CHARACTERIZATION OF FLAVONOIDS FROM CANDIDATE *STRIGA* WEED CONTROLLING FOOD LEGUMES; *Cicer arietinum* L. AND *Vigna radiata* L.

By
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DECLARATION

I hereby declare that the research work carried out and presented in this thesis is original. It has not been submitted partly or fully to any other University or Institution for the award of any diploma or degree.

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This thesis has been submitted for examination with our approval as Kenyatta University (KU) and International Center of Insect Physiology and Ecology (ICIPE)

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DEDICATION

This thesis is dedicated in memory of the late Principal Allan Arondo Okeyo and his daughter Mercy.

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ABBREVIATIONS AND ACRONYMS

2-GV	2-glucosylvitexin
2, 4-D, MCPA	2-methyl-4-chlorophenoxyacetic acid
6-GV	6-glucosylvitexin
λ_{max}	Maximum wavelength
ACE	Normal pressure HPLC column
BCED	Behavioural and Chemical Ecology Department
C_{18}	Octadecyl carbon chain
CH_2Cl_2	Dichloromethane
DRIP	Dissertation research internship programme
EtOAc	Ethyl acetate
GR24	Germination stimulant (2-methyl-4-((2-oxo-2,3,3a,8b-tetrahydro-4H-indeno[1,2-b]furan-3-ylidene)methoxy)but-2-en-4-olide)
HCO_2H	Formic acid
Hex	Hexane
HPLC	High Performance Liquid Chromatography
ICIPE	International Center of Insect Physiology and Ecology
IR	Infra red
ISM	Integrated *Striga* management
L-DOPA	L-3, 4-dihydroxyphenylalanine

LC-MS	Liquid Chromatography-Mass spectrometry
LOD	Limit of detection
MeOH	Methanol
MSD	Mass Spectrometer Detector
MT	Metric Tonnes
m/z	mass-to-charge ratio
NMR	Nuclear Magnetic Resonance
PDA	Photodiode array
pH	Hydrogen ion concentration
RP	Reverse phase
SA	South Asia
SSA	Sub-Saharan Africa
TIC	Total Ion Current
TLC	Thin Layer Chromatography
UV	Ultra violet

ABSTRACT

In Africa, intercropping of some legumes with cereal crops has been found to cause remarkable reduction of *Striga* infestation and improved production of cereal crop. *Desmodium uncinatum* controls *Striga* infestation in intercrop with cereals through an allelopathic mechanism, which involves post-germination growth inhibitors exuded from the roots. Allelopathy is a natural and environmentally friendly technique, which has a potential to be a new approach for weed control. A standardized profiling method based on Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Liquid Chromatography coupled with Mass Spectrometry (LC/MS) was used to identify flavonoids in extracts of chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*), potential trap crops for *Striga* weed. Nine flavonoids including mono- and diglycosyl derivatives of fisetin, baicalein, rahamnetin, isorhamnetin, formonometin, quercetin, isosakuranetin, and sakuranetin were tentatively identified. The detected phenolics were present at concentrations greater than 0.001% of the dry materials. Many of these phenolic compounds have been reported to have human health benefits. These glycosylated flavones are reported for the first time in these two species. The comprehensive analysis of the polar secondary metabolites in these leguminous plants was helpful for understanding their inhibitory chemistry and proposed biosynthesis by C-glucoside traits. All the isolated and characterized compounds in the food legume *C. arietinum* were O-glucosylated. While, Food legume *V. radiata* should be examined further to see if it can attain *Desmodium's* allelopathic ability since it possess inhibitory chemistry and proposed biosynthesis by C-glucosylation.

CHAPTER ONE

INTRODUCTION

1.1 Background

Striga is one of the most important pests that affect food production in the tropics (Esilaba, 2006). The weed problem is compounded by its reproductive capacity; a single plant can produce over 50,000 seeds, which can remain viable in the soil for 15-20 years (Kureh *et al.,* 2003). In Kenya, the parasite is a serious pest that mainly threatens maize production, resulting in yield losses of between 65 and 100%. Decline in soil fertility is one of the main causes for the increase in *Striga* incidence (Esilaba, 2006). *Striga hermonthica* (Del.) Benth is most common on heavy soil particularly in the densely populated parts of Lake Victoria region of western Kenya while, *S. asiatica* is found in the coast province and seriously damage upland rice (Esilaba, 2006).

Research efforts have identified several control options that are effective in reducing *Striga* damage and emergence. These include host plant resistance, use of trap-crops and the improvement and maintenance of soil fertility through cereal legume rotation/intercropping or application of organic or inorganic nitrogen (Kureh *et al.,* 2003). However, due to ecological concerns, environmental health problems and increase in the number of herbicide-resistant weeds resulting from the use of synthetic herbicides, considerable efforts in designing weed

management strategies of using allelopathic compounds as bioherbicides to suppress weeds is gaining consideration (Zaji, 2011).

Trap crops are non-host plants which stimulate the germination of *Striga* seeds, but are not parasitized by the weed (Kureh *et al.,* 2003). They could cause suicidal germination of the weed, which reduces the seed bank in the soil or when intercropped in maize reduce attachment to the host (Othira *et al.,* 2008). Some legume varieties, for example chickpea, mung beans, cowpea (*Vigna unguiculata* L.), groundnut (*Arachis hypogaea* L.) and soybeans **(***Glycine max*) have potential to cause suicidal germination of *S. hermonthica*, reduce attachment to host and improve fertility of soil (Kureh *et al.,* 2003) by enriching the soil with nitrogen and organic matter.

The life cycle of *Striga* ssp. is composed of five stages; germination, haustorium initiation, penetration of host tissue, physiological compatibility and parasite growth and maturation. Apart from normal seed germination requirements, the weed requires a chemical stimulation for germination to occur and a second chemical signal to initiate haustorium formation, which connects *Striga* roots to its host for resource acquisition (Othira *et al.,* 2008). Although trap crops release chemicals that stimulate *Striga* ssp. seed germination they do not produce haustorium signals, neither are they attacked by the parasite (Othira *et al.,* 2008).

Even though the witch-weed, *Striga* ssp. is capable of photosynthesis once it emerges, it relies on host plant for a significant portion of its carbon supply (Rich

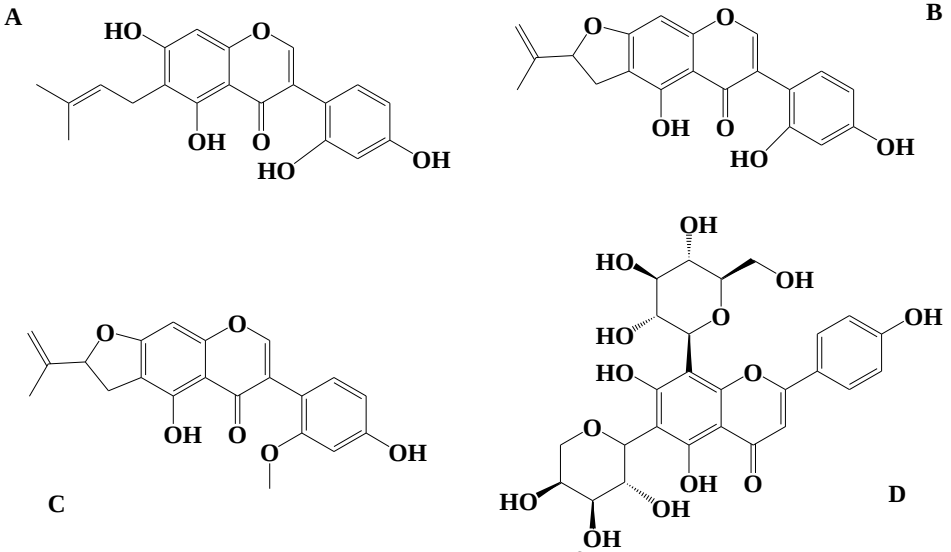
et al., 2008). Beyond the burden of losing food and water to these parasites, host plants suffer from a characteristic malady resembling the symptoms of severe drought, including leaf scorching and increased root: soot ratios as if a hex had befallen the crop the result of hormonal perturbation or toxic produced by the parasite (Rich, 2008). Infestation is generally much less severe where water and soil fertility are optimal for crop growth (Rich, 2008).

Some understanding has been gained of the secondary metabolism involved in the mechanism by which *desmodium* suppresses *Striga* (Tsanuo *et al.,* 2003). Phytochemical studies of various plants of *desmodium* species have progressively isolated phenolic components (Tsai *et al.,* 2011). From *Desmodium uncinatum* root, 2''-O-glucosylvitexin, vitexin, isovitexin and apigenin have been isolated (Tsanuo *et al.,* 2003; Guchu, 2007).

Tsanuo *et al.,* (2003) and Hooper *et al.,* (2010) isolated three isoflavanones, 5, 7, 2',4'- tetrahydroxy-6-(3-methylbut-2-enyl)-isoflavanone, (**A**) 4",5"-dihydro-5,2',4'-trihydroxy-5"-isopropenyl furano-(2",3",7,6)-isoflavanone (**B**) and 4", 5"-dihydro-2'-methoxy-5,4'-dihydroxy-5"-isopropenylferano-(2",3",7,6)-isoflavone (**C**) (Figure 1.1) from the root exudates of *D. uncinatum*. Isolated fractions containing compound B induced germination of *Striga* seeds while fractions containing compound C inhibited radical growth. Pickett *et al.,* (2007) and Hooper *et al.,* (2009) characterized another key haustorium growth inhibitor, di-C-glycosylflavone-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranosylapigenin, also

known as isoschaftoside (**D**) from polar fraction of *D. unicinatum* root exudates (Khan *et al.*, 2010). (Figure 1.1)

The active ingredients in *desmodium* roots exudates that have been characterized are suspected to be present in traditional food legumes. If similar traits are found in the food legumes, then conventional breeding could then be used to select and enhance the traits. Alternatively, if these traits are lacking, a gene could be transferred directly from *Desmodium* into food legume via genetic modification to enable legumes suppress *Striga* ssp. In the long term, it may be possible to transfer the same trait to cereal crops through heterologous gene expression, principally to the open pollinated varieties, rather than hybrids for easier accessibility to small scale farmers (Pickett *et al.*, 2010).



5,7, 2',4'-tetrahydroxy-6-(3-methylbut-2-enyl) isoflavanone (**A**), 4", 5"-dihydro-5,2',4'-trihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavone (**B**), 4",5"-dihydro-2"-methoxy-5,4'-dihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavone (**C**) and isochaftoside (**D**)

Figure 1.1; Isolated compounds from *D. uncinatum* I

1.2 Statement of the problem and justification

The interaction of the parasite and the host is chemically mediated and represents a clear example of allelopathy: the parasite recognizes certain chemicals exuded by the roots of their potential host in the vicinity to which to get attached. Depending on the parasite the haustarium development can also be chemically mediated (Macías *et al.,* 2003). There is relatively little overlap between the molecular target sites of commercial herbicides and those known for natural phytotoxins. Although a relatively large number of highly phytotoxic allelochemicals are derived from the terpeniod pathway (Macías *et al.,* 2003) there are several categories of the secondary metabolites implicated in plant allelopathy. *C. arietinum* L, and *V. radiata* L. are found to have allelopathic substances (Yasmin *et al.,* 1999, Muhammad *et al.,* 2011). Allelochemical structures and modes of action are diverse and may offer potential lead compounds for the development of future herbicides or pesticides.

This study sought to isolate, purify and characterize secondary metabolites of substantial amounts in *C. arietinum* and *V. radiata* leaves and roots extracts. This will enable assessment of the aqueous phase of the two plants. This information could also be useful when considering the possibility of transferring relevant biochemical traits involved to edible legumes, such as chickpea and mung beans.

1.3 Hypothesis

Mung bean (*V. radiata*) and Chickpea (*C. arietinum*) accumulate several unidentified flavonoids in different concentrations in leaves and roots

1.4 Objectives

1.4.1 General objective

To isolate and characterize polar secondary metabolites from the leaf and root tissues of Chickpea (*C. arietinum* L.) and from the root of Mung beans (*V. radiata* L.).

1.4.2 Specific objectives

i. To fractionate and isolate the secondary metabolites from leaf and root tissues of *C. arietinum* and from the root of *V. radiata* extracts, using column chromatography, TLC and RP-HPLC.

ii. To characterize isolated compounds from *C. arietinum* and *V. radiata* using RP-HPLC and LC-MS.

1.5 Significance of study

Many small-holder farmers in Africa, particularly those without livestock, prefer intercropping cereal crops with food legumes. Other than *Desmodium* spp., no legume, including; cowpea (*Vigna unguiculata* L.), greengram (*Vigna radiata* L.), groundnut (*Arachis hypogaea* L.), beans (*Phaseolus vulgaris* L.) and crotalaria (*Crotalaria ochroleuca* G. Don.), has demonstrated such potent suppression of *Striga* spp. (Khan *et al.,* 2007). With sufficient understanding of the mechanism by which *D. uncinatum* suppresses *S. hermonthica,* including the structural identity of some of the key mediating secondary metabolites involved, new ways of developing or transferring relevant biochemical traits involved to edible legumes

and possibly cereal crops themselves could be explored. This is expected to provide greater flexibility to farmers and to contribute even more to stabilizing and improving cereal production in the poorest farming regions (Khan *et al.,* 2008).

Intercropping maize with certain legumes reduces the infestation of maize and sorghum by *Striga*. As a consequence of this, not only is the maize yield increased but soil fertility is improved by nitrogen fixation and reduced soil erosion (Hooper *et. al.,* 2009).

The biosynthesis of isoschaftoside, which is key to the mechanism of parasitism prevention, requires specific *C*-glycosyltransferase (CGT) enzymes that convert precursors present in plants to the highly active post-germination inhibitors (Hooper *et al.,* 2009). The possible impacts to human health in the production of *C*-glycosylflavones in human edible legumes must be considered. *C*-Glycosylflavones are already present in many plants in the human diet and are associated with health foods for their antioxidant properties implicated in prevention of cancer and heart disease. As well as monitoring the concentrations of these compounds in root exudates and in root tissues, the concentration in edible parts of the plant should be examined, too, in order to determine their suitability.

CHAPTER TWO

LITRATURE REVIEW

2.1 Parasitic plant *Striga*

There are at least 30 species of *Striga* but the most problematic species within the genus are; *S. asiatica* (syn. *S. lutea*), *S. gesneriodes* and *S. hermontica* (Csurhes *et al.,* 2012). In Africa, the weed is known as the 'witchweed' because some species only emerge from below ground to produce flowers, spending much of their life underground and out of sight, and robbing their host of nutrients and water (Csurhes *et al.,* 2012).

A parasitic plant is an angiosperm (flowering plant) that directly attaches to another plant via a haustorium (Nickrent *et al.,* 2010). A haustorium is a modified root that forms a morphological and physiological link between the parasite and host (Nickrent *et al.,* 2010). When the parasite seedling forms a haustorium, it obtains a mature, functioning root system by assuming the root system of its host plant (Nickrent *et al.,* 2010). A number of compounds have been shown to function as haustoria initiators in *Striga*. But the signals required for germination and haustoria formation are inherited and produced independently by host roots (Ejeta *et al.,* 1993).

Generally, *Striga* germination is controlled by a group of sesquiterpene derivatives including strigol (Ejeta, 2007). Endogenous ethylenes play a key role in the

response of *Striga* to germination stimulants (Ejeta, 2007). Host produced compounds that are involved in haustorial formation have not been identified. Yet it is known that the chemistry of haustorial induction is distinct from that of germination stimulants (Ejeta, 2007). Kenetin, simple phenolic compounds and quinines like 2,6-dimethoxy-1,4-benzo-quinone (DMBQ) were found to be active haustorial initiators (Riopel and Timko, 1995).

Striga flowers and sheds seeds within the life cycle (Figure 2.1) of its host. The seeds are tiny (<0.3mm) so are easily dispersed by wind, water, animals etc. and one plant can produce 50,000-200,000 of them in a season. Annual increase in the size of *Striga* seed bank in soil is very high at infection densities of 20 plants/m^2 (Manyong *et al.,* 2009). The typical life cycle (Figure 2.1) lasts 90-120 days at a minimum temperature of 20^o C and optimal of 25-30^o C.

The main cereal hosts; maize, sorghum, millet and upland rice are parasitized with *Striga* species throughout the 40% of arable land of sub-Saharan Africa. The weed possess threat to food security particularly for resource-poor households in the densely populated drier areas of Sub-Saharan Africa (Press *et al.,* 1999). The association of *Striga* with infertile soils, particularly of low nitrogen status, is well documented but the mechanism for nitrogen influence is little known (Press *et al.,* 1999).

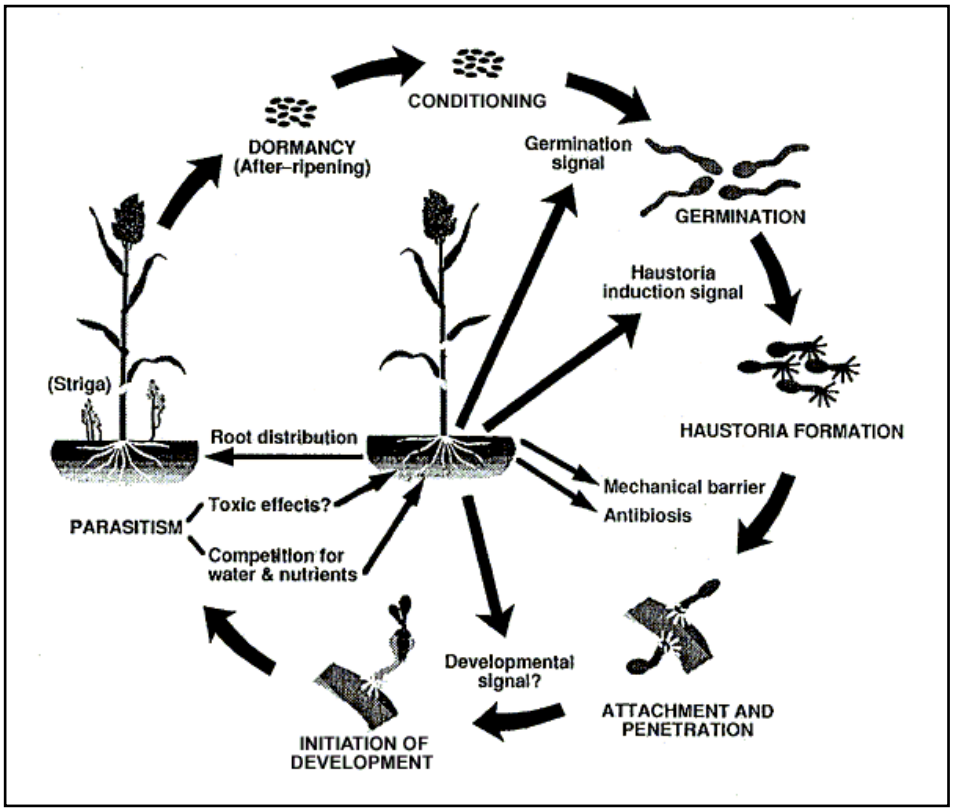


Figure 2.1; General life cycle of *Striga* (Courtesy Ejeta *et al.*, (1993))

2.1.1 Reducing *Striga* reproduction

Striga seed production can be reduced by killing *Striga* plants before they have a chance to produce a seed crop (Manyong *et al.*, 2009), shading from the intercrop canopy, hand weeding, herbicides and biological control remove *Striga*. A highly effective treatment for depleting the *Striga* seed bank include suicidal germination stimulants (such as ethylene fumigation), application of toxic fumigants like ethyl bromide, and soil solarization (covering the soil with plastic sheets for about 1 month in the dry season hence heating and killing the seeds) (Manyong *et al.*, 2009).

No single *Striga* control practice is applicable to all situations, but each has its comparative advantages and disadvantages. Some practices are suited to particular agro-ecology while others are best adopted by resource endowed households (Woomer *et al*., 2006). However, for *Striga* control practices to be useful in small scale farms first, different technologies may be combined. Secondly, packages that capture a technology, or combination of technologies, may be assembled for site-specific distribution and marketing in *Striga*-infested areas (Woomer *et al*., 2006).

2.1.1.1 Cropping systems

Some crops can be used to induce suicidal germination of *Striga,* such as non host crops grown for grain and forage or as soil building fallows. These crops, also called trap crops work by first stimulating the germination of *Striga* before exterminating it (Manyong *et al.,* 2009). However, the ability of these crops to stimulate *Striga* ssp. seed germination should be determined in the laboratory before recommending the cultivar for use in control of the weed (Manyong *et al.*, 2009). The effectiveness of the non host crops to stimulate suicidal germination of high numbers of *Striga* seeds in the soil depends on its ability to stimulate *Striga* ssp. seed germination, its rooting density (more superficial roots are more effective), and the planting density (more plants produce more roots early in the season when *Striga* attacks) (Manyong *et al.,* 2009).

It has been established that *S. hermonthica* does not seem to thrive in non cultivated situation (Berner *et al.,* 1997). One way to deal with it is to avoid growing the susceptible crop. However, *Striga* seed will still lie dormant under the

fallow for 10 years or more. A better way is the use of leguminous non host crops which stimulate *Striga* germination, but do not support its growth (Berner *et al.*, 1997). These non hosts can significantly deplete the soil seed bank by inducing suicidal germination of *Striga*. These crops can be grown in rotation with cereals, or intercropped with them. *Striga* damage can be reduced if legumes are sown at the same time as the cereal. However, this causes nutrient competition between crops, which may reduce yield even more. Therefore, new types of legumes, legume varieties, cereal variety, sowing dates, spacing, excreta, need be investigated to solve these problems (Berner *et al.*, 1997).

