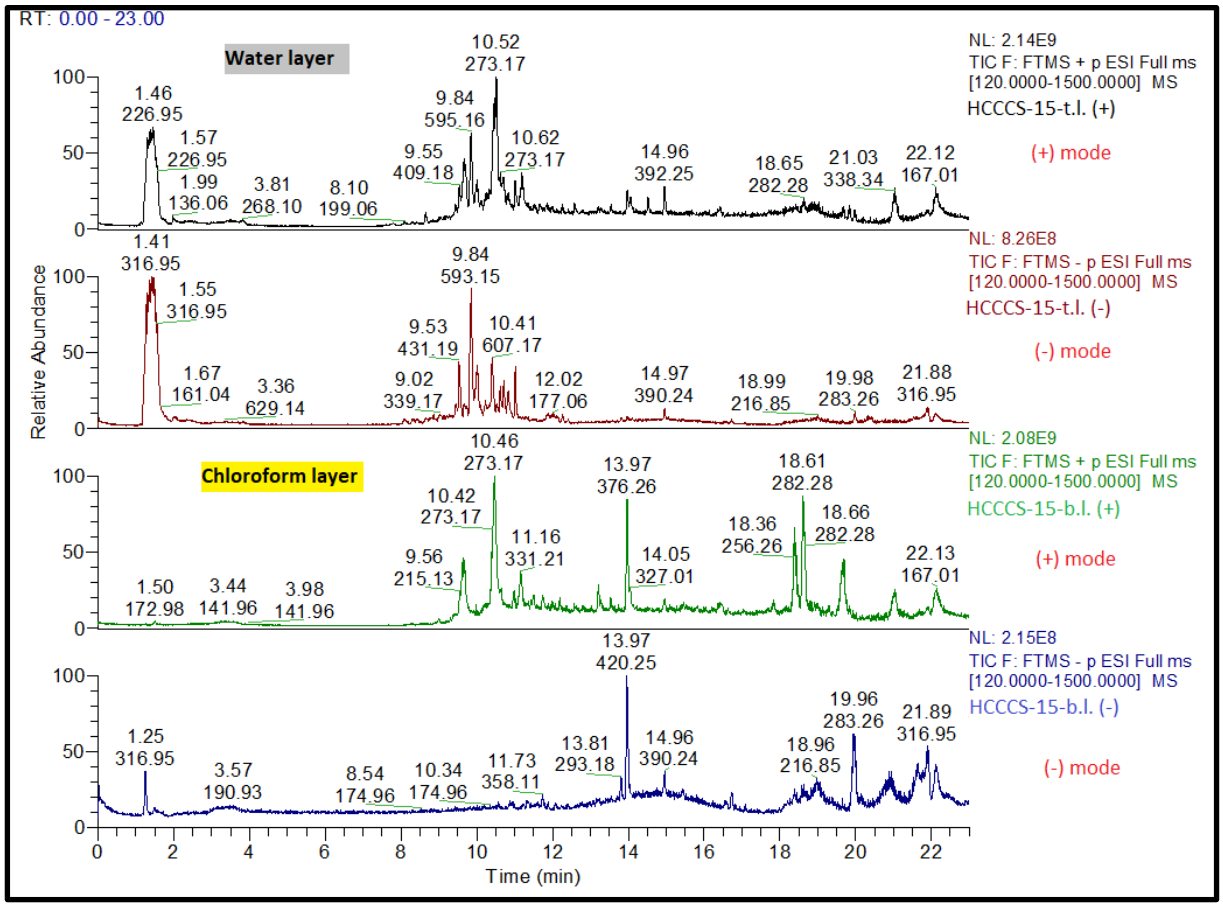


Figure A.68. LC-MS chromatograms of the fractions present in the top and bottom layer of the HCCCS-15 solvent system with (+) and (-) ionisation modes. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.

