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Characterization of flavonoids from candidate *striga* grass in controlling diet legumes *Cicer arietinum* and *Vigna radiata*

ANYANGO ANNA AKUMU[1], HUDSON NYAMBAKA[2,♥], SERGE PHILIBERT KUATE[3], BALDWYN TORTO[3]
[1]School of Pure and Applied Sciences of Kenyatta University. Nairobi, Kenya
[2]Department of Chemistry, Kenyatta University. Nairobi, Kenya.♥email:hudsonyambaka@yahoo.co.uk
[3]Department of Behavioural and Chemical Ecology, International Center of Insect Physiology and Ecology. Nairobi, Kenya

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Abstract. *Akumu AA, Nyambaka H, Kuate SP, Torto B.2018. Characterization of flavonoids from candidate* striga *grass in controlling diet legumes* Cicer arietinum *and* Vigna radiata*. Biofarmasi J Nat Prod Biochem 16: 83-98.* In Africa, intercropping of some legumes with cereal plants has been noticed to generate a remarkable reduction of *Striga* invasion and enhanced cereal crop production. *Desmodium uncinatum* administers *Striga* invasion in intercrop with cereals through an allelopathic technique, including post-sprouting accretion inhibitors emanating from the roots. Having the potential to be a new technique for grass administering, Allelopathy is a natural and environmentally friendly method. A standardized profiling technique based on Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Liquid Chromatography coupled with Mass Spectrometry (LC/MS) was utilized to determine flavonoids in extracts of chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*), potential trap plants for *Striga* grass. Nine flavonoids involving mono-and diglycosyl derivatives of fisetin, baicalein, rhamnetin, isorhamnetin, formononetin, quercetin, isosakuranetin, and sakuranetin were temporarily distinguished. The obtained phenolics were available at concentrations higher than 0.001% of the dry materials. Many of these phenolic mixtures have been claimed to possess human health advantages. These glycosylated flavones are noted for the first time in these two species. The comprehensive analysis of the polar secondary metabolites in these leguminous plants was useful for comprehending their inhibitory chemistry and proposed biosynthesis by C-glucoside characteristics. All the segregated and described mixtures in the diet legume *C. arietinum* were O-glucosylated. At the same time, Food legume *V. radiata* should be observed further to see if it can attain *Desmodium's* allelopathic ability since it possesses inhibitory chemistry and proposed biosynthesis by C-glucosylation.

Keywords: *Cicer arietinum*, control, flavonoids, legumes, Striga, *Vigna radiata*

INTRODUCTION

Striga is one of the most significant pests that affect food production in the tropics (Esilaba 2006). The grass problem is aggravated by its reproductive capability; a single plant can deliver over 50,000 grains, which can still be practicable in the soil for 15-20 years (Kureh et al. 2003). The parasite is a serious pest that primarily impends maize production in Kenya, resulting in harvest losses of between 65 to100%. Degradation in soil fertility is one of the principal reasons for the increment in *Striga* incidence (Esilaba 2006). *Striga hermonthica* (Del.) Benth is most common on heavy soil, particularly in the densely populated parts of the Lake Victoria region of western Kenya; simultaneously, *S. asiatica* is noticed in the coast province and seriously damages upland rice (Esilaba 2006).

Research efforts have distinguished several administering options that effectively reduce *Striga* damage and emergence. These include host plant resistance, utilization of trap plants, 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, because of ecological concerns, environmental health problems, and an increment in herbicide-resistant grass resulting from synthetic herbicides, considerable efforts in designing grass administering strategies of utilizing allelopathic mixtures as bioherbicides is considerable subdue grass is receiving examination (Zaji 2011).

Trap plants are non-host plants that induce Striga grains sprouting but are not parasitized by the grass (Kureh et al., 2003). They could generate suicidal grass sprouting, which decreases the soil's grain bank or attachment to the host when intercropped in maize (Othira et al. 2008). Some legume diversities, for example, chickpea, mung beans, cowpea (*Vigna unguiculata* L.), groundnut (*Arachis hypogaea* L.), and soybeans **(***Glycine max* Merrr.), possess the potential to generate suicidal sprouting of *S. hermonthica,* decrease attachment to host and increase the fertility of the soil (Kureh et al. 2003) by adding to the soil with nitrogen and organic matter.

The life cycle of *Striga* ssp. consists of five stages; sprouting, haustorium initiation, penetration of host tissue, physiological compatibility, and parasite accretion and maturation. Apart from normal grain sprouting requisites, the grass needs a chemical stimulation for sprouting and a second chemical signal to trigger haustorium formation, connecting *Striga* roots to its host for fount procuration (Othira et al. 2008). However, trap plants release chemicals that induce *Striga* ssp. Grain sprouting does not produce haustorium signals, nor are they attacked by the parasite (Othira et al. 2008).

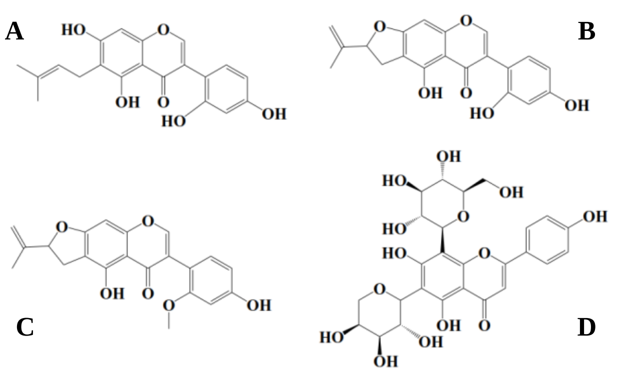

Figure 1. Segregated mixtures from *D. uncinatum* I.5,7, 2',4'-tetrahydroxy-6-(3-methylbut-2-enyl) isoflavanone (**A**), 4", 5"-dihydro-5,2',4'-trihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavone (**B**), 4",5"-dihydro-2"-methoxy-5,4'-dihydroxy-5"-isopropenylfurano-(2",3",7,6)-isoflavone (**C**) and isochaftoside (**D**)

Even though the witch-grass, *Striga* ssp., is competent for photosynthesis once it grows, it depends on the host plant for an important portion of its carbon provision. Beyond the encumbrance of dispossessing diet and water to these parasites, host plants undergo a characteristic malady resembling severe drought symptoms, involving leaf scorching and increased root: shoot ratios as if a hex had befallen the crop the result of hormonal perturbation or toxic produced by the parasite. Invasion is generally much less severe, where water and soil fertility are optimal for crop accretion (Rich and Ejeta 2008).