In Africa savannas, cropping systems are cereal-based mainly, such as sorghum, millet and maize. Since the crops are susceptible to *Striga*, they serve to build up *Striga* seeds in the field. Increasing the frequency of legume crops in the system would contribute greatly towards depletion of the *Striga* seed bank and providing other benefits such as nitrogen, disease/pest control, fodder, high protein grain, excreta (Berner *et al.*, 1997).

Trap crops whose exudates induce suicidal germination of *Striga* seeds but are themselves not parasitized could be useful to control *Striga*. This is because in addition to reduction in *Striga* seed bank, soil fertility and the livelihood of farmers could be improved. *Desmodium uncinatum*, a forage legume has been found to reduce infestation by allelopathic root exudates that stimulate germination of *Striga* seeds and inhibition of radical growth (Bationo *et al.*, 2011).

This process is being applied into Integrated *Striga* management (ISM) "Push–pull" strategy. Where, highly attractive trap plants like Napier grass (pull) are planted around the host stand which is intercropped with repellant (push) plant like *Desmodium* spp. The strategy gave up to 15-20% increase in maize yield. Reduced *Striga* infestation by "push pull" strategy has been attributed to three factors; soil shading, addition of nitrogen contributed and allelopathic mechanism associated with *D. unicinatum* (Othira *et al.,* 2008). Three fodder legumes, *Mucuna gigantic*, *Stylosanthes gugyanensis* and *Desmodium* spp. have been shown to be better *Striga* germination stimulant producers than maize and also potential trap crops (Ndung'u *et al.,* 2000).

2.1.1.2 Herbicides against *Striga*

A variety of herbicides can be used for killing emerged *Striga*. Examples include, glyphosate, paraquate dimethetryne, 2,4-D, MCPA, Triclopyr and 20% urea solution. Oxyfluorfen and dinitroaniline compounds form a barrier in the top few cm of soil and kill *Striga* as it emerges. This class of herbicide can be applied just after tillage when damage to the host is minimal and many other weeds are simultaneously controlled (Berner *et al.,* 1997). However, by directing the spray below the crop plant canopy, crop damage is minimal (Berner *et al.,* 1997).

The pre- and post emergence herbicides do not prevent crop yield loss, because they go to work after *striga* has already attached and damaged the host (Manyong *et al.,* 2009). The economics of herbicide use must be carefully analyzed in cases

involving resource poor, small scale farmers. It could be difficult to convince farmers to invest in a chemical that does not increase yield in the current season (Manyong *et al.*, 2009).

2.1.1.3 Maize with *Striga* resistance

The development of crop plants with resistance to *Striga* has been limited because of the complexity of interactions between host, parasite, and the physical environment (Ejeta, 2007). The ideal mechanism for dealing with *Striga* is immunity of host plants to *Striga* attack because it completely protects the plant. However, to ensure that the test traits in the plants are not simply escapes, a retest should be carried out. Another problem is that immunity is not often durable because it involves the functions of a few genes. Single-gene immunity may become ineffective within a few years cancelling all the made progress (Berner *et al.*, 1997).

Striga can also be reduced by employing genotypes that stimulate *Striga* growth and allow it to attach but slow its growth, delay its emergence and reduce its vigor. Another mechanism whose reaction is found in genotypes that stimulate *Striga* to germinate and allow it to attach, grow and reproduce normally, but not suffer much from the intoxication effect is also used. However, this reaction does not reduce parasitism or *Striga* seed production, which will attack the following crop (Berner *et al.*, 1997).

2.1.2 Geographic distribution of *Striga* in Sub-Saharan Africa

The various species of *Striga* distributed in Africa are shown in Table 2.1. Five of the *Striga* spp. that cause devastating effects on food crops are *S. hermontica*, *S. asiatica*, *S. forbesii*, *S. aspire* and *S. angustifolia.* Dugje *et al.* (2006) reported that there are three major *Striga* species in Nigeria; *S. hermonthica* infest sorghum, rice and maize, *S. aspera* infest rice and *S. gesnerioides* infest cowpea. In Guinea *S. aspera* occurs in rice, while *S. hermonthica* and *S. asiatica* are infectious in the free draining upland areas (Atera *et al.,* 2011). In West Africa *S. aspera* is predominant and is spreading to Ethiopia and Tanzania together with *S. hermontica* (Atera *et al.,* 2011).

Striga spp. grows in places where annual rainfall ranging from 25-150 cm per year. Notable, is a decrease in infection at high rainfall areas (Mohamed *et al.,* 1998). However, *S. forbisii* occurs in wet and water logged areas infecting irrigated crops (Mohamed *et al.,* 1998). In Cote d' Ivoire, Tanzania, Northern Cameroon, Northern Nigeria, Benin, Togo and west–wards reports indicate that *S. hermonthica* and *S. aspera* infest rice. Also reported is that *S. hermonthica* infests rice in western Kenya (Harahap *et al.,* 1993). Along the Indian Ocean islands *S. asiatica* causes losses in upland rice.

Table 2.1; Distribution and occurrence of *Striga* in Sub-Saharan Africa

***Striga* species**	**Host plants**	**Distribution**
S. aequinoctialis	-	Angola, Guinea, Liberia, Sierra Leone
S. angolensis	-	Angola
S. angustifolia	Sorghum, Sugarcane	Malawi, Tanzania, Zanzibar, Zambia
S. asiatica	Rice, Sorghum	Angola, Kenya, Lesotho, Malawi, Mozambique, Sudan, Namibia, Tanzania, Madagascar, South Africa, Zanzibar, Zambia, Botswana, Burundi, Democratic Republic of Congo
S. aspera	Rice, Maize, Sorghum, Finger millet, Wild grasses, Sugarcane	Burkina Faso, Cameroon, Central Africa Republic, Ethiopia, Gambia, Guinea, Cote d' Ivoire, Nigeria, Niger, Mali, Ghana, Senegal, Sudan
S. bilabiata	-	Angola, Burkina Faso, Burundi, Guinea Bissau, Niger, Nigeria, Cameroon, Democratic Republic of Congo, Ethiopia, Guinea, Kenya, Cote d' Ivoire, Nigeria, Niger, Mali, Malawi, South Africa, Tanzania, Uganda, Zambia
S. brachycalyx	-	Burkina Faso, Democratic Republic of Congo, Ghana, Cote d' Ivoire, Nigeria
S. chrysantha	-	Central African Republic
S. dalzielii	-	Guinea, Mali, Nigeria
S. elegans	-	Angola, Botswana, Kenya, Malawi, Mozambique, Namibia, South Africa, Swaziland, Tanzania, Zambia, Zimbabwe

S. forbesii	Sorghum, Maize, Sugarcane, Rice	Angola, Botswana, Democratic Republic of Congo, Ethiopia, Kenya, Malawi, Mozambique, Sudan, South Africa, Swaziland, Tanzania, Uganda, Zambia, Zimbabwe
S. gracilima	-	Malawi, Tanzania
S. hallaei	-	Gabon, Democratic Republic of Congo
S. hermonthica	Maize, Rice, Sorghum, Pearl millet, Finger millet, Sugarcane	Angola, Cameroon, Central African Republic, Democratic Republic of Congo, Cote d' Ivoire, Chad, Djibouti, Eritrea, Ethiopia, Gambia, Guinea Bissau, Kenya, Namibia, Niger, Nigeria, Senegal, Sudan, Tanzania, Togo, Uganda
S. hissuta	-	Angola, Burkina Faso, Central African Republic, Democratic Republic of Congo, Kenya, Ethiopia, Mozambique, Somalia, Nigeria, Seychelles, Tanzania, Zambia
S. junodii	-	Mozambique, South Africa
S. klingii	-	Ethiopia, Kenya Tanzania
S. laterica	Sugarcane	Ethiopia, Kenya Tanzania
S. lepidagathidis	-	Guinea, Senegal, Guinea Bissau
S. lutea	-	Burkina Faso, Democratic Republic of Congo, Kenya, Mali, Nigeria, Sierra Leone
S. macrantha	-	Angola, Central African Republic, Chad, Guinea, Cameroon, Liberia, Mali, Senegal, Sierra Leone
S. passargei	-	Burkina Faso, Ghana, Guinea, Nigeria, Togo, Sudan, Tanzania, Zambia
S. pinnatifida	-	Ethiopia

S. primuloides	-	Ghana, Cote d'Ivoire, Mali, Nigeria
S. pubiflora	Sugarcane	Kenya, Mozambique, Tanzania
S. yemenica	-	Ethiopia

--No infection Source; Atera *et al.* (2011)

2.2 Allelopathy

Allelopathy refers to the direct or indirect chemical effects of one plant on the germination, growth, or development of neighboring plants (Yasmin *et al.,* 1999). Biochemical interaction between plants is both inhibitory and stimulatory, through the release of secondary substances into the environment by decomposition of plant residues, root exudates or leaching by rain (Yasmin *et al.,* 1999). Both crops and weeds are capable of producing these compounds.

Allelochemicals are defined as bio-communicators, suggesting the possibility of active mixtures because of the increasing number of finding in which single compounds are not active or not as active as mixtures (Khalid *et al.*, 2002). Many crops such as cucumber, barley, wheat, rye sunflower, mung beans, chickpea and sorghum are found to have allelopathic substances (Yasmin *et al.,* 1999). The allelopathic compounds can be used as natural herbicide and others pesticides; they are less destructive of the global ecosystem than are synthetic agrochemicals (Khalid *et al.,* 2002).

Allelopathy could be used in various ways: to manipulate the crop-weed balance by increasing the toxicity of the crop plants to weeds thereby reducing weed

germination in the direct area of the crop, which is the most difficult area to control physically (Hasanuzzaman, 2011). Allelopathic effects might also depend on a number of other factors including crop varieties, specificity, autotoxicity, crop on crop effects and environmental factors (Hasanuzzaman, 2011) that might be important in any given situation.

2.2.1 Plant exudates as signals in allelopathic interactions

Allelochemicals are commonly present in conjugated form in almost all parts and in tissues, like leaves, stems, flowers, fruits, seeds and roots of plants (Othira *et al.,* 2008). Knowledge on secondary metabolites which trigger microbe responses in the rhizosphere are relatively limited (Steinkellner *et al.,* 2007). However, phytotoxic compounds in plant root exudates share some structural components, such as aromaticity, presence of hydroxyl and/or ketone groups. Some of these structures include flavonoids, quinines, quinolines and hydroxamic acids (Bais *et al.,* 2006).

The chemical signal may elicit dissimilar responses from different recipients. Chemical components of root exudates may deter one plant while attracting another or two very different plants may be attracted with differing consequences to the plant (Bais *et al.,* 2006). Some legumes contain *Striga* germination stimulants but are non hosts (Kureh *et al.,* 2003).

There are two groups of compounds in the root exudates which have been reported as key signaling molecules for plant microbe interaction in the soil. Flavonoids in

legumes (Figure 2.2) are primarily known for interactions with microbes such as nitrogen fixing rhizobium, arbuscular mycorrhizal fungi, *Fusarium* sp, as signaling compounds while strigolactones (Figure 2.3) are known as germination inducers of seeds of parasitic plants *Striga* and *Orabanche* (Steinkellner *et al.,* 2007).

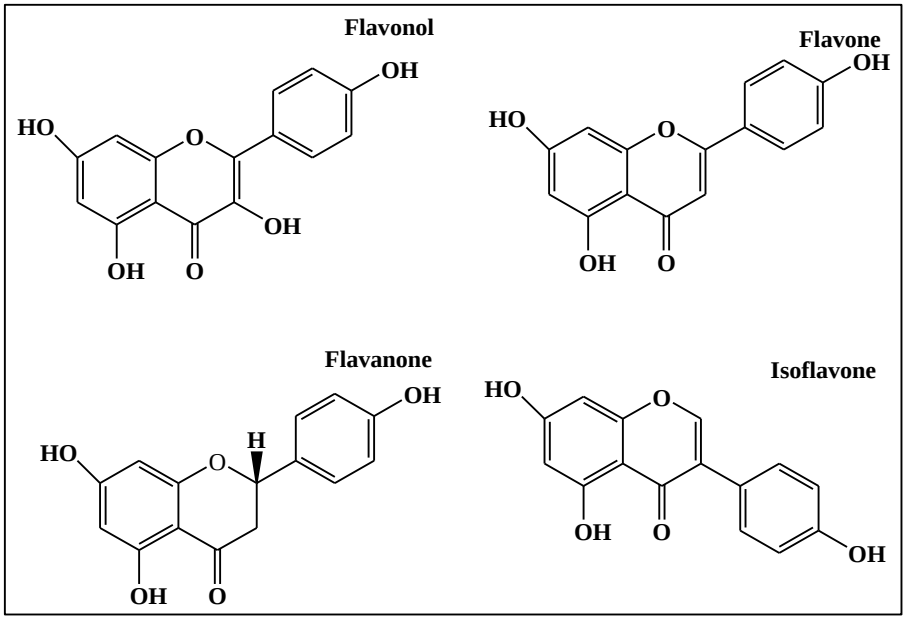


Figure 2.2; Chemical structures flavonoid

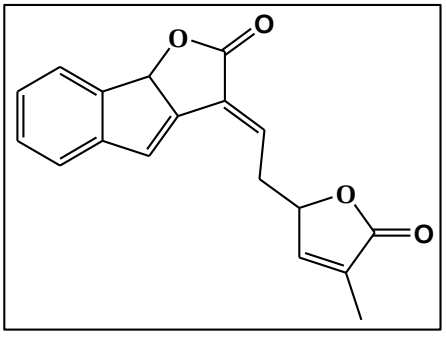


Figure 2.3; Synthetic strigolactone analogue GR24

2.2.2 Allelopathy in *C. arietinum* and *V. radiata*

Allelopathic effects of chickpea aqueous extract have been reported with root and shoot length greatly affected with concentration dependant doses (Yasmin *et al.*, 1999). The allelopathic effect of plants in agriculture was first reported by Theophrastus in 300 BC who observed the destruction of weed by chickpea (*C. arietinum*) (Thagana *et al.*, 2009). Chemical exudates released from the crop caused soil sickness and crop rotation was suggested to be the only solution (Basra, 1998). In Basra, (1998) Abu-lrmaileh *et al.* (1986) reported that aqueous extracts of *Salvia syriaca L*. shoot retard the seed germination and seeding development of *C. arietinum*.

The allelochemistry produced by green bean (*V. radiata* L) could directly or indirectly hamper or accelerate the growth and development of other organisms. The allelochemistry acts selectively, by influencing certain type of organisms and not others (Weston, 1996). According to Dixon and Whiller (1983), the available source of nitrogen was not of benefit to the cucumber because the chemical competition between two types of mung beans (*V. radiata* cultivars; Sriti and Mucuna ruriens) and cucumber. Mung beans secreted allelochemicals through root exudates in the form of C-glycocyl flavonoid or L-DOPA into the soil causing a decrease of pH of rhizosphere on its roots. This inhibited the root growth of cucumber and inhibited the nutrification by bacteria so that the nitrogen supply in the area of non-rhizosphere was lower and thus the nitrogen stored in its tissue.

Kuo *et al.* (1981) reported that aqueous extract of *Brassica campestris* L. residues inhibit seed germination and growth of *V. radiata* L. According to Waller *et al.* (1995), allelopathy may contribute as much as 10-25% of the growth inhibition of mung bean plants grown following mung bean plants. These plants have been found to be allelopathic, and their surrounding soil toxic. Distribution of the phytotoxic activity showed it to be in the stems and the aerial parts, with the roots causing little inhibition of the mung bean plant. Partitioning of the stem extracts with water and organic solvents showed that water extracts were most inhibitory to the moonbeans and lettuce; and the organic solvents were both inhibitory and stimulatory.

2.3 Phytochemical studies

Three isoflavanones, 5,7,2',4'-tetrahydroxy-6-(3-methylbut-2-enyl)-isoflavanone, (**A**) 4",5"-dihydro-5,2',4'-trihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavanone (**B**) and 4",5"-dihydro-2'-methoxy-5,4'-dihydroxy-5"-isopropenylferano-(2",3",7,6)-isoflavone (**C**) (Figure 1.1) and isoflavone 5,7,4'-trihydroxyisoflavone [genistein (Figure 2.4 **A**)] were isolated from the root exudates of the legume *D. uncinatum* (Jacq.) DC. (Hassanali *et al.,* 2003). Bioassay-guided fractionation of the root extract of *D. uncinatum* revealed isoschaftoside (Figure 1.1 (**D**)) to be the main compound in the most potent fraction inhibiting growth of germinated *S. hermonthica* radicles (Hooper *et al.,* 2010).

1,9-Dihydroxy-3-methoxy-2-methylpterocarpan (**B**), isoflavanones; 5,7-dihydroxy-2',3',4'-trimethoxy-6-(3-methylbut-2-enyl)isoflavanone (**C**) and 5,4'-dihydroxy-7,2'-dimethoxy-6-methylisoflavanone (**D**) (Figure 2.4) below, were isolated from the CH_2Cl_2 root extract of *D. uncinatum* (Jacq.) DC and characterised by spectroscopic methods. In addition, a rare pterocarpan edudiol and two known abietane diterpenes, 7-oxo-15-hydroxydehydroabietic acid and 7-hydroxycallitrisic acid were identified (Guchu *et al.*, 2007). 2''-O-glucosylvitexin, vitexin, isovitexin and apigenin have also been isolated from *D. uncinatum* root (Tsanuo *et al.*, 2003, Guchu, 2007).

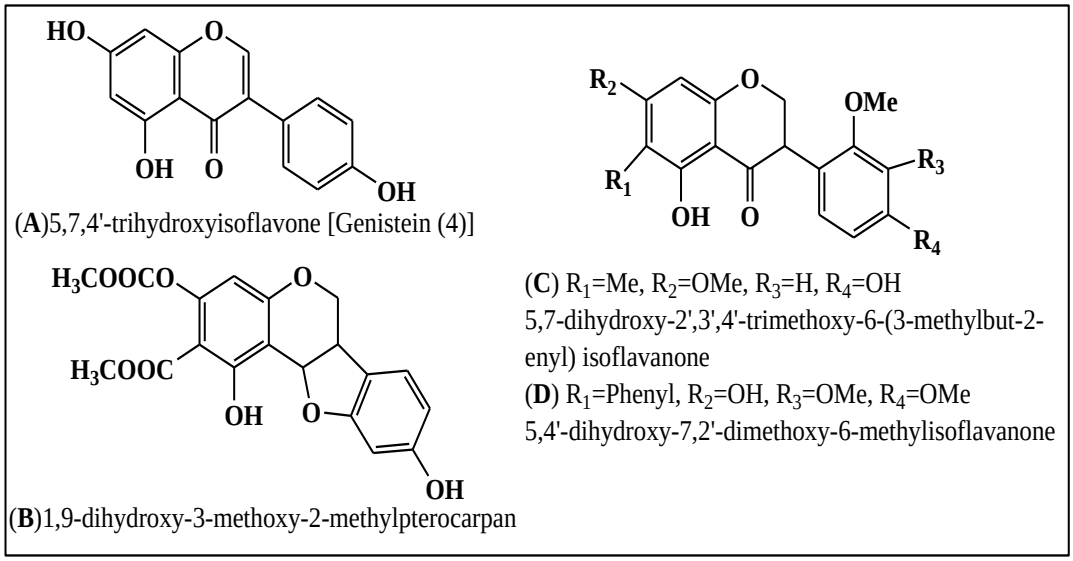
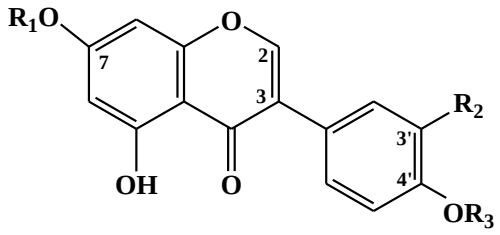


Figure 2.4; Isolated compounds from *D. uncinatum* II

The most common isoflavones identified in human food are those in soybeans, mung beans and chickpeas (Figure 2.5) are daidzein (7,4′-trihydroxyisoflavone (**A**); genistein (5,7,4′-trihydroxyisoflavone (**B**); glycitein (7,4′-dihydroxy-6-

methoxyisoflavone (**C**) and daidezin (7-O-glyciside-7-hydroxy-5,4′-dihydroxyisoflavone (**D**), and an isomer of daidzein, Puerarin (8-O-glyciside-4′-hydroxyisoflavone (**E**) (Barnes, 2010).

Zhao *et al.* (2008) isolated seven isoflavones from sprouted chickpea seeds including; (Figure 2.5) biochanin A (5,7-dihydroxyflavone-4′-methoxyflavone (**F**); calycosin (7,3′-dihydroxy-4′-methoxyisoflavone (**G**); formononetin (7-hydroxy-4′-methoxyisoflavone (**H**); Genistein (5,7,4′-trihydroxyisoflavone (**B**); trifolirhizin (maackiain-3-O-β-d-glucopyranoside, ononin (7-O-β-d-glucosyl-7-hydroxy-4′-methoxyisoflavone (**I**) and sissotrin (7-O-β-d-glucosyl-5,7-dihydroxy-4′-methoxyisoflavone (**J**).