Appendix 10. Chapter 10 Appendix

Appendix 10.1. LC-MS Analysis of the Blank Sample

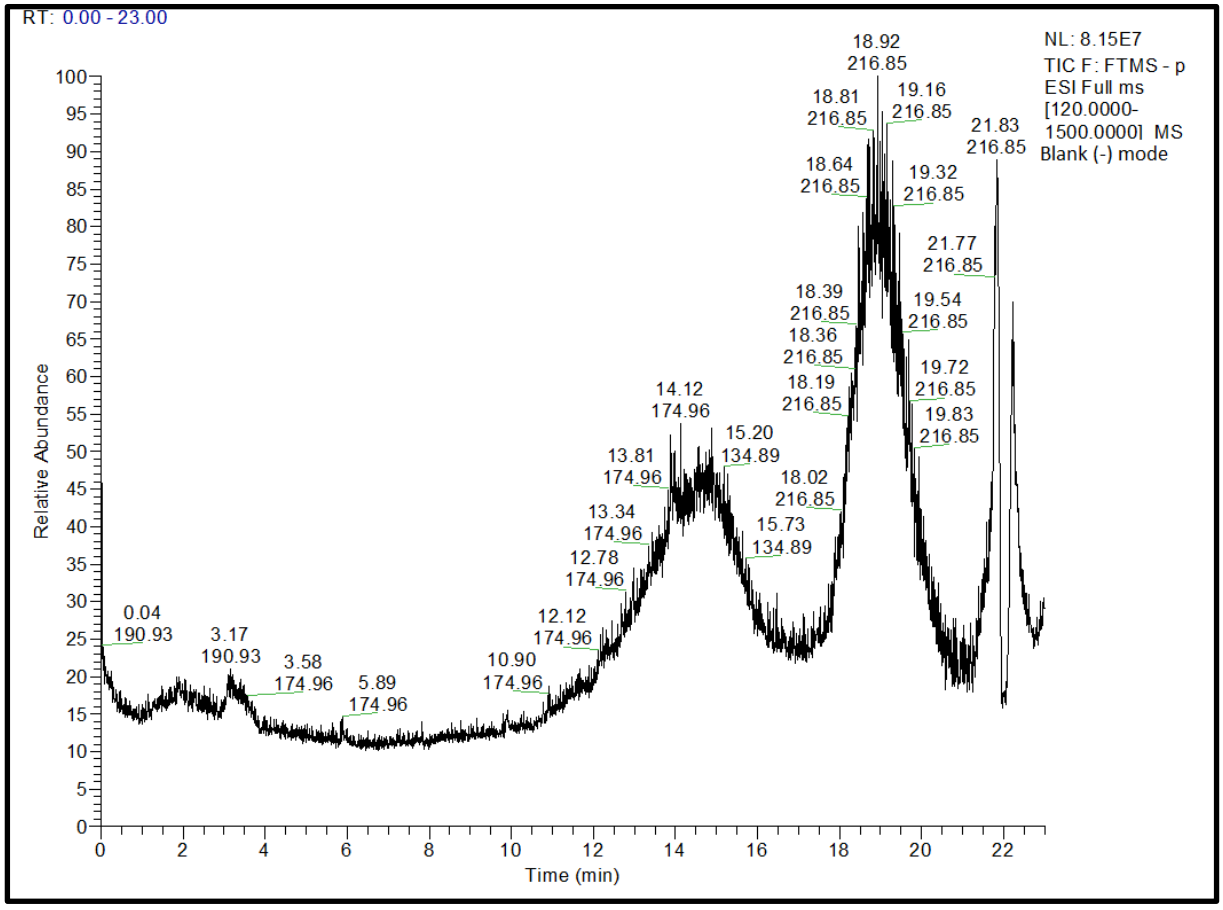


Figure A.69. LC-MS chromatograms of the blank sample with (+) and (-) ionisation mode. Labels above peak represent the RT and m/z of base peak; t.l.= top layer, b.l. = bottom layer.