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

Tsanuo et al. (2003) and Hooper et al. (2010) segregated 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**) from the root exudates of *D. uncinatum*. Segregated fractions comprising mixture B induced sprouting of *Striga* grains, while fractions comprising mixture C impeded radical accretion. Pickett et al. (2007) and Hooper et al. (2009) described another key haustorium accretion impeder, di-C-glycosylflavone-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranosylapigenin, which is also known as isoschaftoside (**D**) from a polar fraction of *D. unicinatum* root exudates (Khan et al. 2010) (Figure 1).

The active components in *desmodium* root exudates described are suspected of having existed in traditional diet legumes. If resemblant characteristics were noticed in the diet legumes, then conventional breeding could be utilized to pick out and increase the characteristics. On the other hand, if these characteristics were lacking, a gene could be transferred directly from *Desmodium* into diet legumes via a genetic modification to allow legumes to subdue *Striga* spp. In the long term, it is likely to transfer the same characteristic to cereal plants through heterologous gene expression, especially to the open-pollinated diversities, rather than hybrids for more convenient availability to little scale farmers (Pickett et al. 2010).

The goals of this research were (i) to fractionate and segregate the secondary metabolites from leaf and root tissues of *C. arietinum* and the root of *V. radiata* extracts, utilizing column chromatography, TLC, and RP-HPLC; (ii) to describe the segregated mixtures from *C. arietinum* and *V. radiata* utilizing RP-HPLC and LC-MS.

MATERIALS AND METHODS

Study design

This was an experimental design in which chemical mixtures in aqueous extracts of leaves and roots of the legumes *C. arietinum* and *V. radiata* were segregated utilizing column chromatography and RP-HPLC and then described utilizing the RP-HPLC and LC-MS.

Samples and sampling procedure

Certified grains of *Cicer arietinum* L. (Desi (SCP 2), Kabuli (SCP 1) types), and *Vigna radiata* L. (KS 20) was gained from Simlaw Seeds Co., Nairobi, Kenya. The grains were cultivated in July 2011 at the Duduville campus of ICIPE, Nairobi. The plants were uprooted after 45 days to gain leaves and roots. The samples were cleaned in streaming water and then dried by air in a ventilated hood in the lab to wipe the moisture before extraction.

Chemicals and reagents

All the analytical reference mixtures involving isoschaftoside, 2-glucosylvitexin, 6-glucosylvitexin-, isovitexin, vitexin, naringenin, and apigenin were of analytical grade. Other utilized analytical chemical reagents were naturstoffreagenz A, normal silica gel, C_{18} silica gel, silica gel/TLC-plates, and silica gel C_{18} TLC-plates. While organic solvents, namely; methanol, ethyl acetate, formic acid, sulphuric acid, hexane, and butanol, were all gained from Sigma Aldrich, USA.

Instrumentation

A high-performance liquid chromatography (HPLC) comprising a quaternary pump, column compartment, auto-sampler, and variable wavelength photodiode array detector (Data system, version 1.24 ACE, Shimadzu Corporation England) was utilized. 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) were utilized on the process of analysis and separation. The mobile phase comprised formic acid in water (solvent **A**) and methanol (solvent **B**). A seven-step gradient analysis for a total run time of 65 min was utilized as follows: beginning with 95% solvent A and 5% solvent **B**, enhancing 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 kept at 20ºC utilizing 10-50µl injection volumes. The analytes were observed with PDA detection at between 225-350 nm.

The Liquid Chromatography-Mass Spectrometer utilized a quaternary LC pump (Model 1200) coupled to an Agilent MSD 6120-Single quadrupole mass spectrometer with an electrospray source. 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) were applied in the analysis. The mobile phase was solvent **A**: 1% formic acid in H_2O and solvent **B**: 1% formic acid in MeOH. Analysis conditions, including a three-step isocratic analysis for a total run time of 29.91 min, were utilized as follows; beginning with 75% solvent **A** and 25% solvent **B**, enhancing 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 needed in full-scan positive-ion mode utilizing a 100 to 800 *m/z* scan range.

Nine seemingly uncontaminated samples making concentrations higher than 0.001% in the plants were examined by LC-MS to ascertain their respective masses. LC-MS data were output utilizing 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) completed with an ion spray fount (ISV=5500, orifice voltage=50) in positive ion mode. Multiple reactions observing mode was utilized, observing 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 utilized as the collision gas, and nitrogen was utilized as the nebulizer gas and orifice curtain. The system was completed with a Synergi 4µ Hydro-RP 80 Å (250×2 mm) column fitted with a 4.0× 3.0 mm i.d. guard column (Phenomenex) at a flow rate of 1ml/min. The mobile phase was Solvent **A**: 0.1% formic acid in H_2O and Solvent **B**: 0.1% formic acid in MeOH. The analysis included a five-step gradient analysis for a total run time of 25 min and was utilized as follows; beginning with 90% solvent **A** and 10% solvent **B**, enhancing to 50% solvent **B** over 15 min, then 80% solvent **B** over 5 min to 95% solvent **B** over 2min and finally isocratic for 3 min. The injection volume was 20 µl, and the scanning wavelength ranged from 190-800nm. Both positive and negative ions between *m/z* 100 and *m/z* 1415 were monitored.