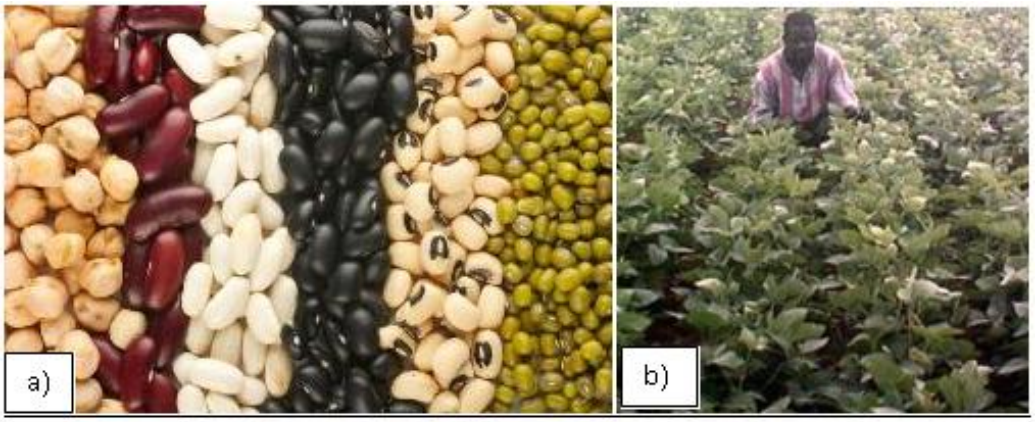
Structure of isoflavones and conjugates mentioned in the text. **Compound A**, Daidzen: R_1=R_2=R_3=H. **Compound B**, Genistein: R_1=R_2=R_3=H. **Compound C**, Glycitein: R_1=R_2=R_3=H. **Compound D**, Daidezin: R_1=O-glyciside-H; R_2=R_3=H. **Compound E**, Puerarin: R_1=R_2=R_3=H. **Compound F**, Biochanin: R_1=R_2=H; R_3=CH_3. **Compound G**, Calycosin: R_1=H; R_2=OH; R_3=CH_3. **Compound H**, Formononetin: R_1=R_2=H; R_3=CH_3. **Compound I**, Ononin: R_1=Oβ-D-glycosyl-H; R_2=H; R_3=CH_3. **Compound J**, Sissotrin: R_1= O-β-D-glycosyl-H; R_2=H; R_3= CH_3.

Figure 2.5; isolated isoflavones from *C. arietinum* and *V. radiata*

2.4 Legumes

Legumes have been recognized and valued as "soil building" crops with beneficial effects on soil's biological, chemical and physical conditions (Berner *et al.*, 1997). Growing legumes will improve soil quality through enhancing the N-supplying power of soils, increasing the soil reserves of organic matter, stimulating soil biological activity, improving soil structure, reducing soil erosion by wind and water, increasing soil aeration, improving soil water-holding capacity and making the soil easier to till (Berner *et al.*, 1997). The legumes are of two types, the fodder and the grain legumes. Grain legumes have smaller and shorter-lived effects on soil quality than perennial forage legumes.

The amounts of nitrogen fixed by grain legumes and their influence on soil physical conditions are limited by their typically small and shallow root system and short growth period. The major portion of plant nitrogen accumulates in the seed at maturity but, most of the fixed nitrogen is removed from the soil with the harvest of the grain. However, during the growth of grain legumes, considerable amounts of nitrogen are leaked from roots into the soil. Also, the residues from these crops have higher nitrogen content than cereal straw and they break down more readily, releasing nitrogen into the soil (Berner *et al.*, 1997).



a) Edible seeds from plants in the legume family (From Sondi 2012)
b) Crop rotation with nitrogen-fixing legumes like soybean (From Berner *et al.*, 1997)

Figure 2.6; Grain legume

More than 30 species of grain legume are grown across the tropics for enhancing food security improving income and helping to maintain soil fertility. The major six legumes (Figure 2.6 (a)) in Sub-Saharan Africa (SSA) and South Asia (SA) include chickpea (*Cicer arieatinum*), common beans (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea*), pigeon pea (*Cajanus cajan*) and soybean (*Glycine max*) (Abate *et al.*, 2012). Mung beans (*Vigna radiata*) are also grown for food security though in minor quantities. Grain legumes can be grown in rotation or as inter-crops with venerable cereals to increase amount of nitrogen in the soil (Figure 2.6 (b)). Under good moisture conditions these legumes are capable of fixing large amounts of nitrogen and can significantly improve the nitrogen supply for succeeding crops (Berner *et al.*, 1997).

An estimated 141 million households in SSA and SA regions or a total of more than 724 million small holders grow one or more of the six tropical grain legumes valued at more than US$ 31 billion each year (Abate *et al.,* 2012). Although they account for 16% of the global area grown with the grain legumes, their export is low with a contribution of only 6% to global production (Abate *et al.,* 2012). Except for chickpea, the bulk of production in SSA is consumed locally. Nearly 32% of the 315,000 MT of chickpea produced in the years 2005-07 was exported while the remaining 68% was used locally, with about 2% of soybean and 1% of groundnut exported (Abate *et al.,* 2012). Increase in the production of these legumes over the past decades is mainly due to expansion of the farming area rather than improved production techniques (Abate *et al.,* 2012). Demand for the six legumes in the region is expected to increase annually by about 3% until 2020, while production increase is projected to be around 7% for chickpea, common bean and pigeon pea and 2.5% for cowpea, groundnut and soya bean (Abate *et al.,* 2012). Production of the legumes in Africa is characterized by landholdings which are highly fragmented with extremely depleted soil due to over-exploitation resulting from increased production pressure, shortage of land and lack of investment in natural resources management by small holder farmers (Abate *et al.,* 2012).

2.4.1 Chickpea (*Cicer arietinum* L.)

The chickpea (*Cicer arietinum*) (Figure 2.7) also known as Asha, garbanzo bean, Indian pea, ceci bean or Bengal gram is an edible legume of the family Fabaceae,

subfamily Faboideae (Mansfeld, 2011). There are two main kinds of chickpea; the first type is Desi chickpea. It has small, darker seeds and a rough coat, cultivated mostly in the Indian subcontinent, Ethiopia, Mexico and Iran. The second is Kabuli chickpea, which is light colored, has large seeds and a smoother coat, mainly grown in Southern Europe, Northern Africa, Afghanistan, Pakistan and Chile, also introduced during the 18th century to the Indian subcontinent. Chickpeas are an excellent source of the essential trace element molybdenum (Mansfeld, 2011).

Desi chickpea is usually sown in clay soils which are neutral to alkaline. Chickpeas are almost always grown as mono-crops but are also found mixed with maize in Kenya and with wheat, barley and tef (Eragrostis tef). They are seldom irrigated or fertilized and pesticide use is minimal (Yadav *et al.*, 2007).

Figure 2.7; Chickpeas

Chickpeas are excellent source of fiber, folic acid, and manganese. They are also good source of macro nutrients such as proteins, containing almost twice the amount of protein compared to cereal grains as well as minerals such as iron, magnesium, copper, and zinc (Dejene, 2010). It is a food crop that can withstand extreme drought conditions because it extracts water deep in the soil profile, which is the basis for drought tolerance. The crop is multipurpose and may be used as human food and animal feed (Thagana *et al.,* 2009).

2.4.2 Mung beans (*Vigna radiata* L.)

Mung bean (*Vigna radiata* L.) green gram (Figure 2.8) comprises about 80 species and occurs throughout the tropics. *V. radiata* belongs to the subgenus Ceratotropis. Other cultivated Asiatic *Vigna* species in this subgenus include *V. aconitifolia* (Jacq.); Maréchal or moth bean, *V. angularis* (Willd.); adzuki bean, *V. mungo* (L.); Hepper black gram or urd bean, *V. trilobata* (L.); Verdc. or pillipesara and *V. umbellata* (Thunb.); also rice bean. Hybrids have been obtained between many of these species. The species have often been confounded, especially *V. radiata* and *V. mungo* (Mogotsi *et al.,* 2006). Other names given to this crop are green gram or golden gram (Moosavi, 2011).

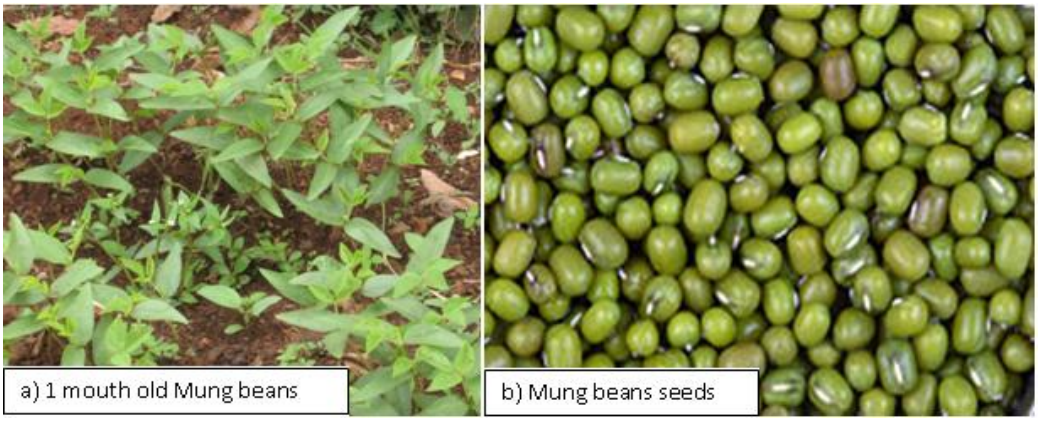


Figure 2.8; Mung beans

Mung bean is cultivated in many tropical African countries. In certain areas of Kenya, especially the Eastern Province, it is the principal cash crop (Mogotsi *et al.,* 2006). Mature mung bean seeds or flour enter a variety of dishes such as soups, porridge snacks, bread, noodles and even ice-cream. In Kenya, mung bean is most commonly consumed as whole seeds boiled with meat or vegetables and eaten as a relish with thick maize porridge ("ugali") and pancakes ("chapati") (Mogotsi *et al.,* 2006).

Mung beans are rich in protein content of about 25% of the seed by weight (Moosavi, 2011). The amino acid profile of mung bean is similar to other beans crop. Immature pod and young leaves are eaten as vegetable. Plant residues and cracked or weathered seeds are feed to livestock. Mung bean is sometimes grown for fodder, green manure or as a cover crop. The seeds are a traditional source of cures for paralysis, rheumatism, cough, fevers and liver ailments (Mogotsi *et al.,*

2006). Weed infestation is, however, one of the major factors limiting mung beans growth and development (Moosavi *et al.,* 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was an experimental design in which chemical compounds in aqueous extracts of leaves and roots of the legumes *C. arietinum* and *V. radiata* were isolated using column chromatography and RP-HPLC, and then characterized using the RP-HPLC and LC-MS.

3.2 Samples and sampling procedure

Certified seeds of *Cicer arietinum* L. (Desi (SCP 2), Kabuli (SCP 1) types) and *Vigna radiata* L. (KS 20) were obtained from Simlaw Seeds Co. Nairobi. The seeds were planted on July, 2011 at Duduville campus of ICIPE, Nairobi. The plants were uprooted after 45 days to obtain leaves and roots. The samples were cleaned in running water then air dried in a ventilated hood in the lab to remove the moisture before extraction.

3.3 Chemicals and reagents

All the analytical reference compounds including isoschaftoside, 2-glucosylvitexin, 6- glucosylvitexin-, isovitexin, vitexin, naringenin and apigenin were of analytical grade. Other analytical chemical reagents used include naturstoffreagenz A, normal silica gel, C_{18} silica gel, silica gel/TLC-plates and silica gel C_{18} TLC-plates. While organic solvents; methanol, ethyl acetate, formic

acid, sulphuric acid, hexane and butanol were all obtained from Sigma Aldrich, USA.

3.4 Instrumentation

A high performance liquid chromatography (HPLC) consisting of quaternary pump, column compartment, auto-sampler and variable wavelength photodiode array detector (Data system, version 1.24 ACE, Shimadzu Corporation England) was used. The analysis and separation employed an analytical ACE, RP-C_{18} column (4.6mm i.d. × 2500 mm, 5µm particle size) and a preparative ACE, RP-C_{18} column (10mm i.d. × 2500 mm, 5µm particle size) (Hichrom LTD, England). The mobile phase consisted of 5% formic acid in water (solvent **A**) and methanol (solvent **B**). A seven step gradient analysis for a total run time of 65 min was used as follows: starting from 95% solvent A and 5% solvent **B**, increased to 15% solvent **B** over 3min, then 25% solvent **B** over 10 min, to 30% solvent **B** over 20 min, to 95% solvent **B** over 13 min and finally for 7 min 5% solvent **B**. The column compartment was maintained at 20^{o}C using 10-50µl injection volumes. The analytes were monitored with PDA detection at between 225-350 nm.

The Liquid Chromatography-Mass Spectrometer used consisted a quaternary LC pump (Model 1200) coupled to an Agilent MSD 6120-Single quadruple mass spectrometer with electrospray source. The analysis employed a Zorbax Eclipse Plus C_{18} column (4.6 × 100mm i.d., 3.5µm particle size) and a Zorbax narrow bore SB-C_{18} RRHT, (2.1 × 50 mm i.d, 1.8µm particle size) (Agilent Technologies, USA). The mobile phase which was solvent **A**: 1% formic acid in H_2O and solvent

B: 1% formic acid in MeOH. Analysis conditions involved a three step isocratic analysis for a total run time of 29.91 min was used as follows; starting from 75% solvent **A** and 25% solvent **B**, increasing to 100% solvent **B** over 25 min, then back to 25% solvent **B** over 2.9min. Injection volumes were 10-30 µl and signals were acquired in full-scan positive-ion mode using a 100 to 800 *m/z* scan range.

Nine apparently pure samples which gave concentrations greater than 0.001% in the plants were analyzed by LC-MS to determine their respective masses. LC-MS data were produced using a Hewlett-Packard 1090 photodiode array detector (Agilent Technologies) and a Perkin-Elmer SCIEX API III triple-quadrupole mass spectrometer (Perkin-Elmer, Toronto, Ontario, Canada) equipped with an ion spray source (ISV=5500, orifice voltage=50) in positive ion mode. Multiple reactions monitoring mode was used, scanning the mass of the molecular (parent) ions in the first quadrupole (Q1) and scanning for the fragmented ions and flavonoids (daughter ions) in the third quadrupole (Q3). Argon was used as the collision gas, and nitrogen was used as nebulizer gas and orifice curtain. The system was equipped with a Synergi 4µ Hydro-RP 80 Å (250×2 mm) column fitted with a 4.0× 3.0 mm i.d. guard column (Phenomex) at a flow rate of 1ml/min. The mobile phase which was Solvent **A**: 0.1% formic acid in H_2O and Solvent **B**: 0.1% formic acid in MeOH. Analysis involved a five step gradient analysis for a total run time of 25 min was used as follows; starting from 90% solvent **A** and 10% solvent **B**, increasing to 50% solvent **B** over 15 min, then 80% solvent **B** over 5min. to 95% solvent **B** over 2min and finally isocratic for 3 min. The injection

volume was 20 µl and the scan wavelength ranged from 190-800nm. Scans were performed for both positive and negative ions between *m/z* 100 and *m/z* 1415.

3.5 Laboratory procedures

3.5.1 Extraction

Using a WTC binder 7200 drying oven, air dried roots and leaves of *C. arietinum* L. and *V. radiata* L. were further dried at temperatures of 40^{o} C for 48 hours. The brittle dry material of each plant was ground in Retsch (GmbH 5667 HAAN West-Germany) grinder, weighed (Table 3.1) then extracted with 50:50 MeOH/CH_2Cl_2 at room temperature for 3 days in the ratio of one gram herbage: 7.5 ml solvent. Filtered to separate the filtrates from residue then using the residue the extraction was repeated twice using the same solvent system. The filtrates were evaporated *in vacuo*, weighed. Each extract was analyzed using TLC and HPLC then further purified using HPLC.

3.5.2 Fractionation and isolation

TLC analysis was performed on silica plate (DC-Alufolien-Kieselgel $25F_{254nm}$), and C_{18} (Alugram RP-18w/UV_{254}) using mobile phase consisting of EtOAC-H_2O-HCO_2H (18:1:1), Hex-EtOAC (20:80 and 80:20). The compounds were viewed under UV light and visualized after spraying with 5% sulphuric acid in methanol to enhance spot detection. Then the TLC plates dried at oven temperatures ranging between 80-100^{o}C.

Different solvent systems were investigated in a bid to develop column chromatography system to fractionate the constituents present in the extract and the mobile phase showing a better separation was selected. Fractions were further purified by high performance liquid chromatography (HPLC).

3.5.3 Preliminary experiments

Fingerprint chromatographic profiles of the two types of *C. arietinum*; Kabuli and Desi Chickpeas were analyzed using TLC plates and HPLC instrument. Comparison of the biochemical composition of aqueous extracts in the roots and leaves of the two plants was done based on the difference in concentration and presence or absence of secondary metabolites. Also HPLC fingerprint profiles of *V. radiata* leaves and roots were obtained.

A medium normal phase column of *C. arietinum* leaves was eluted using different solvent systems of varying concentration and polarity from hexane, EtOAc, CH_2Cl_2, MeOH then Water, so as to determine a suitable solvent system for extracting polar compounds from the legumes. The results were analyzed by TLC and HPLC. The solvent system at which polar secondary metabolites were eluted at substantial concentration was suggested for use in extraction.

On the other hand, the extraction of *C. arietinum* from leaves was done by suspending the leaves in water and partitioned with hexane, ethyl acetate then butanol. Each extract was analyzed using TLC and HPLC in order to identify the

best solvent providing a better separation of the compounds and also, used to estimate the relative quantities of various constituents in the extracts.

3.5.4 Characterization of isolated compounds

Isolates from each plant part were assigned codes in the order of their elution time/retention time. For example, CAL 1 is the first isolated peak from the crude extract of *C. arietinum* leaf. The first two letters abbreviates the botanical name of the plant and the third letter abbreviates the part of the plant from which the isolate was obtained.

The isolated compounds were characterized using the HPLC by analysis of the peak purity of the isolated peaks, comparison of their UV maximum wavelength absorbance with standards also one to another and using retention time relative to that of known standards. LC-MS analysis was used to obtain molecular ions and characteristic fragments using MS data of the legumes' pure extractable.

Chemical test for flavonoid glycosides was done using filter papers dipped in ethanol solution of the individual compound then expose to ammonia vapour. Formation of yellow spot on filter paper indicated presence of flavonoid glycoside (Ahmed, 2007).

Using the HPLC, the isolated compounds' retention times and UV λ_{max} were compared with those of known flavonoid glycosides to ascertain their nature at a LOD of 0.25 μg/ L compared to the chemical tests for flavonoid glycoside with a greater LOD.

3.5.5 Spectrophotometric characterization

The initial spectral scan of phenolics from nine samples of the leaves and roots of *C. arietinum* and *V. radiata* was done by combining the data from PDA and MS to allow facile and efficient identification. Three screening strategies were used as follows:

Firstly, where reference compounds were available as standards, the presence or absence of a particular biophenol was assessed by comparison of retention time and UV–visible spectra, with that of the reference. Where the retention times were found to be similar, co-injection and comparison of the UV-visible spectra was done. The presence was further confirmed by molecular mass data.

Secondly, when reference compounds were not available, the TIC traces in positive mode at soft ionization and strong ionization conditions were scanned for appropriate pseudomolecular ions $[M+H]^+$. Confirmation was performed by examining UV–visible spectra and mass spectral fragmentation data at the expected elution time, depending on pre-identified compounds in the sample, the structure of the target compound, and the literature data.

Thirdly, major peaks in TIC and UV chromatograms that were not identified by either of the other screening processes were screened for novel compounds by generating mass spectra. In all, screening was conducted for nine biophenols from different classes. The main aim was to demonstrate the power of combined use of HPLC–PDA and ESI-MS in profiling legume extracts.

Samples were injected into an LC-MS system, allowing spectral analysis followed by mass determination to determine the moieties attached to the glycoside.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Method validation and preliminary results

4.1.1 Sample yield

Each of the ground leaves and roots part of the *C. arietinum* and *V. radiata* plants were weighted and recorded. Further, the parts of plants were extracted using 50/50 MeOH/CH_2Cl_2 and evaporated *in vacuo* then recorded. (Table 4.1)

Table 4.1; Yield of the crude sample and extracts

Sample name	Powder (grams)	Extract (grams)	% yield
C. arietinum leaves	346.3	71.6	21
C. arietinum roots	59.0	6.3	11
V. radiata leaves	275.8	25.8	10
V. radiata roots	67.7	3.2	5

The yield was not optimal for *V. radiata* due to cat worm attack on the plantation. The extract weights above were used to determine the capacity and amount of packing material used in column chromatography.

4.1.2 Fingerprints

Two different types of chickpea seeds were planted separately on the field, the plantations were pretty similar in the first 30 days. But on flowering, the Desi (SCP 1) chickpea had purple flowers while Kabuli (SCP 2) chickpeas' were white. Figure 4.2 are TLC and HPLC chromatograms of the different parts of the plants.

Figure 4.2 A; TLC card I, comparison of composition in *C. arietinum* leaves and roots (SCP 1 & SCP2)

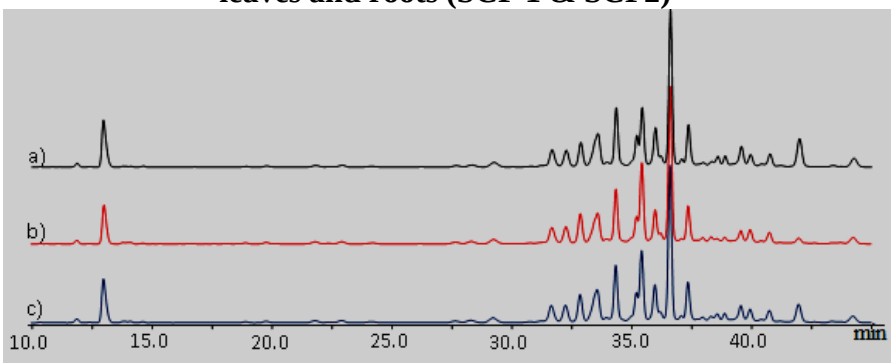


Figure 4.2 B; HPLC *C. arietinum* Leaves profiles of a) Kabuli (SCP 2); b) Desi (SCP1); c) Mixture

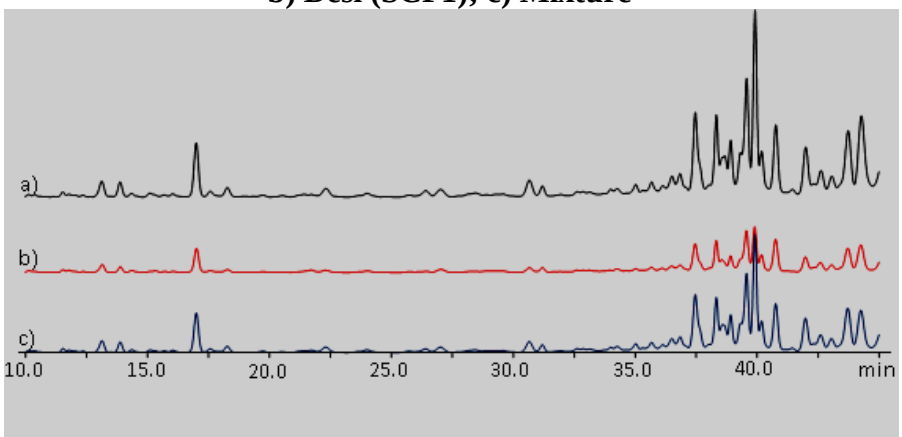


Figure 4.2 C; HPLC *C. arietinum* Roots profiles of; a) Kabuli (SCP 2); b) Desi (SCP1); c) mixture

There were variation in amounts of compounds present in the two plants, similarity, on the TLC retention factors of eluted compounds and fingerprint HPLC profiles between the two types of plants indicated the same aqueous chemical constituents. Therefore the similar plants parts were mixed and analyzed as leaves and roots of *C. arietinum* L.

Similarly, for a view of the aqueous secondary metabolites present in the *V. radiata* plant see HPLC profile of leaves and roots at 270 nm and 350 nm absorbance below (Figures 4.3 A and B).

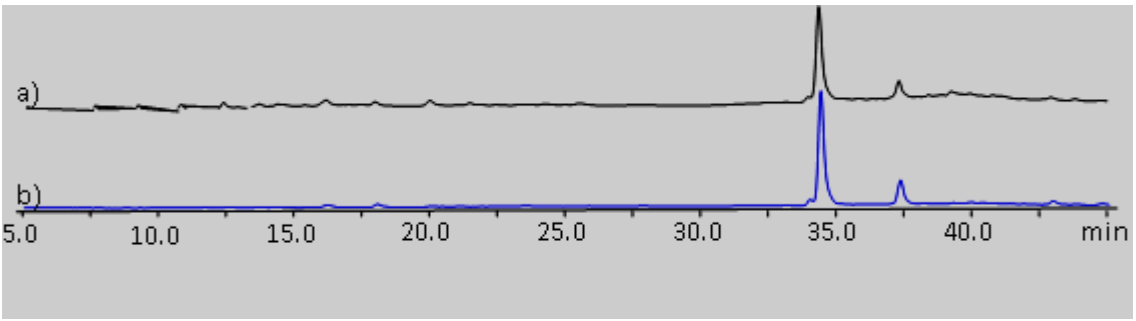


Figure 4.3 A; HPLC profiles of *V. radiata* leaves at a) at 270 nm absorbance b) at 350 nm absorbance

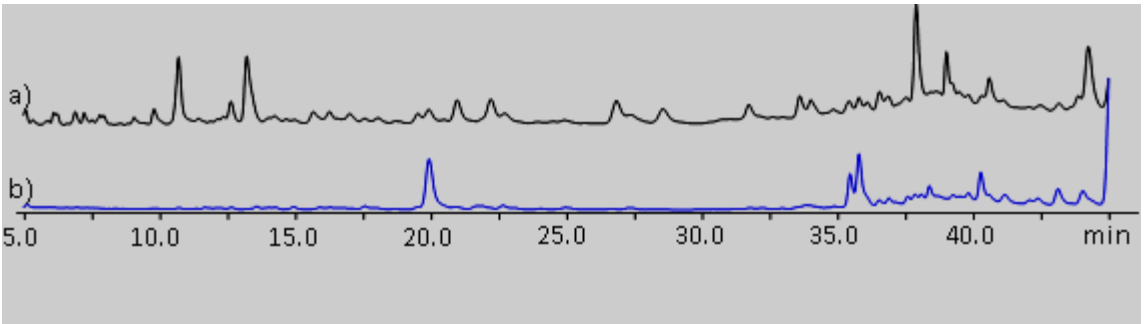


Figure 4.3 B; HPLC profiles of *V. radiata* roots at a) at 270 nm absorbance b) at 350 nm absorbance

Apparently, few are the polar compounds in substantial amount in the two parts of the plant; *V. radiata*. Even though it was possible to isolate the present compounds using the HPLC, the process was tricky because the plant has a raged profile (especially the root profile; the 270 nm profile is very different from the 350 nm one) and most compound are in very small amounts.

4.1.3 Solvent system

Different solvent systems were investigated for extraction so as to ensure maximum collection of polar secondary metabolite from the plants. A 9.6 g portion of *C. arietinum* leaves had been extracted using 80/20 MeOH/H_2O. 1 g of the extract was been suspended in water and partitioned with hexane, ethyl acetate and finally butanol (see page 31 paragraph 2). The weights of fractions were recorded (Table 4.2).

Table 4.2; Weights of 1g *C. arietinim* leaf partitioned

Fraction	**Weight in (mg)**	**Percentage weight (%)**
Hexane	27.8	2.8
Ethyl Acetate	50.0	5
Butanol	20.0	2
Water	25.1	2.5

Alongside the crude sample, the separation in four fractions was analyzed on silica gel C_{18} TLC-plate (Figures 4.4 A), mobile phase; EtOAc-H_2O-HCO_2H 18:1:1. Further, HPLC chromatograms (Figure 4.4 B) were obtained.

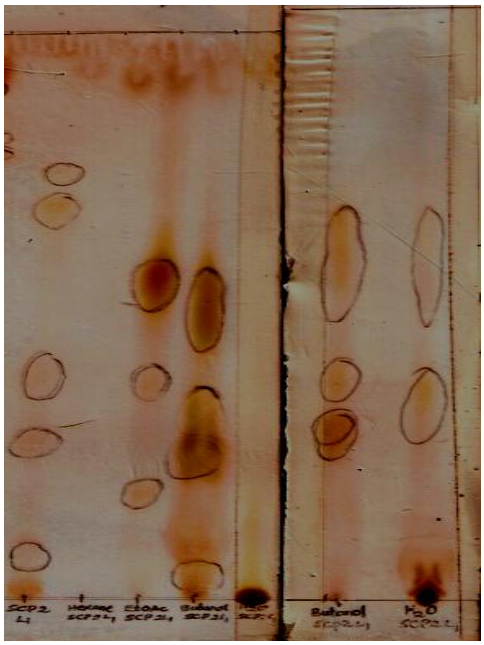


Figure 4.4 A; TLC card II, *C. arietinum* leaf fractionation using fractionating funnel

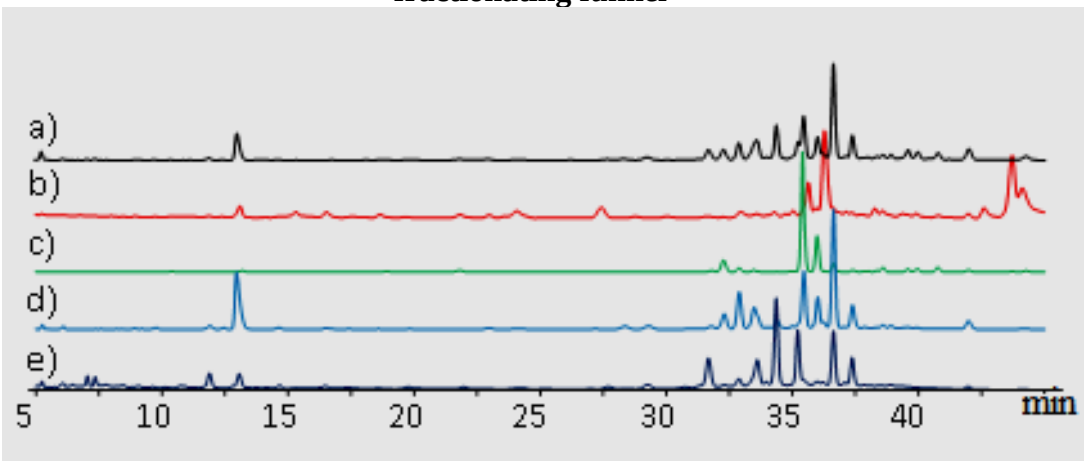


Figure 4.4 B; Fractionating funnel's HPLC profile at 270 nm of *C. arietinum* leaf a) Crude sample; b) Hexane fraction; c) Ethyl acetate fraction; d) Butanol fraction; e) Water fraction

Similarly, 1.4 g of *C. arietinum* leaves extract was dissolved in 50/50 MeOH/CH_2Cl_2. The soluble part of the sample (1.0 g) was fixed in 2 g of silica gel. Using a ratio of 1/30 sample: silica gel a 42.3 g capacity column was packed with 31.0 g silica gel dissolved in hexane. The mobile phase varied from hexane, ethyl acetate, dichloromethane, methanol to water. 30 fractions of 20 ml each were obtained. A TLC C_{18} card was spotted after fractionation and using mobile phase; EtOAc-H_2O-HCO_2H 18:1:1 spots were developed (Figure 4.5 A). Eight fractions were pooled as indicated on the TLC card below then analyzed using the HPLC (Figure 4.5 B).

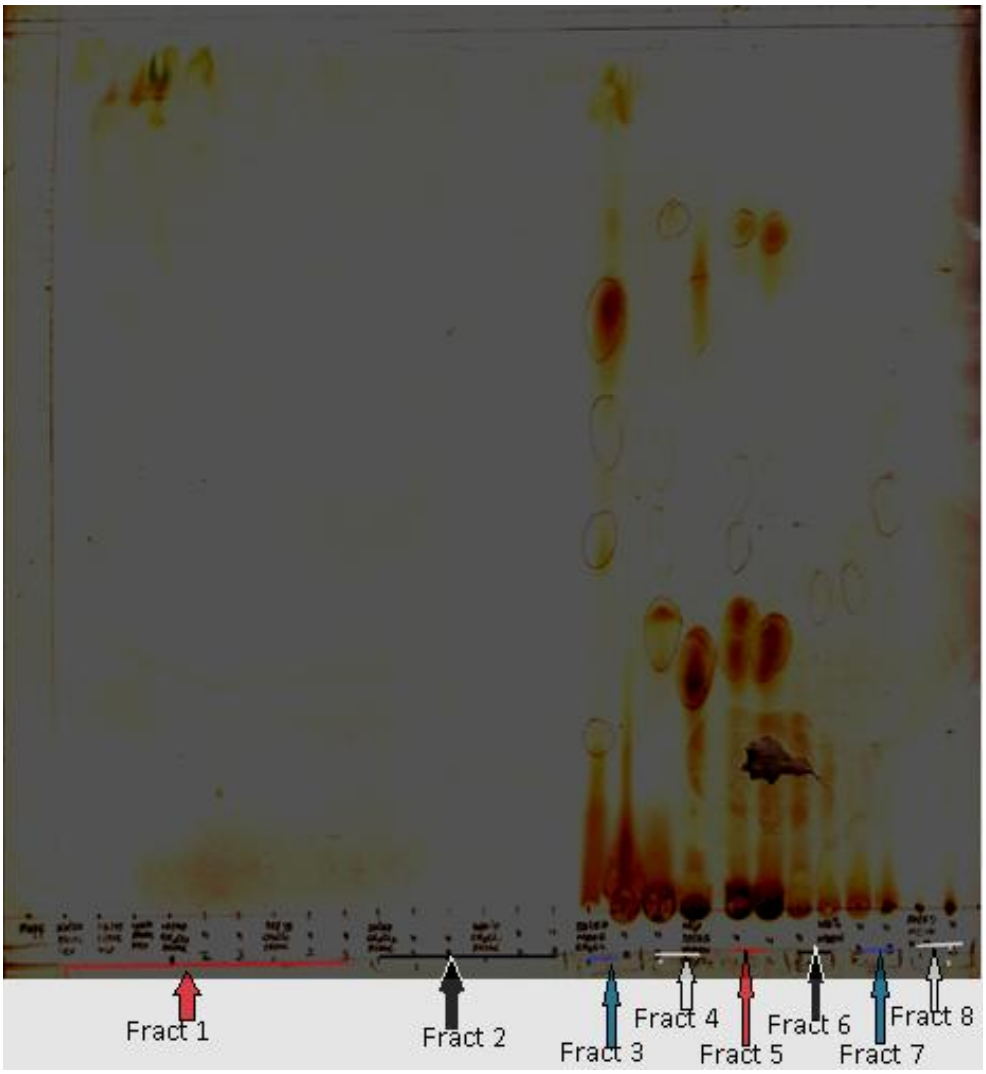


Figure 4.5 A; TLC card III, Preliminary column chromatography fractionation of *C. arietinum* leaf

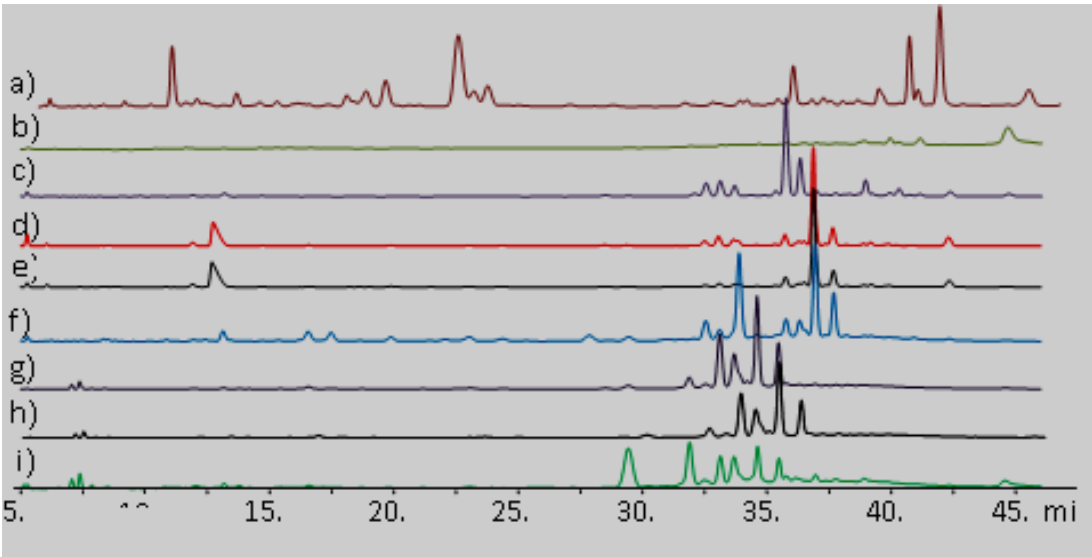


Figure 4.5 B; Preliminary analysis using HPLC profiles at 270 nm of *C. arietinum* leaves fractions a) Crude sample; b) Fraction 1 c) Fraction 2; d) Fraction 3; e) Fraction 4; f) Fraction 5; g) Fraction 6; h) Fraction 7; i) Fraction 8

From these experiments, it was observed that there was not any separation of compounds using neither fractionating funnel nor normal phase column chromatography. From the hexane fraction, no polar compounds were found. While in the butanol and water fractions, all the compounds present in the crude sample were present at low amounts. Ethyl acetate gave an increased concentration of some compounds present in the original sample.

From the column chromatography, it was observed that most polar compounds are eluted by methanol dichloromethane solvent systems. Therefore extraction of the samples was done using 50/50 MeOH/CH_2Cl_2 and column chromatography was used for fractionation and purification of the samples. It was recommended that before using the HPLC for isolation of peaks column chromatography should be

done at least twice for every sample for purification, increased amounts of present compounds and higher resolution.

4.1.4 Spectroscopic study of reference biophenols

A series of flavonoid standards (Figure 4.6), representative of different flavonoid groups previously reported in allelopathic *D. uncinatum* and other legumes, using HPLC-PDA and RP-LC-ESI-MS were examined to ascertain their structure/spectra (Appendix table A). From HPLC-PAD, various correlations between structure and UV spectrum were extracted (Table 4.3).

Table 4.3; Reference biophenols

Cording	Flavonoid standards	Retention time T_R (min)	Maximum absorbance (λ_{max}) (nm)	Molecular weight $[M]^+$
1	Genistein	8.89	212, 225, 229, 280	270
2	6- glucosylvitexin	17.83	219, 239, 334	594
3	Isoschaftoside	21.99	218, 239, 270, 336	564
4	Vitexin	24.64	214, 267, 343	432
5	2-glucosylvitexin	26.80	207, 239, 268, 334	594
6	Isovitexin	30.51	214, 239, 267, 343	432
7	Naringenin	36.68	213, 288	273
8	Luteolin	38.93	225, 256, 292, 349	286
9	Apigenin	41.74	212, 238, 267	270
10	Quercetin Dihydrate	50.98	238, 258	302

The most notable effects of flavonoids is the λ_{max} (band I) increases in the series: flavones (apigenin) < flavonol (quercetin). The λ_{max} also provided an indication of the substitution within the same class as seen for glucosylvitexins, vitexin and isovitexin in which the absorption λ_{max} was not affected in isomers of the same group (e.g. vitexin and isovitexin) Table 4.3.

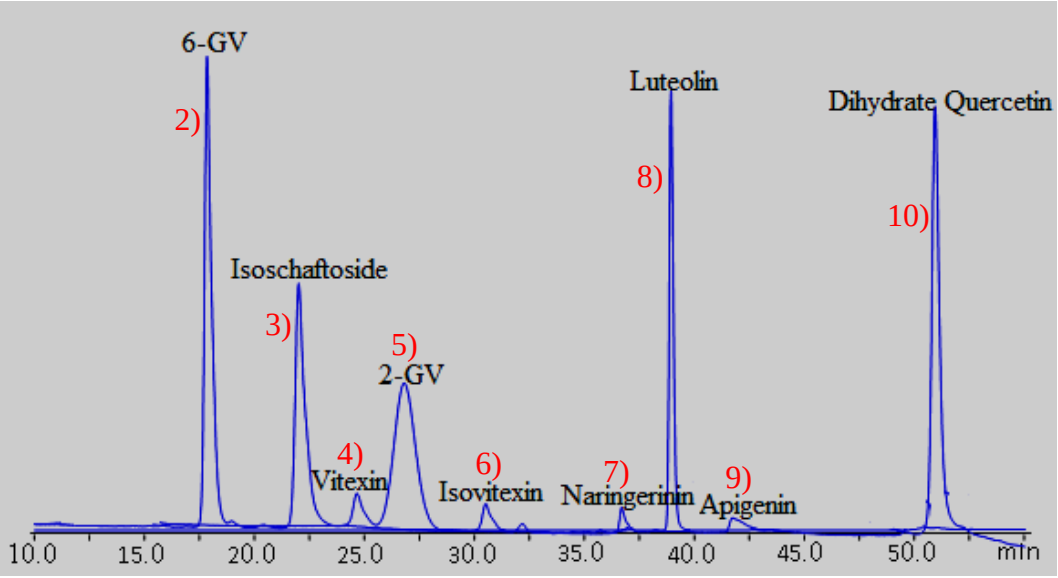


Figure 4.6; Simultaneous HPLC chromatogram detected with a photodiode array detector set at 350nm (A) Peak identification was listed in Table 4.3 (B) Peak structures listed in Appendix A

4.2 Fractions and isolates of secondary metabolites

4.2.1 *C. arietinum* roots

4.2.1.1 Column chromatography I

Flash fractionation column chromatography was used for fractionation of *C. arietinum* roots (Table 4.4).