Laboratory procedures

Extraction

Utilizing 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 40º C for 48 hours. Each plant's brittle, dry material was ground in Retsch (GmbH 5667 HAAN West-Germany) grinder, weighed (Table 1), then distilled 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 the residue, then the residual extraction was repeated twice utilizing the same solvent system. The filtrates were evaporated *in vacuo* and weighed. Each distillate was examined utilizing TLC and HPLC and then purified utilizing HPLC.

Fractionation and isolation

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

Other solvent systems were examined in a bid to build up a column chromatography system to fractionate the constituents present in the distillate, and the mobile phase representing a better separation was picked out. The purifications of fractions were further performed by high-performance liquid chromatography (HPLC).

Preliminary experiments

Fingerprint chromatographic profiles of the two types of *C. arietinum*, Kabuli and Desi Chickpeas, were examined utilizing TLC plates and HPLC instruments. A comparison of the biochemical composition of aqueous extracts in the roots and leaves of the two plants was carried out based on the variation in concentration and existence or absence of secondary metabolites. Also, HPLC fingerprint profiles of *V. radiata* leaves and roots were gained.

A medium normal phase column of *C. arietinum* leaves was eluted utilizing other solvent systems of various concentrations and polarity from hexane, EtOAc, CH_2Cl_2, MeOH then Water to ascertain an appropriate solvent system for extracting polar mixtures from the legumes. TLC and HPLC examined the results. The solvent system at which polar secondary metabolites were eluted at substantial concentration was recommended for utilization in extraction.

On the other hand, the extraction of *C. arietinum* from leaves was performed by suspending the leaves in water and partitioning them with hexane, ethyl acetate, then butanol. Each distillate was examined utilizing TLC and HPLC to determine the best solvent giving a better separation of the mixtures, and utilized to assess the proportional quantities of various elements in the extracts.

Description of segregated mixtures

Isolates from each plant part were given codes in the order of their elution time/retention time. For example, CAL 1 is the first segregated peak from the crude distillate of *C. arietinum* leaf. The first two letters are the abbreviation of the plant's botanical name, and the third letter is the abbreviation of the part of the plant from which the segregation was gained.

The segregated mixtures were described utilizing the HPLC by analyzing the peak fineness of the segregated peaks, comparing their UV maximum wavelength absorbance with standards and one to another, and utilizing retention time proportional to that of known standards. LC-MS analysis was utilized to gain molecular ions and characteristic fragments utilizing MS data of the legumes' pure extractable.

A chemical test for flavonoid glycosides was carried out utilizing filter papers dipped in an ethanol solution of the individual mixture and then exposed to ammonia vapor. The formation of yellow spots on filter paper showed the existence of flavonoid glycoside (Ahmed 2007).

Utilizing the HPLC, the segregated mixtures' retention times and UV λ_{max} were compared with known flavonoid glycosides to determine their nature at a LOD of 0.25 µg/ L compared to the chemical tests for flavonoid glycoside with a higher LOD.

Spectrophotometric description

The initial spectral scan of phenolics from nine samples of the leaves and roots of *C. arietinum* and *V. radiata* was carried out by combining the data from PDA and MS to permit easy and efficient identification. Three screening schemes were utilized as follows:

Firstly, where reference mixtures were accessible as standards, the existence or absence of a particular biophenol was appraised by comparing retention time and UV–visible spectra with that of the reference. Where the retention times were noticed to be alike, co-injection and comparison of the UV-visible spectra were carried out. Molecular mass data further verified the existence.

Secondly, when reference mixtures were not accessible, the TIC traces in positive mode at soft ionization and strong ionization conditions were monitored for relevant, suitable pseudomolecular ions $[M+H]^+$. Verification was carried out by analyzing UV–visible spectra and mass spectral fragmentation data at the expected elution time, depending on pre-identified mixtures in the sample, the structure of the target mixture, and the literature data.

Thirdly, major peaks in TIC and UV chromatograms that were not distinguished by the other screening processes were screened for novel mixtures by generating mass spectra. In all, screening was performed for nine biophenols from different classes. The principal objective was to show the power of mixed utilization of HPLC–PDA and ESI-MS in profiling legume extracts.

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

RESULTS AND DISCUSSION

Method validation and preliminary results

Sample harvest

The ground leaves and roots of the *C. arietinum* and *V. radiata* plants were weighed and noted. Further, the parts of plants were distilled utilizing 50/50 MeOH/CH_2Cl_2 and evaporated *in vacuo*, then noted (Table 1).

The harvest was not optimal for *V. radiata* because of the attack of cat worms on the plantation. The distillate weights above were utilized to ascertain the capacity and amount of packing material used in column chromatography.

Fingerprints

Two different chickpea grains were grown separately on the field; the plantations were similar in the first 30 days. But on flowering, the Desi (SCP 1) chickpea owned purple flowers, while Kabuli (SCP 2) chickpeas had white flowers. Figure 2 shows TLC and HPLC chromatograms of the other parts of the plants.

Table 1. Harvest of the crude sample and extracts

Sample name	Powder (grams)	Distillate (grams)	% harvest
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

Figure 2.A. TLC card I, comparison of composition in *Cicer arietinum* leaves and roots (SCP 1 & SCP2)

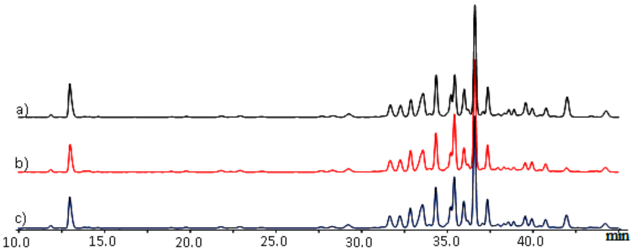

Figure 2.B. HPLC *Cicer arietinum* leaves profiles of a) Kabuli (SCP 2);b) Desi (SCP1); c) Mixture