Table 4.4; Fractionation on the 1st column of *C. arietinum* roots

Fraction	Solvent System	% Conc. of extraction solvent	Weight of dry Sample in (g)	Percentage weight (%)
Crude	-	-	5.5	
0	EtoAc	100	1.77	32.2
1	EtOAc	100	0.18	3.3
2	EtoAc/CH_2Cl_2	75	0.06	1.1
3	$EtOA_C$/CH_2Cl_2	50	0.05	0.9
4	$EtOA_C$/CH_2Cl_2	25	0.03	0.5
5	CH_2Cl_2	100	0.05	0.9
6	CH_2Cl_2/MeOH	95	0.24	4.4
7	CH_2Cl_2/MeOH	90	0.86	15.6
8	CH_2Cl_2/MeOH	80	1.27	23.1
9	CH_2Cl_2/MeOH	50	1.40	25.5
10	CH_2Cl_2/MeOH	25	0.30	5.5
11	MeOH	100	0.17	3.1
12	MeOH/H_2O	50		
13	H_2O	100		

The fractions were analyzed by HPLC (Figure 4.7) detected with a photodiode array detector set at 270 nm.

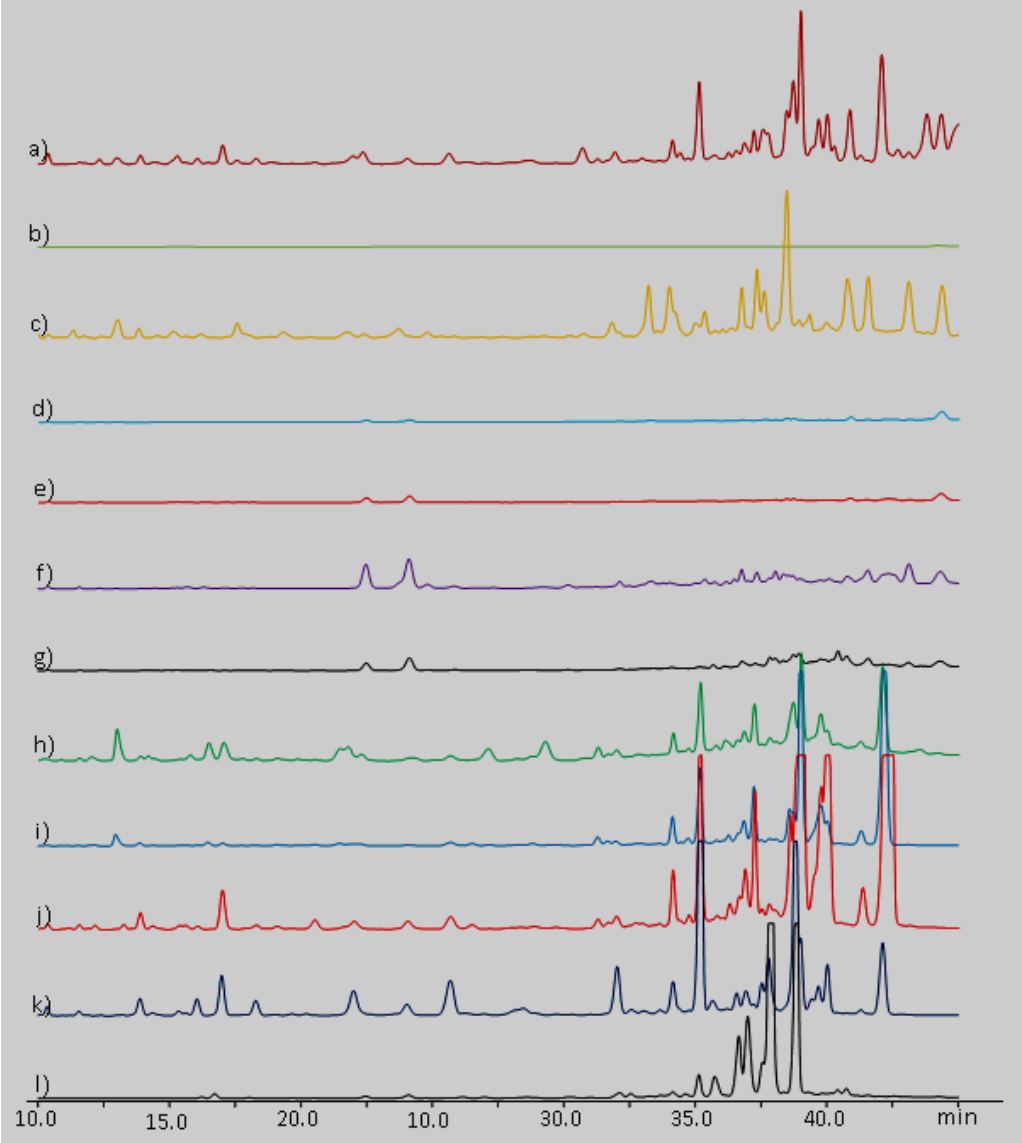


Figure 4.7; HPLC profiles of *C. arietinum* roots fractions from 1st column a) Crude sample; b) 100% EtOAc-0; c) 100% EtOAc-1; d) EtOAc/CH_2Cl_2 75/25; e) EtOAc/CH_2Cl_2 50/50; f) EtOAc/CH_2Cl_2 25/75; g) 100%CH_2Cl_2; h) MeOH/CH_2Cl_2 25/75; i) MeOH/CH_2Cl_2 50/50; j) MeOH/CH_2Cl_2 80/20; k) MeOH/CH_2Cl_2 90/10; l) MeOH/CH_2Cl_2 95/5

Using mobile phases (d, e, f and g) there were no polar compounds observed. The separation was poor in fraction (h) where the compounds present were not

concentrated but where the concentration was better isolation of individual peaks would be tricky because the compounds were very close to each other. In fractions (I, j, k and l) the clarity and concentration of the compounds present was good. Therefore, it was recommended that a smaller column containing a mixture of the four fractions be mounted to further purify the fractions.

4.2.1.2 Column chromatography II

A second 600 ml column was packed and 4 fractions each of 20 ml eluted using 95/5, 90/10, 85/15, 80/20 then 50/50 MeOH/CH_2Cl_2 respectively. Each fraction was spotted on silica gel/TLC-cards (Figure 4.8). The spots were developed using mobile phase consisting of EtOAc-H_2O-HCO_2H (18:1:1). Further HPLC profiles detected with a photodiode array detector set at 270 nm (Figure 4.9) of 6 fractions pooled as indicated on the TLC card were obtained on PDA detector.

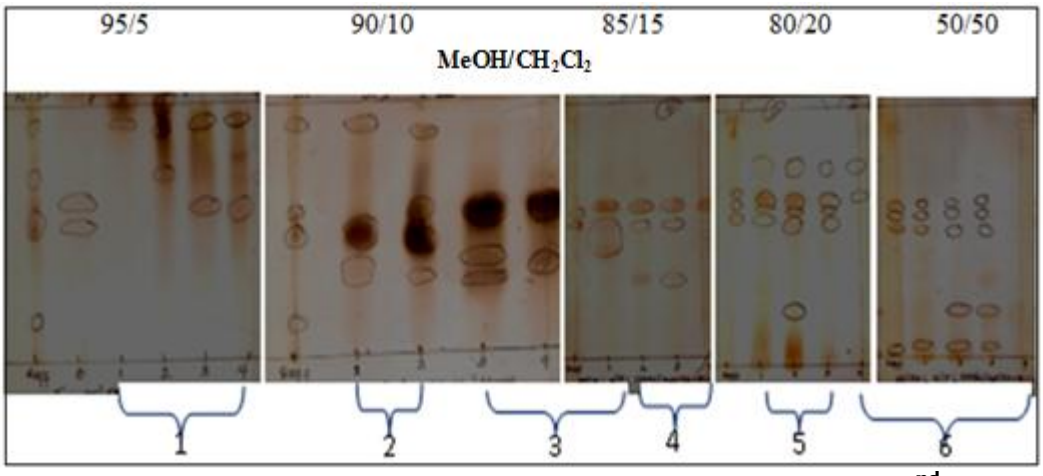


Figure 4.8; TLC card IV, *C. arietinum* root fractions from 2nd Column chromatography

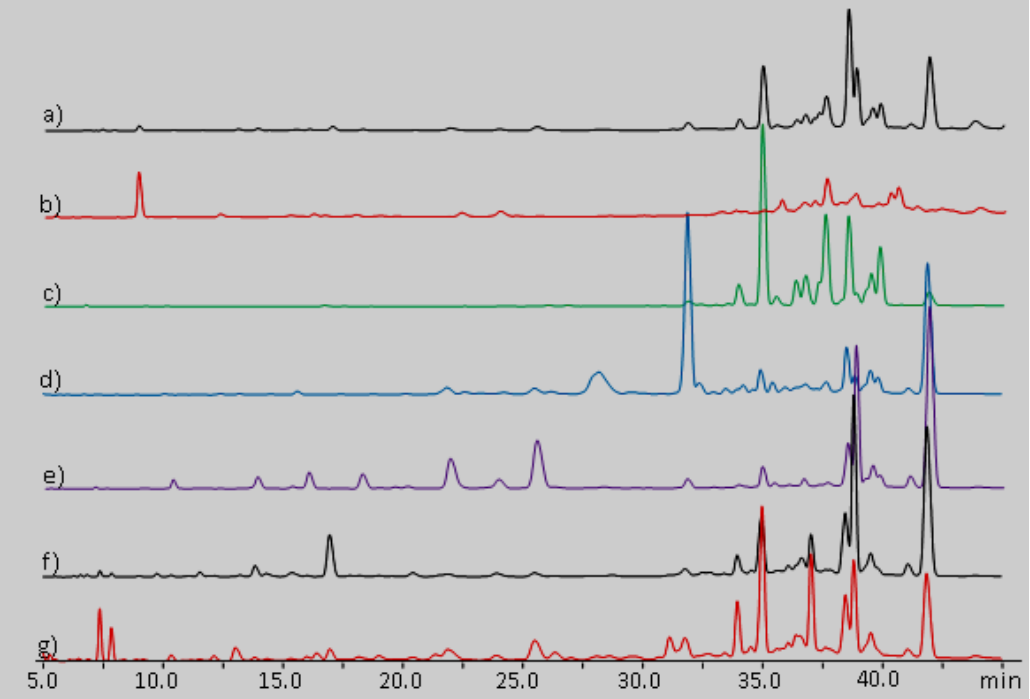


Figure 4.9; HPLC roots profiles of fractions from *C. arietinum* 2nd column a) crude sample; b) Fraction 1; c) Fraction 2; d) Fraction 3; e) Fraction 4; f) Fraction 5; i) Fraction 6

Better resolution was observed in fractions d), e) and f). Using HPLC, well resolved peaks of substantial concentration in the fractions were recommended for isolation.

4.2.1.3 Characterization of isolates

Using a HPLC on PDA detector at 270 nm and a preparative RP-C_{18} column (10 mm i.d. × 250 cm, 5 µm particle size), six fractions was obtained and weights recorded (Table 4.5).

Table 4.5; HPLC, LC-MS and Flavonoid test analysis of *C. arietinum* root extract

Fraction	Weight in (mg)	% of extract	Retention Time (min)	UV λ_{max} (nm)	$[M]^+$	Purity	Ammonia test
CAR 1	0.34	0.005	16.6~17.0	222, 273, 318	470/590	Impure	-ve
CAR 2	0.70	0.010	27.5~27.9	224, 272	484	Impure	-ve
CAR 3	0.30	0.005	27.8~28.6	223, 239, 262	472	Pure	-ve
CAR 4	0.22	0.004	31.5~31.8	228, 260	484	Pure	-ve
CAR 5	13.54	0.215	34.6~35.0	236, 254	430	Pure	+ve
CAR 6	13.06	0.207	38.2~38.6	224, 239, 260, 325	390/468	Impure	-ve

HPLC profiles on the analytical column detected with a photodiode array detector set at 270 nm (Figure 4.10).

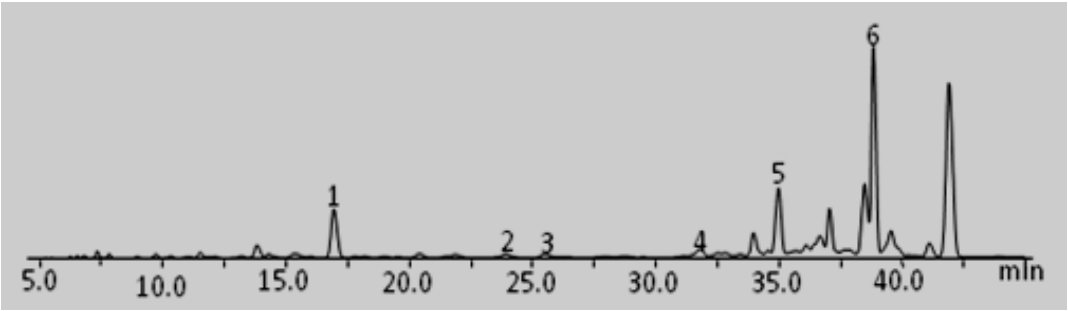


Figure 4.10; Isolated compounds from *C. arietinum* roots

Using the LC-MS, TIC traces in positive mode at soft ionization and strong ionization conditions were scanned and appropriate pseudomolecular ions $[M+H]^+$ obtained. Results were tabulated in Table 4.5.

Analysis using the RP-HPLC revealed all the six extracts; CAR 1, CAR 2, CAR 3, CAR 4, CAR 5 and CAR 6 gave characteristic single peaks at their respective retention times. Implying they were pure enough to be characterized using the LC-MS. There was enough of CAR 5 and CAR 6 for further analysis.

On further analysis, using the LC-MS three of the compounds whose TIC scans gave single peaks could be identified but only CAR 5 had a flavonoid agylcone based on the phenol-Explorer database (Anonymous, 2012), was identified in this study.

4.2.2 *C. arietinum* Leaves

4.2.2.1 Column chromatography I

A 1020 ml capacity column was packed and 40 fractions, 200 ml each of *C. arietinum* leaves collected (Table 4.6) while varying polarity of the eluting solvent from hexane to methanol. The mobile phase was changed only when there was no more observable separation following analysis by TLC.

Table 4.6; Fractionation on the 1st column of *C. arietinum* leaf

Fraction	Solvent System	Percentage Conc.	Weight of dry sample	Percentage Yield
CRUDE			54.33	
1	Hexane	100	1.23	2.30
2	Hexane	100	0.50	0.90
3	Hexane/EtOAc	90	0.23	0.40
4	Hexane/EtOAc	90	0.25	0.50
5	Hexane/EtOAc	90	0.15	0.30
6	Hexane/EtOAc	90	0.22	0.40
7	Hexane/EtOAc	50	0.13	0.20
8	Hexane/EtOAc	50	2.22	4.10
9	Hexane/EtOAc	50	0.38	0.70
10	Hexane/EtOAc	50	0.36	0.70
11	Hexane/EtOAc	50	0.03	0.05
12	Hexane/EtOAc	50	0.02	0.04
13	Hexane/EtOAc	50	0.14	0.30
14	EtOAc	100	0.05	0.09
15	EtOAc	100	0.04	0.07
16	EtOAc	100	0.08	0.15
17	EtOAc	100	0.12	0.20
18	EtOAc	100	0.23	0.40
19	EtOAc	100	0.34	0.60
20	EtOAc/CH_2Cl_2	50	0.30	0.55
21	EtOAc/CH_2Cl_2	50	0.25	0.50

22	EtOAc/CH_2Cl_2	50	0.14	0.30
23	CH_2Cl_2	100	0.04	0.07
24	CH_2Cl_2	100	0.03	0.05
25	CH_2Cl_2	100	0.01	0.02
26	CH_2Cl_2/MeOH	90	0.02	0.04
27	CH_2Cl_2/MeOH	90	0.04	0.07
28	CH_2Cl_2/MeOH	90	3.63	6.70
29	CH_2Cl_2/MeOH	80	4.70	8.70
30	CH_2Cl_2/MeOH	80	5.21	9.60
31	CH_2Cl_2/MeOH	80	5.31	9.80
32	CH_2Cl_2/MeOH	80	5.40	9.90
33	CH_2Cl_2/MeOH	80	2.74	5.0
34	CH_2Cl_2/MeOH	50		
35	CH_2Cl_2/MeOH	50		
36	CH_2Cl_2/MeOH	50		
37	MeOH	100		
38	MeOH	100		
39	MeOH	100		

Using mobile phase EtOAc-H_2O-HCO_2H 18:1:1, (Figure 4.11 (a)) the spots were developed then a repeat of the first 27 fractions was done using EtOAc: Hex; 80:20 (Figure 4.11(b)).

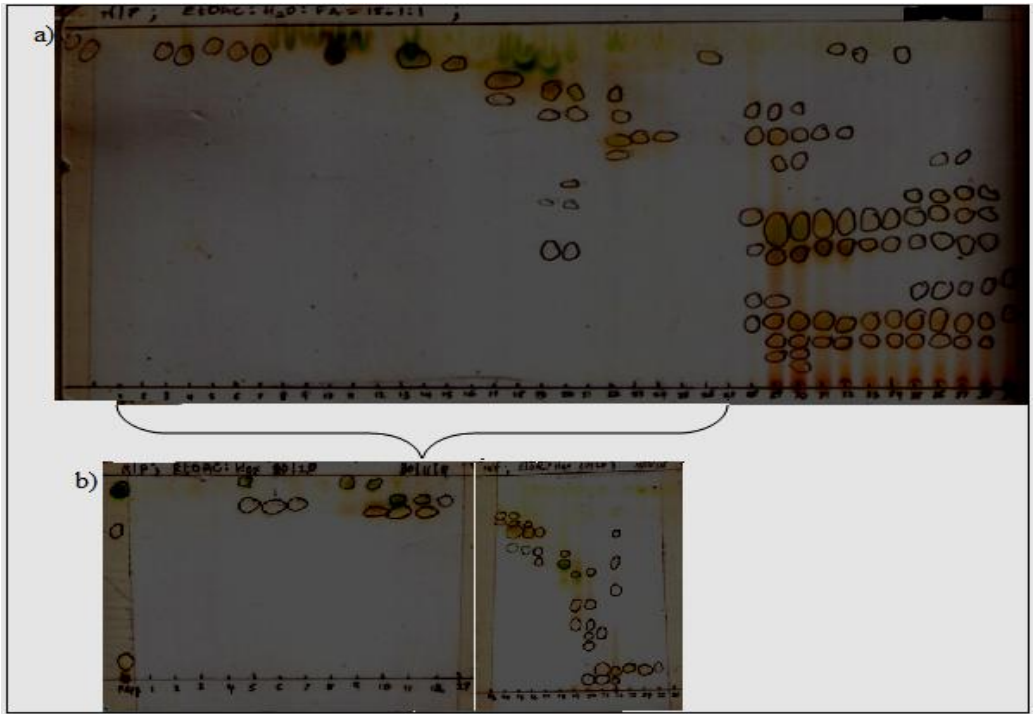


Figure 4.11 (a) & (b); TLC cards V; Fractions from the 1st column chromatography of *C. arietinum* leaf

Only the even numbered fractions on the TLC card were analyzed on HPLC and were detected with a photodiode array detector set at 270 nm (Figure 4.12).

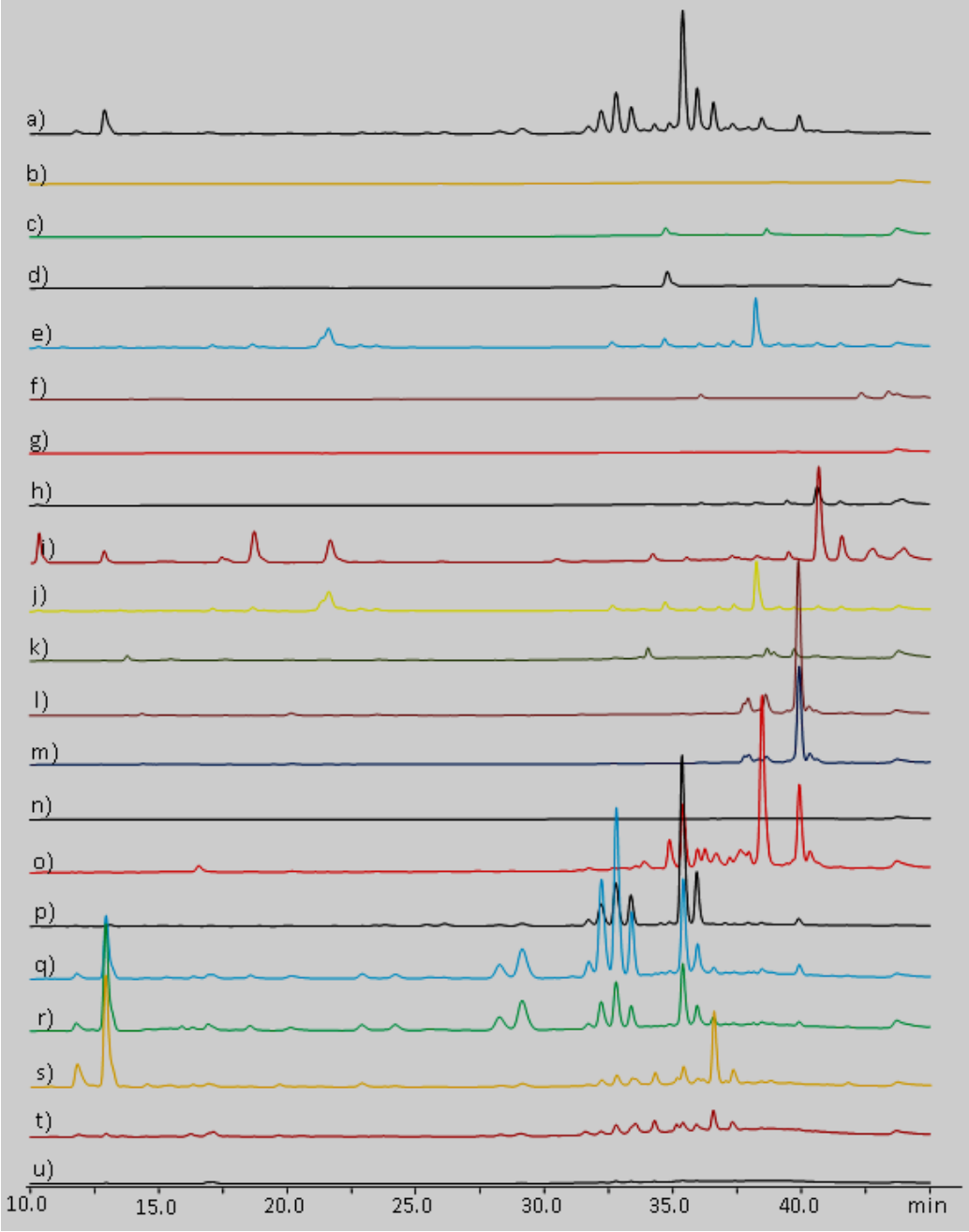


Figure 4.12; HPLC profiles of 1st column chromatography of *C. arietinum* leaf a) Crude sample; b) Fraction 2; c) Fraction 4; d) Fraction 6; e) Fraction 8; f) Fraction 10; g) Fraction 12; h) Fraction 14; i) Fraction 16; j) Fraction 18; k) Fraction 20; l) Fraction 22; m) Fraction 24; n) Fraction 26; o) Fraction 28; p) Fraction 30; q) Fraction 32; r) Fraction 34; s) Fraction 36; t) Fraction38; u) Fraction 40

The compounds were mainly eluted in fraction 29-33. Therefore, these fractions were pooled, dried and a reverse phase column packed and eluted with ethyl acetate, dichloromethane and methanol while slowly changing the percentage concentrations of solvents (Table.4.7).

4.2.2.2 Column chromatography II

The sample from fractions 29-33 weighed 23.4 g, was fixed in C_{18} silica gel and a reverse phase column packed then eluted with ethyl acetate, dichloromethane and methanol while slowly varying the percentage concentrations of solvent system. Results were tabulated in (Table 4.7).

Table 4.7; Fractionation of *C. arietinum* leaf on the 2nd column chromatography

Fraction	Solvent System	Percentage Conc.	Weight of dry sample	Percentage Yield
1	EtoAc	100	0.52	2.2
2	EtOAc	100	0.71	3.0
3	EtoAc/CH_2Cl_2	50	0.33	1.4
4	CH_2Cl_2	100	0.10	0.4
5	CH_2Cl_2	100	0.12	0.5
6	CH_2Cl_2	100	0.02	0.09
7	CH_2Cl_2/MeOH	90	2.15	9.2
8	CH_2Cl_2/MeOH	90	1.34	5.7
9	CH_2Cl_2/MeOH	90	2.07	8.8
10	CH_2Cl_2/MeOH	80	1.37	5.9
11	CH_2Cl_2/MeOH	80	0.86	3.7

12	CH_2Cl_2/MeOH	70	1.49	6.4
13	CH_2Cl_2/MeOH	70	1.55	6.6
14	CH_2Cl_2/MeOH	60	0.21	0.9

14 fractions were spotted on silica gel TLC card IV and spots developed using mobile phase; EtOAc-H_2O-HCO_2H 18:1:1 (Figure 4.13).

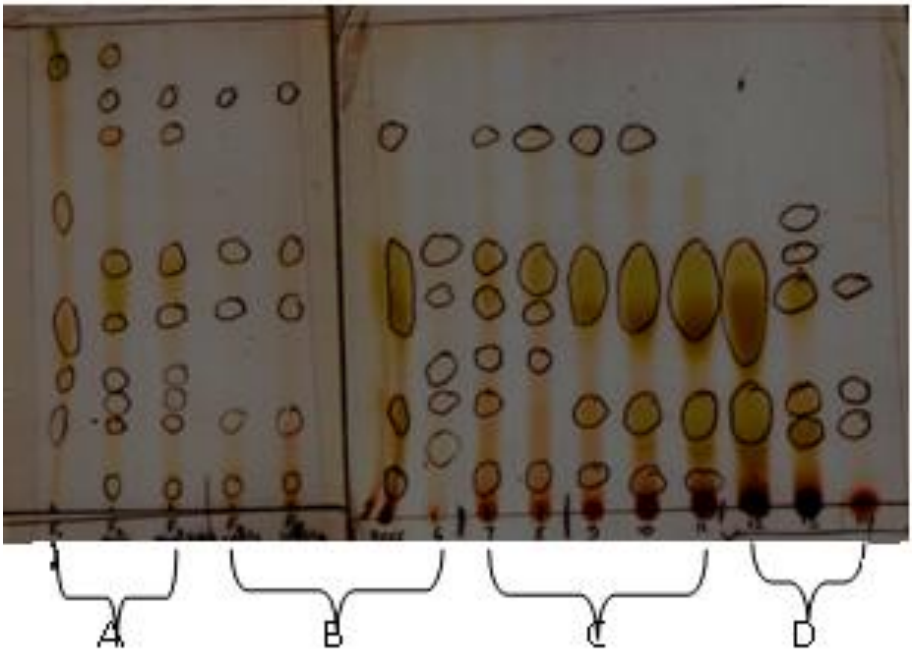


Figure 4.13; TLC cards VI; Fractions from the 2nd column of *C. arietinum* leaf

HPLC analysis of four relatively similar fractions pooled from the 14 fractions as indicated on the TLC card (Figure 4.14).

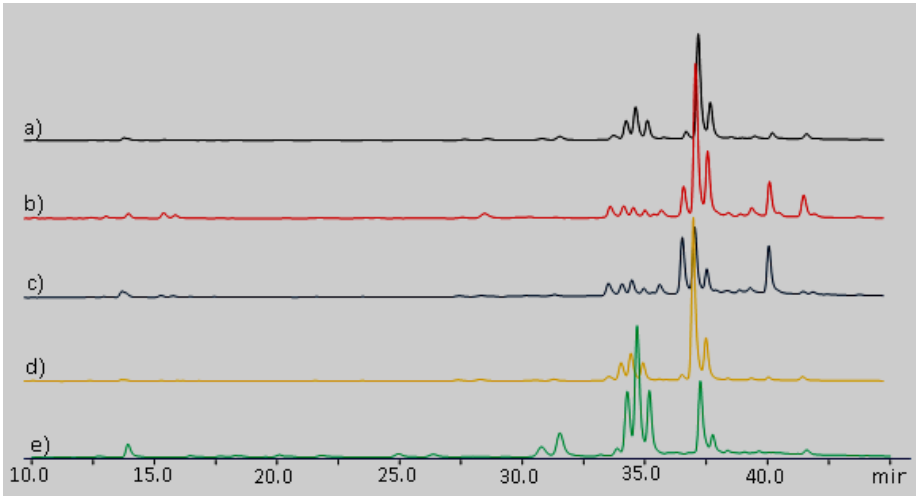


Figure 4.14; HPLC profiles of fractionations from 2nd column chromatography of *C. arietinum* leaf II a) Crude sample; b) Fraction A; c) Fraction B; d) Fraction C; e) Fraction D

Majority of the compounds with reference to the crude sample were available in fraction D with better separation. The fraction whose constituents include; fractions 12, 13 and 14 which had been eluted using mobile phase of concentration varying from 30/70 to 40/60 MeOH/CH_2Cl_2. This fraction was recommended for further fractionation using the HPLC.

4.2.2.3 Characterization of isolates

A shorter gradient elution method was optimized from the original 65 min method that lasted 49 minutes. 11 extracts were obtained, Figure 4.15 shows the HPLC peak profile detected with a photodiode array detector set at 270 nm.

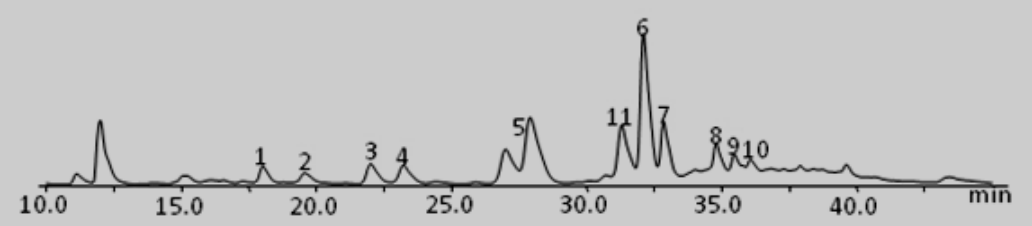


Figure 4.15; Isolated peaks from *C. arietinum* leaf's fraction D

Using the LC-MS, TIC traces in positive mode at soft ionization (25^{o}eV) and strong ionization (40^{o}eV) conditions were scanned and appropriate pseudomolecular ions $[M+H]^{+}$ obtained. Weights of purified samples and their respective molecular ions are summarized in Table 4.8.

Table 4.8; HPLC, LC-MS and Flavonoid test analysis of *C. arietinum* leaf extracts

Fraction	Weight in (mg)	%age of extract	RT (min)	UV λ_{max} (nm)	$[M]^{+}$	Purity	NH_3 test
CAL 1	0.54	0.0008	17.9~18.3	224, 239, 265, 345	610	Pure	-ve
CAL 2	1.80	0.0025	19.3~19.7	222, 238, 255, 352	640	Pure	+ve
CAL 3	1.70	0.0024	21.7~22.1	225, 238, 257, 353	626	Pure	+ve
CAL 4	4.00	0.0056	22.9~23.3	230, 265, 325, 337	610	Pure	+ve
CAL 5	4.50	0.0063	25.6~26.0	236, 265,347	-	Impure	+ve
			26.0~26.4	244, 351			
CAL 6	1.83	0.0026	29.1~29.5	232, 254, 354	-	Impure	+ve
			29.8~30.2	236, 265, 347			
CAL 7	1.70	0.0024	32.5~32.9	238, 255, 352	609	Pure	+ve
CAL 8	2.40	0.0034	34.5~34.9	230, 265, 337	448	Pure	+ve
CAL 9	0.33	0.0005	36.5~36.9	220, 244, 264, 348	448	Pure	+ve

CAL 10	0.09	0.0001	37.7~38.1	224, 239, 265, 348	-	Impure	-ve
CAL 11	0.25	0.0004	29.4~29.8	218, 244, 348	610	Pure	-ve

Analysis using the RP-HPLC revealed nine extracts; CAL 1, CAL 2, CAL 3, CAL 4, CAL 7, CAL 8, CAL 9, CAL 10 and CAL 11 were of substantial concentration and gave characteristic single peaks at their respective retention times. Implying they were pure enough to be characterized using the LC-MS. But CAL 5 and CAL 6 each produced twin peaks that were inseparable using HPLC and therefore were not pure enough to be characterized using the LC-MS.

Using the LC-MS seven of the above compounds whose TIC scans gave single peaks each and had flavonoid agylcone based on the phenol-Explorer database, (Anonymous, 2012) were identified in this study. Though pure, CAL 1 and CAL 10 were not identified in this study because their agylcone are not of any reported flavonoid.

4.2.3 *Vigna radiata* roots

4.2.3.1 Column chromatography

A 270.7 ml normal phase column was packed and 2.5 g of *V. radiata* root extract was eluted using solvents of varying polarity from hexane to methanol. The results were tabulated in (Table 4.9).

Table 4.9; Fractionation of *V. radiata* root using column chromatography

Combined fraction	Fraction	Solvent System	% Conc.	Combined weight (g)	% Yield
	1	Hexane/EtOAc	50		6.4
	2	Hexane/EtOAc	50		
Fraction A	3	Hexane/EtOAc	50	0.16	
	4	Hexane/EtOAc	50		2.4
	5	Hexane/EtOAc	50		
	6	Hexane/EtOAc	50		
	7	EtOAc	100		
Fraction B	8	EtOAc	100	0.06	
	9	EtOAc	100		10.0
	10	EtOAc/CH_2Cl_2	50		
	11	EtOAc/CH_2Cl_2	50		
	12	EtOAc/CH_2Cl_2	50		
	13	CH_2Cl_2	100		
Fraction C	14	CH_2Cl_2	100	0.24	
	15	CH_2Cl_2	100		0.05
	16	CH_2Cl_2/MeOH	90		
	17	CH_2Cl_2/MeOH	90		
	18	CH_2Cl_2/MeOH	80		
	19	CH_2Cl_2/MeOH	80		
Fraction D	20	CH_2Cl_2/MeOH	80	0.0012	
	21	CH_2Cl_2/MeOH	80		13.2
Fraction E	22	CH_2Cl_2/MeOH	80	0.33	

	23	CH_2Cl_2/MeOH	80		
	24	CH_2Cl_2/MeOH	80		
	25	CH_2Cl_2/MeOH	80		
	26	CH_2Cl_2/MeOH	50		
	27	CH_2Cl_2/MeOH	50		
	28	CH_2Cl_2/MeOH	50		
	29	CH_2Cl_2/MeOH	30		
	30	CH_2Cl_2/MeOH	30		
	31	CH_2Cl_2/MeOH	30		
	32	CH_2Cl_2/MeOH	30		
	33	CH_2Cl_2/MeOH	30		48.0
	34	CH_2Cl_2/MeOH	30		
	35	MeOH	100		
	36	MeOH	100		
	37	MeOH	100		
Fraction F	38	MeOH/H_2O	50	1.2	

While collecting the fractions, the samples were spotted on pieces of silica gel TLC cards. Using appropriate mobile phases; 20:80, 80:20 Hex: EtOAc and EtOAc-H_2O-HCO_2H 18:1:1 the spots were developed (Figure 4.16).

Figure 4.16; TLC card VII; fractionation of *V. radiata* root by column chromatography

Comparing the number of fractions per spot and the pigmentation of the eluted spots per sample (Figure 4.16) above, the fractions were pooled into seven fractions. Then using the HPLC set at 270 nm, the samples were analyzed (Figure 4.17). The peaks in fraction F were isolated using the HPLC instrument.

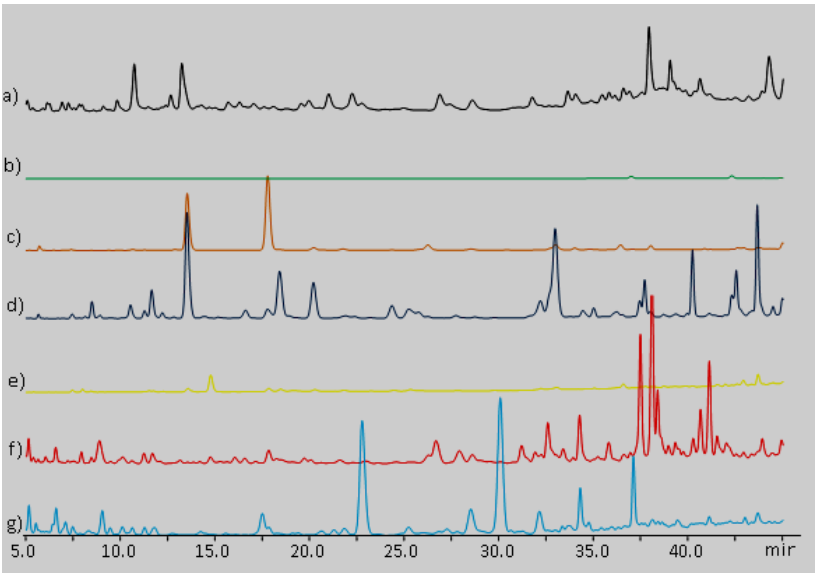


Figure 4.17; HPLC profiles of *V. radiata* root's fractions a) Crude sample; b) Fraction A; c) Fraction B; d) Fraction C; e) Fraction D; f) Fraction E; g) Fraction F

4.2.3.2 Characterization of isolates

Using a short method that lasts 30 minutes, eight peaks were each isolated from the roots of *V. radiata* (Table 4.10).

Using the LC-MS, TIC traces in positive mode at soft ionization and strong ionization conditions were scanned and appropriate pseudomolecular ions $[M+H]^+$ obtained. Results were tabulated in Table 4.10 below.

Table 4.10; HPLC, LC-MS and flavonoid test analysis of *V. radiata* root extracts

Fraction	Weight in (mg)	% of extract	RT (min)	UV λ_{max} (nm)	Molecular mass $[M]^+$	Purity	NH_3 test
VRR 1	1.64	0.05	5.1~5.5	227, 257, 324	-	Impure	-ve
VRR 2	1.50	0.05	9.8~10.2	226, 244, 272	-	Impure	-ve
VRR 3	2.90	0.09	10.5~10.9	230, 247, 295	322, 484, 623	Impure	-ve
VRR 4	1.70	0.05	12.8~13.2	229, 257	-	Impure	-ve
VRR 5	2.60	0.08	13.9~14.3	226, 250	-	Impure	-ve
VRR 6	1.70	0.05	16.4~16.8	219, 256, 282, 319	594	Pure	-ve
VRR 7	1.75	0.06	18.0~18.4	226, 314	146, 311	Impure	-ve
VRR 8	2.41	0.08	19.7~21.0	240, 299, 335	382, 461, 487, 562, 592	Impure	-ve

Analysis using the RP-HPLC revealed all the eight fractions; VRR 1, VRR. 2, VRR 3, VRR 4, VRR 5, VRR 6, VRR 7 and VRR 8 were of substantial concentration and gave characteristic single peaks at their respective retention times (figure 4.18). Implying they were all pure enough to be characterized using the LC-MS.

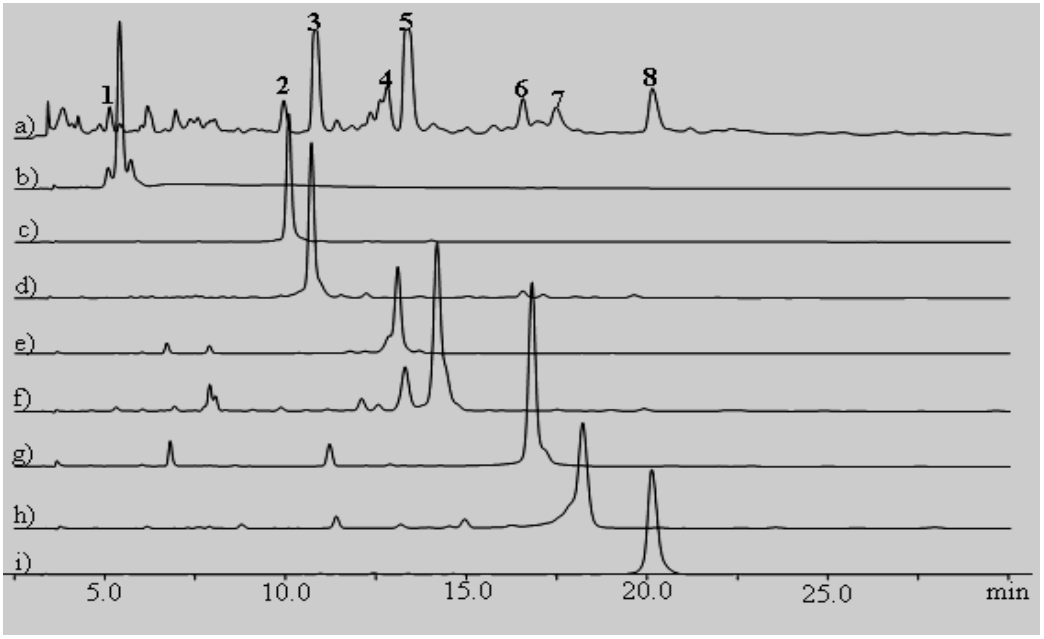


Figure 4.18; HPLC profile of isolated peaks from *V. radiata* roots' fraction F a) Fraction F; b) VRR 1; c) VRR 2; d) VRR 3; e) VRR 4; f) VRR 5; g) VRR 6; h) VRR 7; i) VRR 8

Though all these fractions recorded negative results on the ammonium test, VRR 6 had flavonoid agylcon based on the data base.

Using the LC-MS only VRR 6 TIC scans gave a single peak and had flavonoid agylcone based on the phenol-Explorer database, (Anonymous, 2012). It was the

only one of the fractions identified in this study. The rest of the isolates' TIC scans gave multiple peaks hence several molecular masses were observed for these fractions.

Though not identified, VRR 3 gave 3 peaks on the TIC scans of which, the peak that had a molecular mass of 484 portrayed characteristic C-Glucosylation traits. Fragmentation patterns revealed by the MS method (Figure 4.19) provided structural information about inter glycosidic linkages and aglycone substitution (Andersen, 2006). Usually, fragments 162 and 132 amu is obtained when the sugar is linked to the aglycone through an O-link. But a direct linkage to the aglycone gives characteristic fragmentation of 120 or 90 amu (Andersen, 2006). The integrity of the aglycone structure is preserved at the expense of the glycan for C-glucosides (Raymond *et al,* 2006) two fragmentations were observed on this sample's glycan.