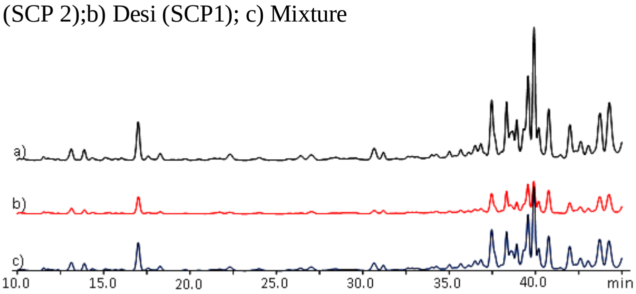

Figure 2.C. HPLC *Cicer arietinum* roots profiles of; a) Kabuli (SCP 2); b) Desi (SCP1); c) mixture

There were varieties in the amounts of mixtures found in the two plants, similarity in the TLC retention factors of eluted mixtures, and fingerprint HPLC profiles between the two types of plants showed the same aqueous chemical elements. Therefore, the resembling plant parts were mingled and examined as leaves and roots of *C. arietinum.*

Likewise, to view the aqueous secondary metabolites found in the *V. radiata* plant, see the HPLC profile of leaves and roots at 270 nm and 350 nm absorbance below (Figures 3.A and B).

Obviously, few are the polar mixtures in substantial amounts in the two parts of the plant; *V. radiata*. Even though it was likely to segregate the present mixtures utilizing the HPLC, the process was complicated because the plant possesses a raged profile (especially the root profile; the 270 nm profile is very dissimilar from the 350 nm one) mixture is in a very small amount.

Solvent system

Other solvent systems were examined for extraction to ensure maximum collection of the polar secondary metabolite from the plants. A 9.6 g portion of *C. arietinum* leaves had been distilled utilizing 80/20 MeOH/H_2O. 1 g of the distillate was suspended in water and partitioned with hexane, ethyl acetate, and butanol. The weights of fractions were noted (Table 2).

Alongside the crude sample, the separation in four fractions was examined on silica gel C_{18} TLC-plate (Figures 4.A), mobile phase; EtOAc-H_2O-HCO_2H 18:1:1. Further, HPLC chromatograms (Figure 4.B) were gained.

Likewise, 1.4 g of *C. arietinum* leaves distillate was liquefied in 50/50 MeOH/CH_2Cl_2. The soluble part of the sample (1.0 g) was fixed in 2 g of silica gel. Utilizing a 1/30 sample: silica gel ratio, a 42.3 g capacity column was packed with 31.0 g silica gel liquefied in hexane. The mobile phase varied from hexane, ethyl acetate, dichloromethane, and methanol to water. 30 fractions of 20 ml each were gained. A TLC C_{18} card was spotted after fractionation and utilizing the mobile phase; EtOAc-H_2O-HCO_2H 18:1:1 spots were developed (Figure 5.A). Eight fractions were pooled as shown on the TLC card below and then examined utilizing the HPLC (Figure 5.B).

These experiments found no separation of mixtures utilizing neither fractionating funnel nor normal phase column chromatography. From the hexane fraction, no polar mixtures were noticed. While in the butanol and water fractions, all the mixtures existing in the crude sample were found at low amounts. Ethyl acetate yielded an enhanced concentration of some mixtures existing in the original sample.

From the column chromatography, it was examined that methanol dichloromethane solvent systems liquefy most polar mixtures. Therefore, extraction of the samples was carried out utilizing 50/50 MeOH/CH_2Cl_2, and column chromatography was utilized for fractionation and purification of the samples. It was suggested that before utilizing the HPLC for the segregation of peaks, column chromatography should be carried out at least twice for every sample for purification, enhanced amounts of present mixtures, and higher resolution.

Spectroscopic study of reference biophenols

A series of flavonoid standards (Figure 6), representative of other flavonoid groups previously reported in allelopathic *D. uncinatum* and other legumes, utilizing HPLC-PDA and RP-LC-ESI-MS, were observed to determine their structure/spectra. From HPLC-PAD, various correlations between structure and UV spectrum were distilled (Table 3).

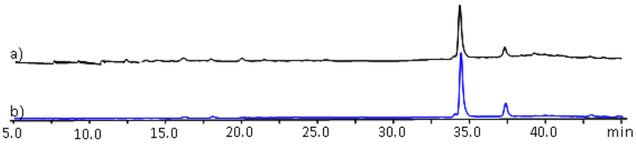

Figure 3.A. HPLC profiles of V. radiata leaves at a) at 270 nm absorbance, b) at 350 nm absorbance.

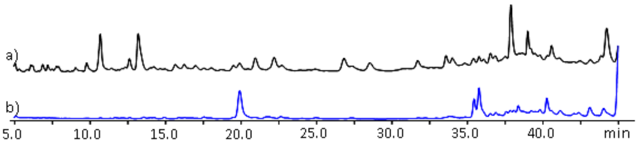

Figure 3.B. HPLC profiles of V. radiata roots at a) at 270 nm absorbance, b) at 350 nm absorbance.

Table 2. Weights of 1g *Cicer arietinum* 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

Figure 4.A. TLC card II, *C. arietinum* leaf fractionation utilizing a fractionating funnel

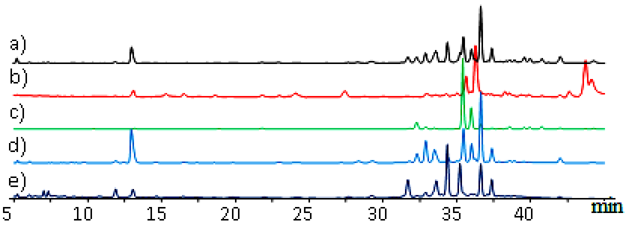

Figure 4.B. Fractionating funnel's HPLC profile at 270 nm of *Cicer arietinum* leaf a) Crude sample; b) Hexane fraction; c) Ethyl acetate fraction; d) Butanol fraction; e) Water fraction.

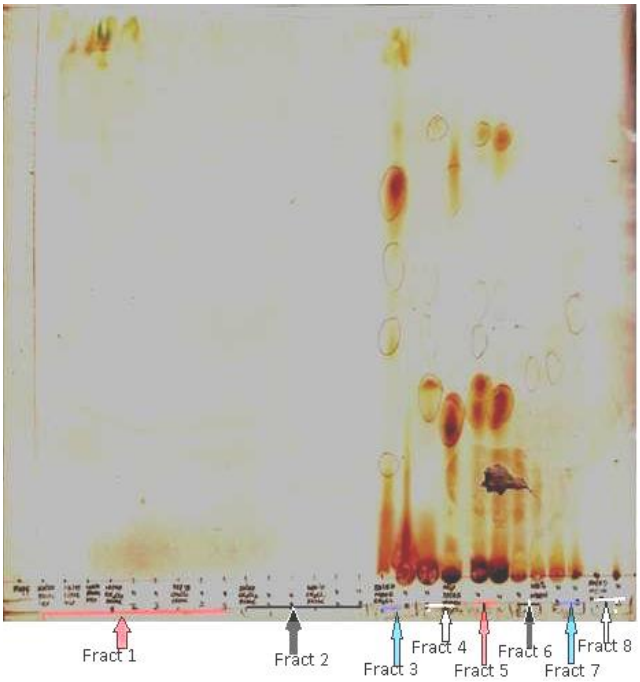

Figure 5.A. TLC card III, Preliminary column chromatography fractionation of *Cicer arietinum* leaf

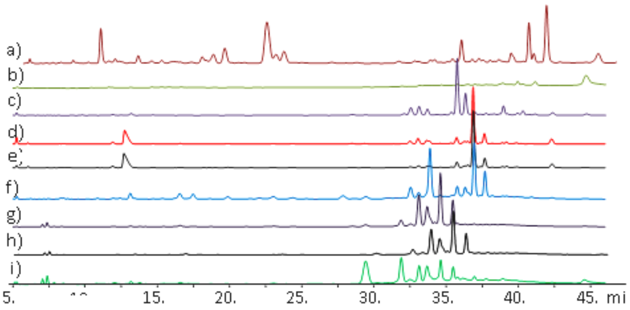

Figure 5.B. Preliminary analysis utilizing HPLC profiles at 270 nm of *Cicer arietinum* leaves fractions a) Crude sample; b) Fraction 1 c) Fraction 2; d) Fraction 3; e) Fraction 4; f) Fraction 5; g) Fraction 6; h) Fraction 7; Fraction 8

Table 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

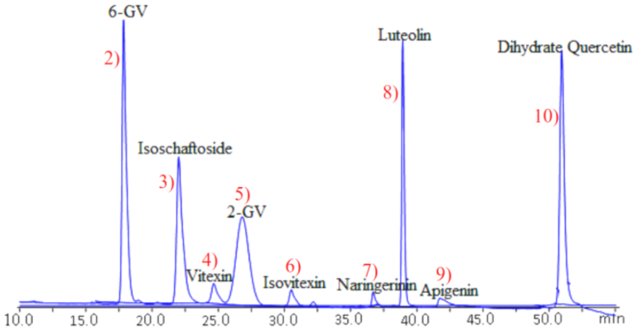

Figure 6. A simultaneous HPLC chromatogram was obtained with a photodiode array detector set at 350nm (A); Peak identification was listed in Table 3 (B)

The most influential flavonoids are the λ_{max} (a band I), and it increases in the series: flavones (apigenin) < flavonol (quercetin). The λ_{max} also indicated the substitution within the same class for glucosylvitexins, vitexin, and isovitexin. The permeation λ_{max} was not affected in isomers of the same group (e.g., vitexin and isovitexin) Table 3.

Fractions and isolates of secondary metabolites

Cicer arietinum roots

Column chromatography I. Flash fractionation column chromatography was utilized to fractionate *C. arietinum* roots (Table 4). The fractions were examined by HPLC (Figure 7) obtained with a photodiode array detector set at 270 nm.

No polar mixtures were examined using mobile phases (d, e, f, and g). The separation was poor in fraction (h), where the mixtures were not concentrated. Still, where the concentration was better, isolation of individual peaks would be complicated because the mixtures were very close to each other. In fractions (I, j, k, and l), the clarity and concentration of the mixtures were good. Therefore, it was suggested that a smaller column comprising a mixture of the four fractions should be mounted to purify the fractions further.

Table 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		

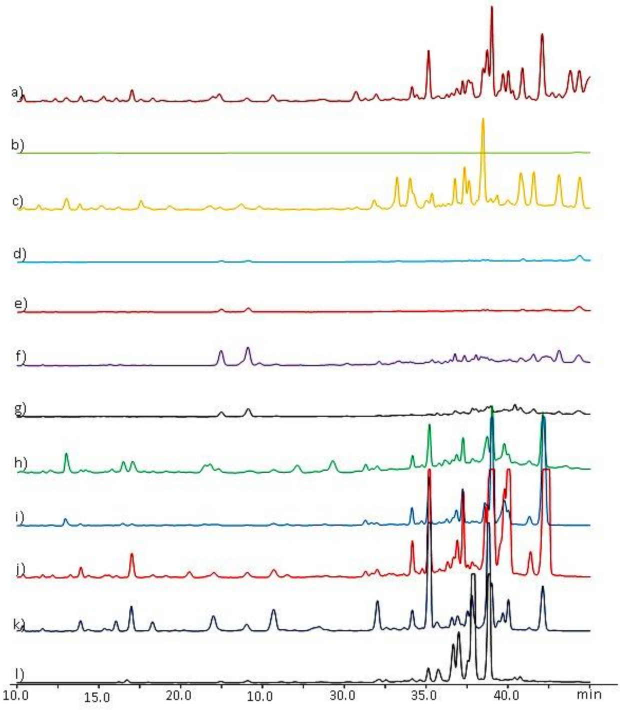

Figure 7. HPLC profiles of *C. arietinum* roots fractions from 1st column. a) Crude sample; b) 100% EtOAc-0; c) 100% EtOAc-1; d) EtOAc/CH_2Cl_2 75/25; e) EtOAc/CH_2Cl_2 50/50; f) EtOAc/CH_2Cl_2 25/75; g) 100%CH_2Cl_2;h) MeOH/CH_2Cl_2 25/75; i) MeOH/CH_2Cl_2 50/50; j) MeOH/CH_2Cl_2 80/20;k) MeOH/CH_2Cl_2 90/10; l) MeOH/CH_2Cl_2 95/5