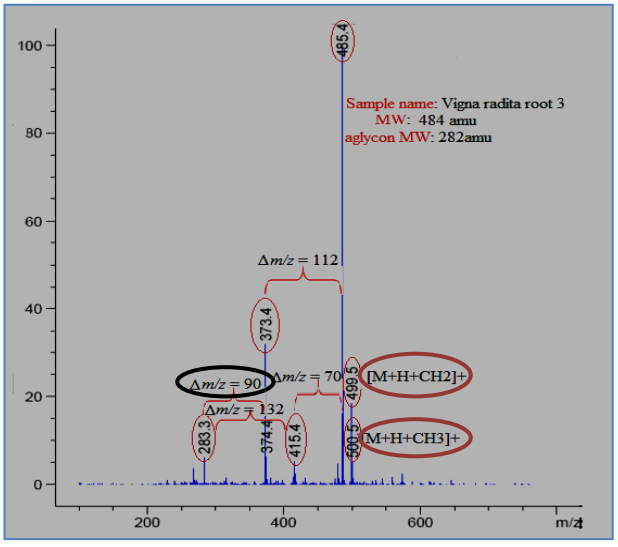


Figure 4.19; MS fragmentation of C-Glucosylated VRR 3

4.3 Identity of the compounds

Some of the peak elution times were consistent with that of a diglycoside available in the *icipe'*s (BCED) library. For this reason, MS scans were made up to 1415 mass/charge (*m/z*), because most diglycosides have an *m/z* of 431 to 700 and single moiety had between 269 and 317 *m/z*. Parent peaks were identified at 641, 627, 611, 610, 595, 449 and 431 *m/z* with daughter peaks at 317, 312, 303 287, 271 and 269 *m/z*. A subsequent direct injection MS-electron scan detected these peaks as well as several others in lesser quantities. These masses were compared with all combinations of known food flavonoid and glycosyl moieties.

Though eleven fractions were obtained from *C. arietinum* leaf (Figure 4.19 A), only the outlined seven compounds were identified from the plant (CAL 2, CAL 3, CAL 4, CAL 7, CAL 8, CAL 9 and CAL 11) (Figure 4.19 A).

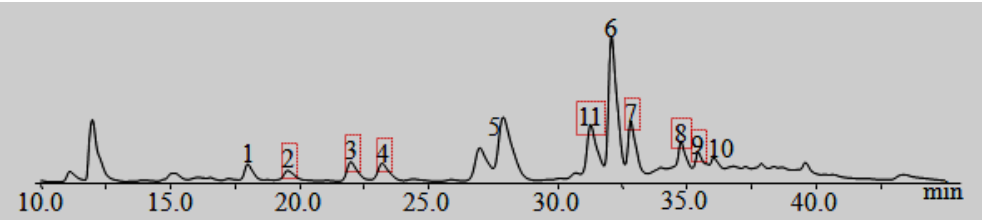


Figure 4.20 A; Isolated and identified compounds from *C. arietinum* Leaf

Six fractions were isolated from *C. arietinum* root (Figure 4.19 B). But only (CAR 5) outlined in (Figure 4.19 B) was identified in this study.

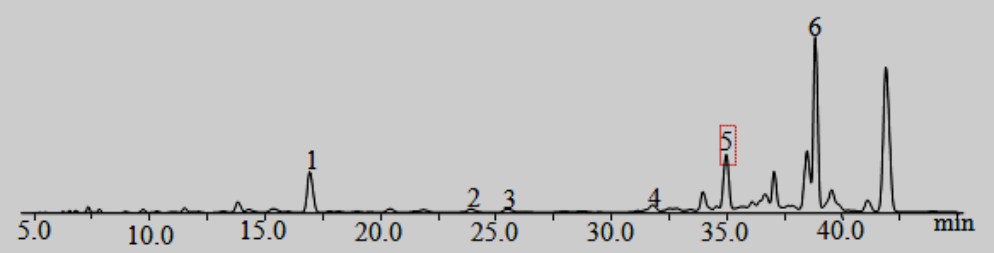


Figure 4.20 B; Isolated and identified compound from *C. arietinum* Root

CAR 5 (Figure 4.20 A), a white solid, had a retention time of 34.1-34.4 min on HPLC. It showed $[M + H]^+$ at *m/z* 431 and $[M + Na]^+$ at *m/z* 453, in the positive ion mass spectrum. Therefore, its molecular mass was inferred to be 430. Its UV spectrum exhibited maximum absorption at 254 nm and a shoulder peak at 300 nm with a weak band I (236 nm) and strong band II (254 nm). The positive MS spectrum of *m/z* 431 showed ions at *m/z* 269 indicating a loss of 162 amu suggesting the presence of a sugar molecule. In the phenol-Explorer database (Anonymous, 2012), Formononetin-7-*O*-glucoside had a molecular mass of 430 amu. Based on these data, CAR 5 was tentatively identified as Formononetin-7-*O*-glucoside.

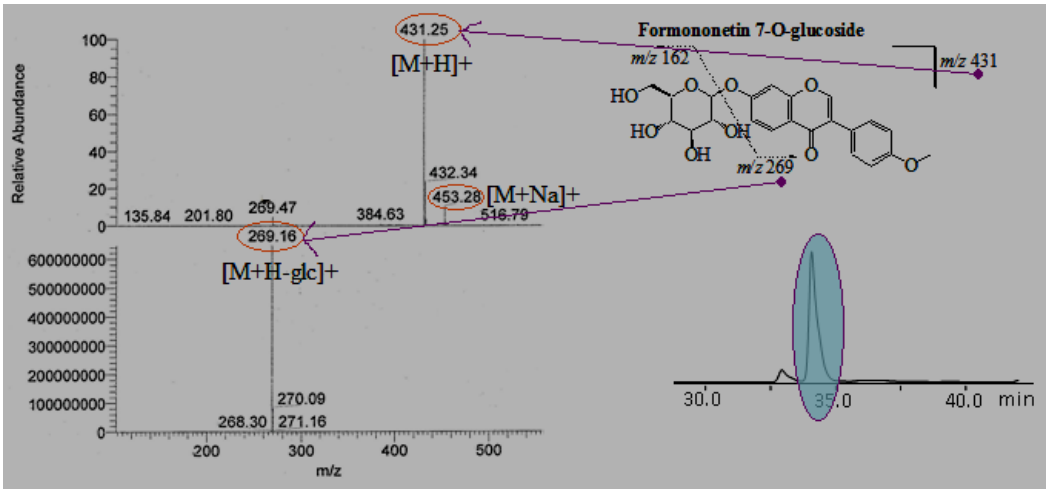


Figure 4.21 A; HPLC profile, LC-MS fragmentation and possible structure of CAL 5

CAL 2 (Figure 4.20 B) a yellow solid had a retention time of 19.4~19.7 min on HPLC. It showed $[M+Na]^+$ at *m/z* 663 and an intense *m/z* 700 ion in the normal mass spectra which dissociated to yield an *m/z* 641 in the ms2 spectra. The resulting 59 amu neutral loss showed the presence of an acetate $[M+H+CH_3COO^{\cdot}]^+$ radical ion (confirming *m/z* 641 as the molecular ion) further dissociation of the *m/z* 641 ion produced an intense *m/z* 479 in the ms3 spectra which indicates the presence of a terminal galactose sugar (due to the 162 amu neutral loss). An additional *m/z* 317 ion fragment was found in the ms3 ion spectra, revealing the presence of another terminal glucose sugar (due to the 162 amu neutral loss). The positive MS spectrum of *m/z* 641 gave fragmentation ions at *m/z* 503, 479 and 317.

Its UV spectrum exhibited maximum absorption at 255 and 352 nm, a shoulder peak at 222 nm, and minimum absorption at 245 nm. These data indicated that its'

first aglycon had a molecular mass of 316. From the precursor ion in the molecule, the presence of two sugar molecules of molecular weights 162 amu is indicated. Rhamnetin-3-O-galactoside-4'-O-glucoside had a molecular mass of 640 amu in the phenol-Explorer database (Anonymous, 2012). Based on these data, the combination of the various chromatographic and spectroscopic methods (Table 4.11) and comparison, CAL 2 was tentatively identified as Rhamnetin-3-O-galactoside-4'-O-glucoside.

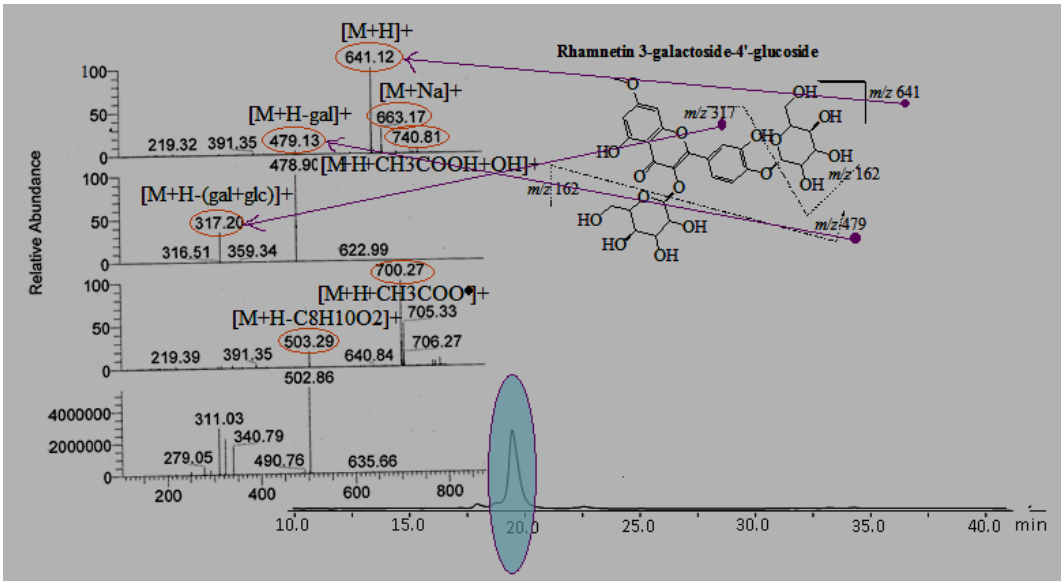


Figure 4.21 B; HPLC profile, LC-MS fragmentation and possible structure of CAL 2

CAL 3 (Figure 4.20 C); yellow solid had a retention time of 21.7~22.1 min on HPLC. It showed $[M+H]^+$ at *m/z* 627 and $[M + Na]^+$ at *m/z* 650 in the positive ion mass spectrum, indicating that its molecular mass was 626. Its UV spectrum

exhibited maximum absorption at 238, 258 and 353 nm, a shoulder peak at 225 nm, and minimum absorption at 246 nm.

The positive MS spectrum of *m/z* 627 gave fragmentation ions at *m/z* 465, 446 and 303. These data indicated that CAL 3 was a diglucoside, and the aglycon had a molecular mass of 302. In the phenol-Explorer database (Anonymous, 2012), Quercetin-3,7-O-diglucoside had a molecular mass of 626. Based on these data, the combination of the various chromatographic and spectroscopic methods (Table 4.11) and comparison CAL 3 was tentatively identified as Quercetin-3,7-O-diglucoside.

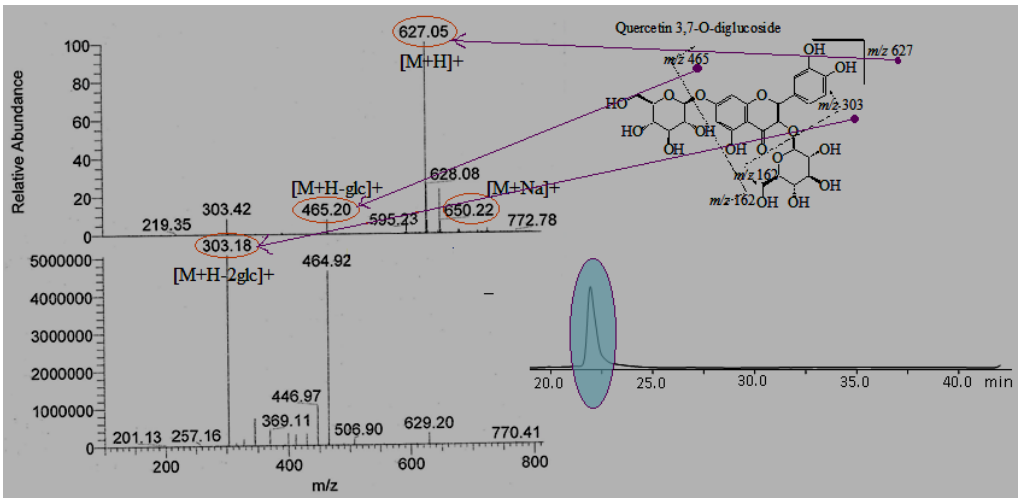


Figure 4.21 C; HPLC profile, LC-MS fragmentation and possible structure of CAL 3

CAL 4 (Figure 4.20 D); a yellow solid had a retention time of 22.9~23.2 min on HPLC. It showed $[M + H]^+$ at *m/z* 611 and $[M + Na]^+$ at *m/z* 633 in the positive ion mass spectrum, indicating that its molecular mass is 610. Its UV spectrum

exhibited maximum absorption at 265, 325 and 337 nm, a shoulder peak at 230 nm, and minimum absorption at 245 nm.

The positive MS spectrum of *m/z* 611 gave fragmentation ions at *m/z* 449, 430 and 287. These data indicated that CAL 4 had an aglycon of molecular mass of 286 and from the precursor ion the presence of a two sugar molecules of molecular weights 162 amu presents in the molecule. Sakuranetin-5,4'-O-diglucoside had a molecular mass of 610 on the phenol-Explorer database (Anonymous, 2012). Based on these data, the combination of the various chromatographic and spectroscopic methods (Table 4.11) and comparison CAL 4 was tentatively identified as Sakuranetin-5,4'-O-diglucoside.

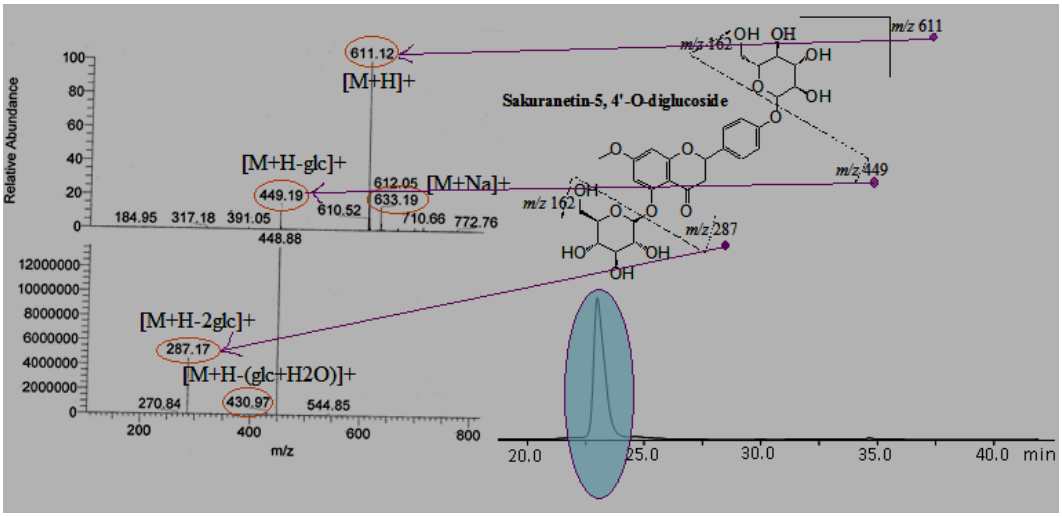


Figure 4.21 D; HPLC profile, LC-MS fragmentation and possible structure of CAL 4

CAL 7 (Figure 4.20 E), a yellow solid, had a retention time of 32.6~33.0 min on HPLC. It showed $[M+Na]^+$ at *m/z* 633, $[M]^+$ at *m/z* 610, [M+H]+ at *m/z* 611 and

$[M + 132]^+$ at *m/z* 742 in the positive ion mass spectrum. Therefore, its molecular mass was confirmed to be 610. Its UV spectrum exhibited maximum absorption at 255 and 352 nm, a shoulder peak at 238 nm, and minimum absorption at 245 nm, suggesting that it was an isomer of CAL 2.

The positive MS spectrum of *m/z* 611 showed fragment ions at *m/z*, 479, 461, 317 and 302; sequential losses of 132 amu, 132+H_2O amu and 162 amu indicated the presence of a pentosyl and a glycosyl moiety respectively. Most likely, the sugar molecules were each attached to the aglycon whose molecular mass is 316 amu. Based on these and data from the phenol-Explorer database (Anonymous, 2012), CAL 7 was tentatively identified as Isorhamnetin-3-O-xyloside-7-O-glucoside.

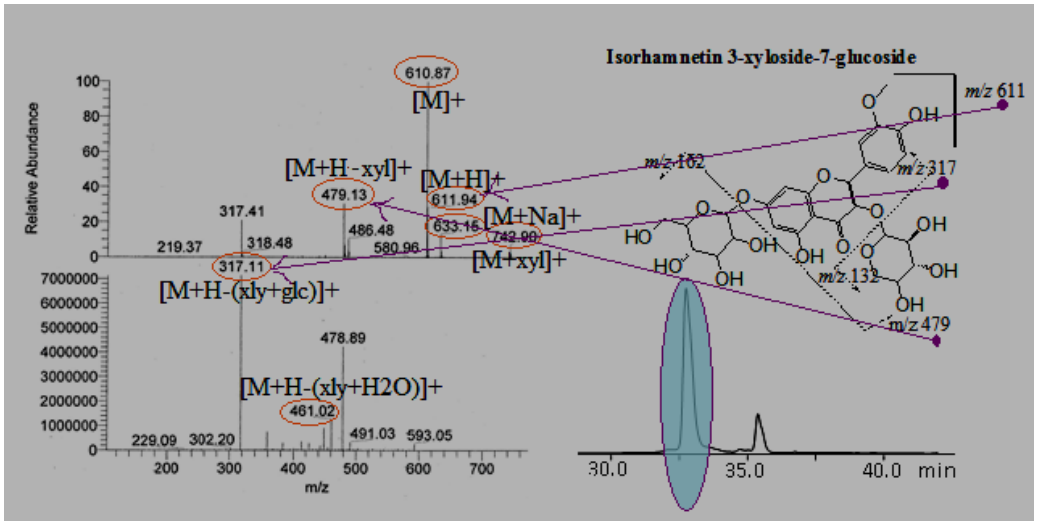


Figure 4.21 E; HPLC profile, LC-MS fragmentation and possible structure of CAL 7

CAL 8 (Figure 4.20 F); a yellow solid had a retention time of 34.6~35.0 min on HPLC. It showed $[M+Na]^+$ at *m/z* 471 and $[M+H]^+$ at *m/z* 449 in the positive ion mass spectrum. Therefore, its molecular mass was confirmed to be 448.

Its UV spectrum exhibited maximum absorption at 265 and 337 nm, a shoulder peak at 230 nm and minimum absorption at 246 nm, suggesting that it is an isomer of CAL 4. The positive MS spectrum of *m/z* 449 showed fragment ion at *m/z* 287; the loss of 162 amu indicated the presence of a glucosyl which was attached to the aglycon whose molecular mass is 286. Based on these data in reference to the phenol-Explorer database (Anonymous, 2012), CAL 8 was tentatively identified as Isosakuranetin-5-O-diglucoside.

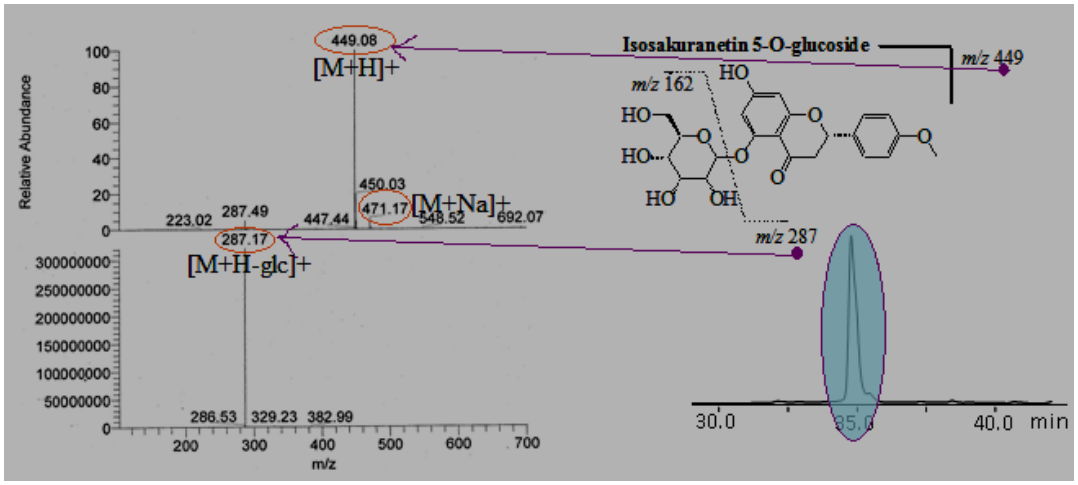


Figure 4.21 F; HPLC profile, LC-MS fragmentation and possible structure of CAL 8

CAL 9 (Figure 4.20 G); a yellow solid, had a retention time of 36.6~36.9 min on HPLC. It showed $[M +H+3CO]^+$ at *m/z* 533, $[M+H+CO]^+$ at *m/z* 476 and [M+H]+ at *m/z* 449 in the positive ion mass spectrum.