Column chromatography II. A second 600 ml column was packed, and 4 fractions each of 20 ml were eluted utilizing 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 8). The spots were developed utilizing a mobile phase comprising EtOAc-H_2O-HCO_2H (18:1:1). Further HPLC profiles were obtained with a photodiode array detector set at 270 nm (Figure 9) of 6 fractions pooled as shown on the TLC card were gained on the PDA detector. The better resolution was examined in fractions d), e), and f). Utilizing HPLC, well-resolved peaks of substantial concentration in the fractions were suggested for isolation.

Description of isolates. Utilizing an HPLC on a PDA detector at 270 nm and a preparative RP-C_{18} column (10 mm i.d. × 250 cm, 5 µm particle size), six fractions were gained, and weights were noted (Table 5). HPLC profiles on the analytical column were obtained with a photodiode array detector set at 270 nm (Figure 10).

Utilizing the LC-MS, TIC traces in positive mode at soft ionization and strong ionization conditions were monitored, and relevant pseudomolecular ions $[M+H]^+$ were gained. Results are presented in Table 5.

Analysis utilizing the RP-HPLC showed that all the six extracts; CAR 1, CAR 2, CAR 3, CAR 4, CAR 5, and CAR 6, presented characteristics of single peaks at their respective retention times. Meaning that they were good enough to be described utilizing the LC-MS. CAR 5 and CAR 6 were enough for further analysis.

On further analysis, utilizing the LC-MS, three of the mixtures that TIC scans presented with single peaks could be distinguished. Still, only CAR 5had a flavonoid aglycone based on the Phenol-Explorer database (Anon 2012) was distinguished in this study.

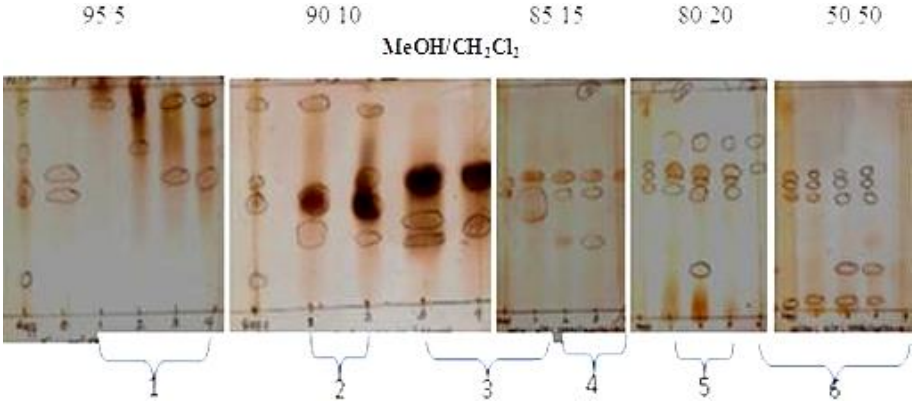

Figure 8. TLC card IV, C. arietinum root fractions from 2nd column chromatography

Table 5. HPLC, LC-MS, and Flavonoid test analysis of *Cicer arietinum* root distillate

Fraction	Weight in (mg)	% of distil-late	Retention Time (min)	UV λmax (nm)	$[M]^+$	Purity	Ammo-nia 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

Cicer arietinum leaves

Column chromatography I. A 1020 ml capacity column was packed, and 40 fractions, 200 ml each, of *C. arietinum* leaves were collected (Table 6) with the varying polarity of the eluting solvent from hexane to methanol. The mobile phase was altered only at the time there was no more examinable decomposition following analysis by TLC.

Utilizing mobile phase $EtOAc\text{-}H_2O\text{-}HCO_2H$ 18:1:1 (Figure 11.A), the spots were spread, then a repeat of the first 27 fractions was carried out utilizing EtOAc: Hex; 80:20 (Figure 11.B).

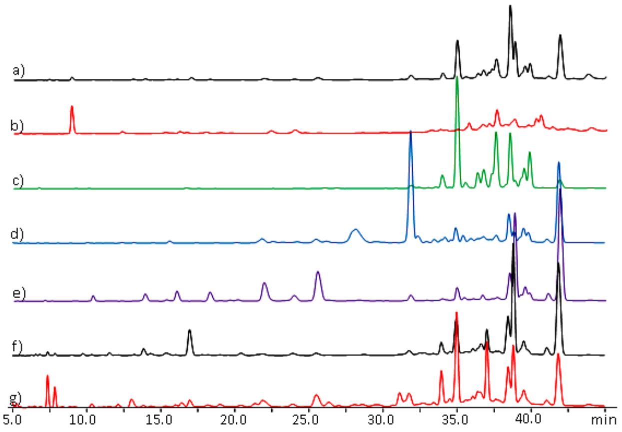

Figure 9. HPLC roots profiles of fractions from *Cicer arietinum* 2nd columncrude sample; b) Fraction 1; c) Fraction 2; d) Fraction 3; e) Fraction 4;f) Fraction 5; i) Fraction 6

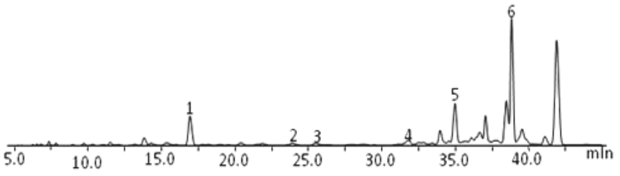

Figure 10. Segregated mixtures from *Cicer arietinum* roots

Table 6. Fractionation on the 1st column of *Cicer arietinum* leaf

Fraction	Solvent system	Percentage conc.	Weight of dry sample	Percentage harvest
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/CH2Cl2	50	0.30	0.55
21	EtOAc/CH2Cl2	50	0.25	0.50
22	EtOAc/CH2Cl2	50	0.14	0.30
23	CH2Cl2	100	0.04	0.07
24	CH2Cl2	100	0.03	0.05
25	CH2Cl2	100	0.01	0.02
26	CH2Cl2/MeOH	90	0.02	0.04
27	CH2Cl2/MeOH	90	0.04	0.07
28	CH2Cl2/MeOH	90	3.63	6.70
29	CH2Cl2/MeOH	80	4.70	8.70
30	CH2Cl2/MeOH	80	5.21	9.60
31	CH2Cl2/MeOH	80	5.31	9.80
32	CH2Cl2/MeOH	80	5.40	9.90
33	CH2Cl2/MeOH	80	2.74	5.0
34	CH2Cl2/MeOH	50		
35	CH2Cl2/MeOH	50		
36	CH2Cl2/MeOH	50		
37	MeOH	100		
38	MeOH	100		
39	MeOH	100		

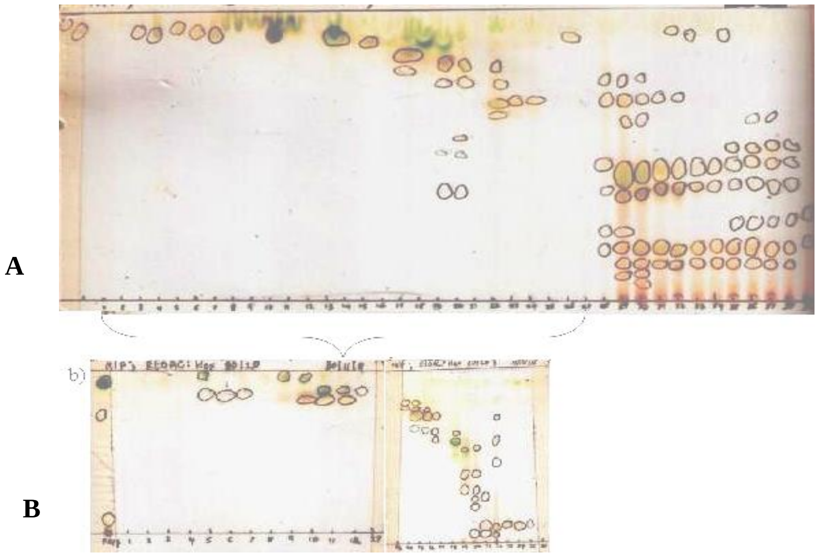

Figure 11.A-B. TLC cards V; Fractions from the 1st column chromatography of *Cicer arietinum* leaf

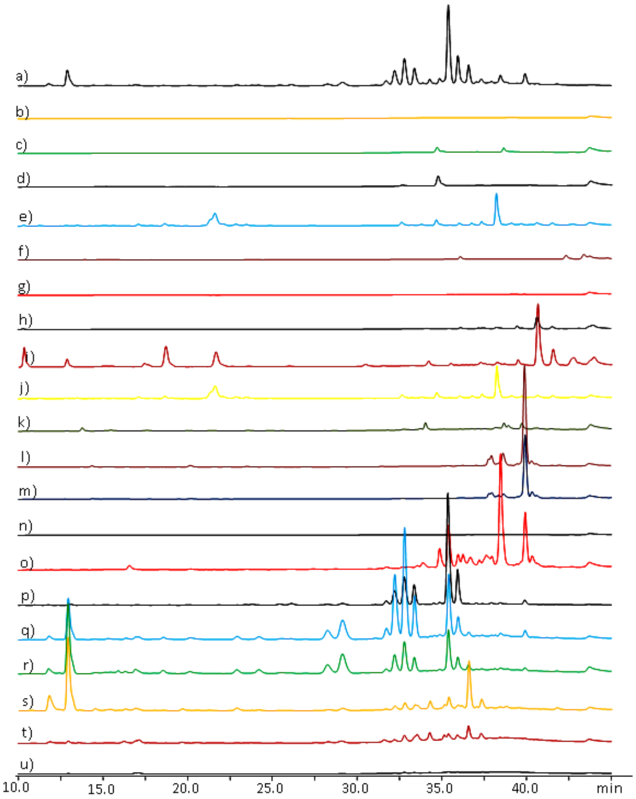

Figure 12. HPLC profiles of 1st column chromatography of *Cicer arietinum* leaf. a) Crude sample; b) Fraction 2; c) Fraction 4; d) Fraction 6; e) Fraction 8; f) Fraction 10; g) Fraction 12; h) Fraction 14; i) Fraction 16; j) Fraction 18; k) Fraction 20; l) Fraction 22; m) Fraction 24; n) Fraction 26;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

Only the even-numbered fractions on the TLC card were examined on HPLC and were obtained with a photodiode array detector set at 270 nm (Figure 12).

The mixtures were primarily eluted in fractions 29-33. Therefore, these fractions were pooled, dried, and a reversed-phase column packed and eluted with ethyl acetate, dichloromethane and methanol while slowly changing the percentage concentrations of solvents (Table 7).

Column chromatography II. The sample from fractions 29-33 weighing 23.4 g was fixed in C_{18} silica gel and a reversed-phase column packed and then eluted with ethyl acetate, dichloromethane and methanol while slowly varying the percentage concentrations of the solvent system. Results are presented in Table 7.

Fourteen fractions were spotted on silica gel TLC card IV, and spots were expanded utilizing mobile phase; EtOAc-H_2O-HCO_2H 18:1:1 (Figure 13).

HPLC analysis of four was relatively similar to the 14 fractions, as shown on the TLC card (Figure 14).