The molecular mass of the compound was confirmed to be 448. Its UV spectrum exhibited maximum absorption at 244, 264 and 348 nm, a shoulder peak at 220 nm, and minimum absorption at 245 nm suggesting that CAL 9 is neither an isomer of CAL 4 nor of CAL 8.

The positive MS spectrum of *m/z* 449 showed fragment ions at *m/z* 287. The loss of 162 amu indicates the presence of a glucosyl fragment and suggest that the glucosyl was attached to the aglycon whose molecular mass is 286. In the phenol-Explorer database on polyphenol content in foods (Anonymous, 2012), luteolin, kaempferol, scutellarein and fisetin have a molecular mass of 286. This compound is neither a luteolin nor scutelarein because it is an isomer or same compound as CAL 11. Based on these data; CAL 9 was tentatively identified as; either, fisetin-3-O-glucoside, Fisetin-7-O-glucoside, Fisetin-4'-O-glucoside Kaempferol-3-O-glucoside or Kaempferol-7-O-glucoside.

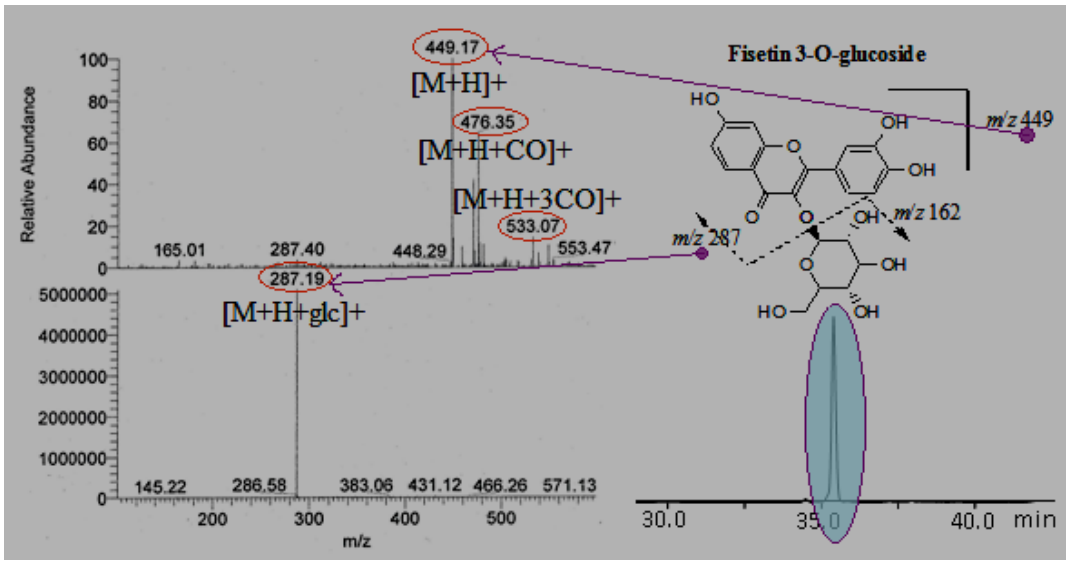


Figure 4.21 G; HPLC profile, LC-MS fragmentation and possible structure of CAL 9

CAL 11 (Figure 4.20 H); a yellow solid, had a retention time of 29.4~29.8 min on HPLC. It showed $[M+Na]^+$ at *m/z* 633 and $[M+H]^+$ at *m/z* 611 in the positive ionization mode. Therefore, its molecular mass was confirmed to be 610.

Its UV spectrum exhibited maximum absorption at 244 and 348 nm, a shoulder peak at 218 nm, and minimum absorption at 238 nm, suggesting that it was an isomer of CAL 9 but not an isomer of CAL 4. The positive MS spectrum of *m/z* 449 showed fragment ions at *m/z* 449 and 287; sequential losses of 162 amu, and 2 (162) amu indicated the presence of two sugar molecules suggesting that the two glucosyl were attached to the aglycon whose molecular mass is 286. Comparing with published data, O-diglucosides of scutellarein is yet to be reported in food. Based on these data in reference to the phenol-Explorer database (Anonymous,

2012), CAL 11 was tentatively identified as either, Fisetin 3,7-O-diglucoside or Kaempferol 3,7-O-diglucoside.

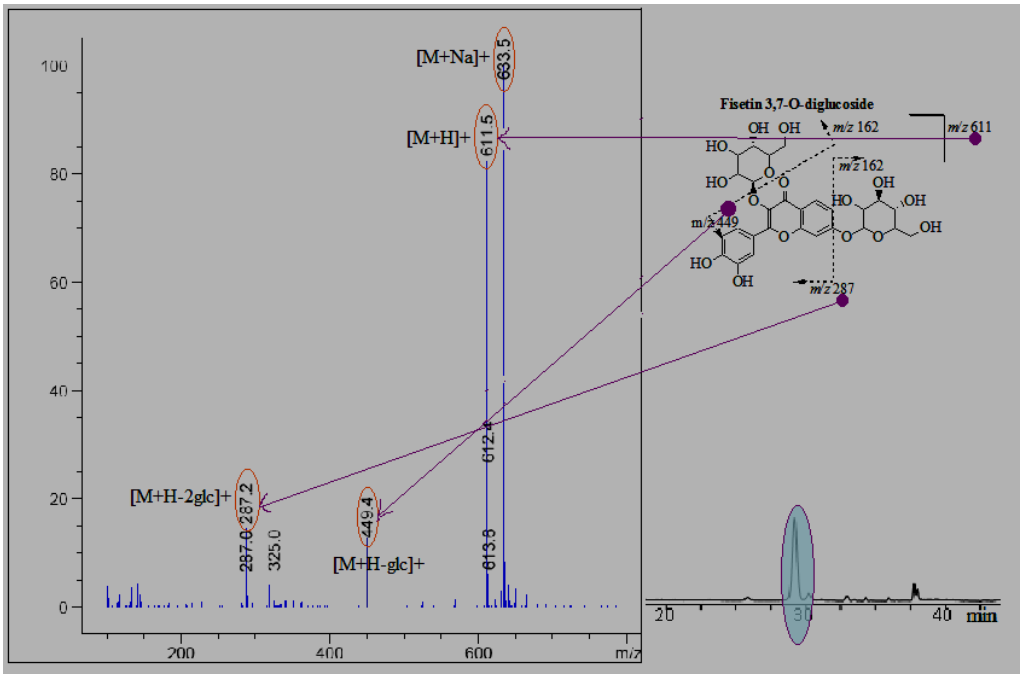


Figure 4.21 H; HPLC profile, LC-MS fragmentation and possible structure of CAL 11

Out of eight fractions isolated from *V. radiata* root (Figure 4.19 C) only (VRR. 6) outlined in (Figure 4.19 C) was identified in this study

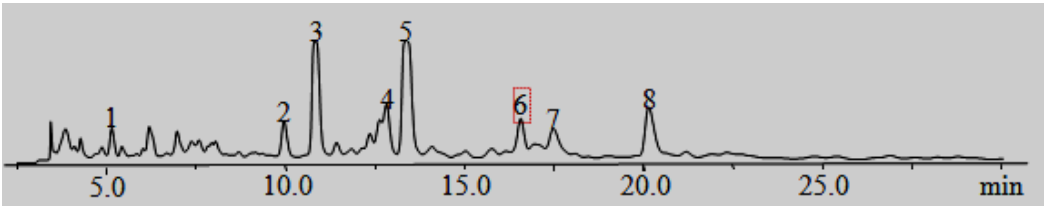


Figure 4.20 C; Isolated and identified compound from *V. radiata* root

VRR 6 (Figure 4.20 I); a red solid had a retention time of 15.1~15.4 min on HPLC. It showed $[M+Na]^+$ at *m/z* 617 and $[M+H]^+$ at *m/z* 595 in the positive ion mass spectrum. Therefore, its molecular mass was confirmed to be 594.

Its UV spectrum exhibited maximum absorption at 256, 282 and 319 nm, a shoulder peak at 219 nm, and minimum absorption at 245 nm. The positive MS spectrum of *m/z* 432 showed fragment ions at *m/z* 271, sequential losses of 162 amu. Indicating that two glucosyl were attached to the aglycon whose molecular mass is 270.

Based on these data and the phenol-Explorer database (Anonymous, 2012), Apigenin, Baicalein, Genistein and Galangin have molecular mass of 270. But comparison of the UV results of the compound with that of Apigenin and Genistein standards shows the aglycone of VRR 6 cannot be Apigenin nor Genistein. Also from the data base glucosides of Galangin are yet to be reported. Therefore, VRR 6 was tentatively identified as Baicalein-7-O-diglucoside.

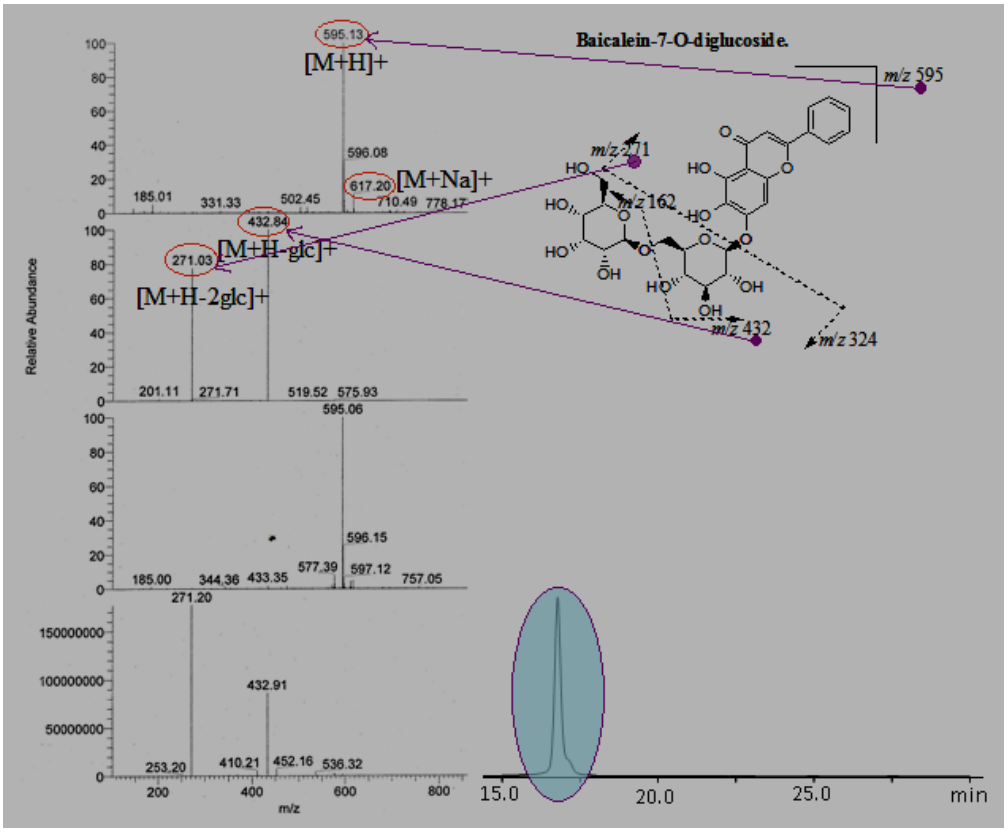


Figure 4.21 I; HPLC profile, LC-MS fragmentation and possible structure of VRR 6

4.4 Summary of Identified compounds

Finally, the combination of the various chromatographic and spectroscopic methods, and comparison with published data (Anonymous, 2012) allowed the tentatively identification several compounds as summarized in Table 4.11.

Table 4.11; Summary of the tentatively identified compounds

Compound/ Possible identification	**Retention Time (min)**	**UV Maximum absorbance (λ_{max}) (nm)**	**ESI-MS fragment ions (% relative intensity)**	**$[M]^+$**
CAR 5 Formononetin 7-O-glucoside	34.1~34.4	236, 254	*m/z* 453 $[M+Na]^+$ (10) and 431 $[M+H]^+$ (100). *m/z* 269 $[M+H-162]^+$ (<5)	430
CAL 2 Rhamnetin 3-O-galactoside-4'-O-glucoside	19.4~19.7	222, 238, 255, 352	*m/z* 740 $[M+CH_3COOH+OH]^+$ (<5) 663 $[M+Na]^+$ (30) and 641 $[M+H]^+$ (100). *m/z* 623 $[M+H-H_2O]^+$ (<5), 479 $[M+H-162]^+$ (<5), 317 $[M+H-2(162)]^+$ (<5)	640
CAL 3 Quercetin 3,7-O-diglucoside	21.7~22.1	225, 238, 258, 353	*m/z* 650 $[M+Na]^+$ (<10) and 627 $[M+H]^+$ (100). *m/z*, 465 $[M+H-162]^+$ (5), 446 $[M+H-(162+H_2O)]^+$ (1), 303 $[M+H-2(162)]^+$ (<5)	626
CAL 4 Sakuranetin-5, 4'-O-diglucoside	22.9~23.2	230, 265, 325, 337	*m/z* 633 $[M+Na]^+$ (15) and 611 $[M+H]^+$ (100). *m/z* 449 $[M+H-162]^+$ (15), 430 $[M+H-(162+H_2O)]^+$ (<2), 287 $[M+H-2(162)]^+$ (5)	610
CAL 7 Isorhamnetin 3-O-glucoside-7-O-xyloside	32.6~33.0	238, 255, 352	*m/z* 742 $[M+132]^+$ (10), 633 $[M+Na]^+$ (15), 611 $[M+H]^+$ (30) and 610 $[M]^+$ (100). *m/z* 479 $[M+H-132]^+$ (30), 461 $[M+H-(132+H_2O)]^+$ (12), 317 $[M+H-(132+162)]^+$ (20)	610
CAL 8 Isosakuranetin 5-O-glucoside	34.6~35.0	230, 265, 337	*m/z* 471 $[M+Na]^+$ (<10) and 449 $[M+H]^+$ (100). *m/z* 287 $[M+H-162]^+$ (5)	448
CAL 9 Fisetin 3-O-	36.6~36.9	220, 244, 264, 348	*m/z* 533 $[M+H+3CO]^+$ (15), 476 $[M+H+CO]^+$	448

glucoside			(65) and 449 $[M+H]^+$ (100). *m/z* 287 $[M+H-162]^+$ (<5)	
CAL 11 Fisetin 3,7-O-diglucoside or Kaempherol 3,7-O-diglucoside	29.4~29.8	218, 244, 348	*m/z* 633 $[M+Na]^+$ (100) and 611 $[M+H]^+$ (84). *m/z* 449 $[M+H-162]^+$ (13) and 287 $[M+H-2(162)]^+$ (15)	610
VRR 6 Baicalein-7-O-diglucoside	15.1~15.4	219, 256, 282, 319	*m/z* 710 $[M+Na+C_6H_5O^{\cdot}]^+$ (<5), 617 $[M+Na]^+$ (15) and 595 $[M+H]^+$ (100). *m/z* 432 $[M+H-163]^+$ (<5) and 271 $[M+H-2(162)]^+$ (<5)	594

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

The secondary metabolites isolated from the leaf of *C. arietinum* L. were eleven and those from the root tissues were six. Characterization of these metabolites using HPLC and LC-ESI-MS revealed that they are all O-glucosylated. It does not possess inhibitory chemistry and proposed biosynthesis by C-glucosylation (Pickett, 2011). The plant accumulates several unidentified secondary metabolites in its leaf and root since all the compounds characterized and identified in this study from the plant have never before been reported from the plant and the ones found in the leaf are not replicated in the root or vice versa.

Eight secondary metabolites were isolated from the root of *V. radiata* L. Out of these, four compounds were characterized by HPLC and LC-ESI-MS. One compound V R R 6 was identified as Baicelein-7-O-diglucoside.

Compound V R R 3 showed characteristic C-glucoside in MS fragmentation though not identified in this study. This implies that *V. radiata* possess inhibitory chemistry and proposed biosynthesis by C-glucosylation (Pickett, 2011).

5.2 Recommendations

The following recommendations are drawn from this study:

By allelopathy, *C. arietinum* may not be an effective system for controlling *Striga* since none of its isolates was characteristically C-glucosylated. But because the C-glycosyltransferase (CGT) enzyme may be present in *V. radiata* L. the plant could be both an effective intercropping food legume and system for controlling *Striga.*

All the flavonoids identified in this study have been reported for the first time in both the food legumes. Since the presence of flavonoids in food legume provide a sound epidemiological correction and lowers the risk of coronary heart diseases (Andersen, 2006) information obtained from this study may be beneficial for human diet and nutrition.

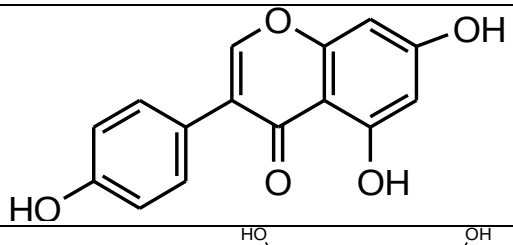
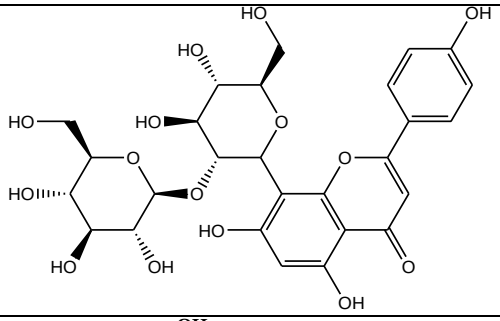
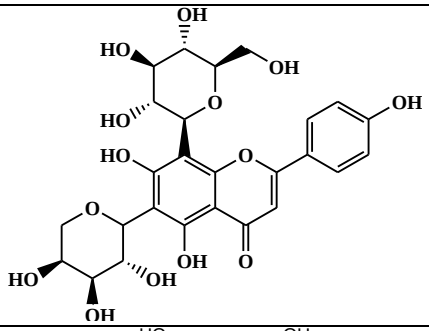
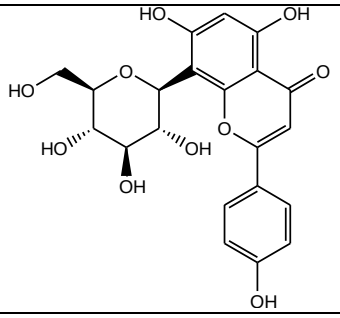
5.3 Areas of further study

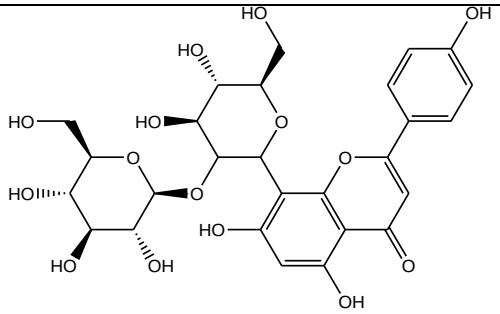
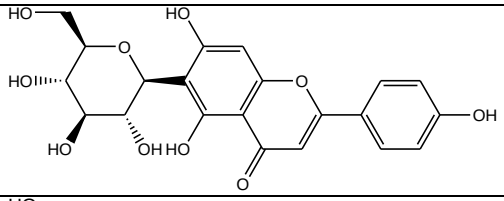
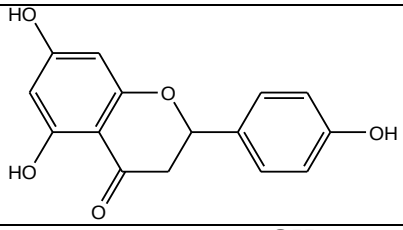
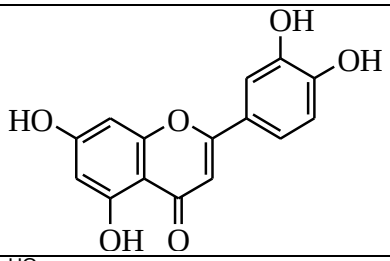
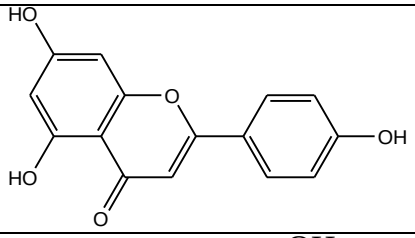
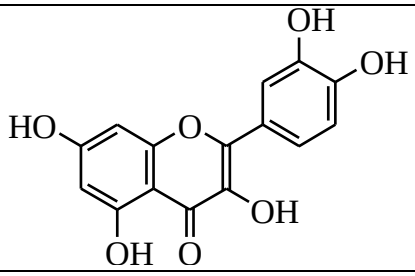
For future research:

i. The extracts from the *V. radiata* plant should be characterized further by NMR so as to reveal their exact identities.
ii. Bioassay of all the C-glucoside extracts from the plant should be done since other metabolism may also be involved in the *Striga*-Legume interaction.
iii. A field study to examine the effects of shade, nitrogen fixation and *V. radiata* on maize yield can also be done depending on the findings of (i & ii) above

APPENDIX A

Structures of the reference biophenols used in the study

Cording	Flavonoid standards	Structures
1	Genistein	
2	6- glucosylvitexin	
3	Isoschaftoside	
4	Vitexin	

5	2-glucosylvitexin	
6	Isovitexin	
7	Naringenin	
8	Luteolin	
9	Apigenin	
10	Quercetin Dihydrate	

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