The majority of the mixtures concerning the crude sample were accessible in fraction D with better separation. The fractions involving fractions 12, 13, and 14 had been eluted utilizing a mobile phase of concentration varying from 30/70 to 40/60 MeOH/CH_2Cl_2. This fraction was suggested for further fractionation utilizing the HPLC.

Description of isolates. A shorter gradient elution technique was optimized from the original 65 min technique that lasted 49 minutes. 11 extracts were gained; Figure 15 shows the HPLC peak profile obtained with a photodiode array detector set at 270 nm.

Table 7. Fractionation of *Cicer arietinum* leaf on the 2nd column chromatography

Fraction	Solvent System	Percentage Conc.	Weight of dry sample	Percentage Harvest
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

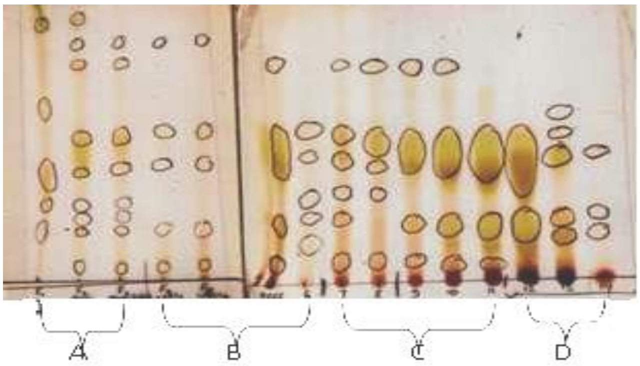

Figure 13. TLC cards VI; Fractions from the 2nd column of *Cicer arietinum* leaf

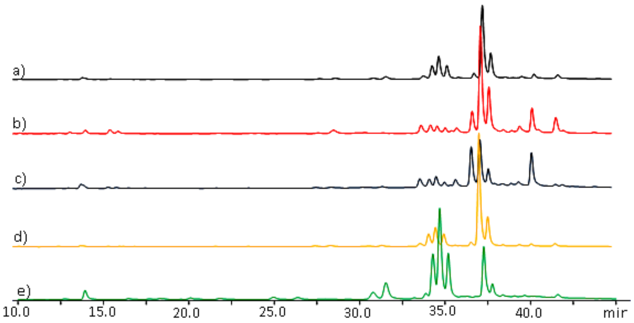

Figure 14. HPLC profiles of fractionations from 2nd column chromatography of *Cicer arietinum* leaf II. a) Crude sample; b) Fraction A; c) Fraction B; d) Fraction C; e) Fraction D

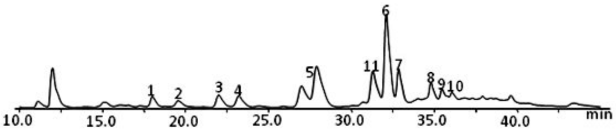

Figure 15. The segregated peaks from *Cicer arietinum* leaf's fraction D

Utilizing the LC-MS, TIC traces in positive mode at soft ionization (25^{o}eV) and strong ionization (40^{o}eV) conditions were monitored, and relevant pseudomolecular ions $[M+H]^+$ were gained. Weights of purified samples and their respective molecular ions are shown in Table 8.

Analysis utilizing the RP-HPLC showed nine extracts; CAL 1, CAL 2, CAL 3, CAL 4, CAL 7, CAL 8, CAL 9, CAL 10, and CAL 11, which were substantial concentrations and gave characteristics of single peaks at their respective retention times. It meant that they were good enough to be described utilizing the LC-MS. But CAL 5 and CAL 6 each produced twin peaks that were inseparable utilizing HPLC, and therefore, they were not good enough to be described utilizing the LC-MS.

Utilizing the LC-MS, seven of the above mixtures, which TIC scans gave single peaks each and had flavonoid aglycone based on the Phenol-Explorer database (Anon 2012), were distinguished in this research. Though pure, CAL 1 and CAL 10 were not distinguished in this research since their aglycone is not of any reported flavonoid.

Vigna radiata roots

Column chromatography. A 270.7 ml normal phase column was packed, and 2.5 g of *V. radiata* root distillate was eluted utilizing solvents of varying polarity from hexane to methanol. The results are presented in Table 9.

In gathering the fractions, the samples were spotted on pieces of silica gel TLC cards. Utilizing relevant mobile phases, 20:80, 80:20 Hex: EtOAc and EtOAc-H_2O-HCO_2H 18:1:1, the spots were expanded (Figure 16).

Comparing the number of fractions per spot and the pigmentation of the eluted spots per sample (Figure 16) above, the fractions were pooled into seven fractions. Then utilizing the HPLC set at 270 nm, the samples were examined (Figure 17). The peaks in fraction F were segregated utilizing the HPLC instrument.

Description of isolates. Utilizing a short technique that lasted 30 minutes, eight peaks were each segregated from the roots of *V. radiata* (Table 10). Utilizing the LC-MS, TIC traces in positive mode at soft ionization and strong ionization conditions were monitored, and relevant pseudomolecular ions $[M+H]^+$ were gained.

Table 9. Fractionation of *Vigna radiata* root utilizing column chromatography

Combined fraction	Fraction	Solvent system	% Conc.	Combined weight (g)	% Harvest
A	1	Hexane/EtOAc	50	0.16	6.4
	2	Hexane/EtOAc	50		
	3	Hexane/EtOAc	50		
B	4	Hexane/EtOAc	50	0.06	2.4
	5	Hexane/EtOAc	50		
	6	Hexane/EtOAc	50		
	7	EtOAc	100		
	8	EtOAc	100		
C	9	EtOAc	100	0.24	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		
	14	CH_2Cl_2	100		
D	15	CH_2Cl_2	100	0.0012	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		
	20	CH_2Cl_2/MeOH	80		
E	21	CH_2Cl_2/MeOH	80	0.33	13.2
	22	CH_2Cl_2/MeOH	80		
	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		
F	33	CH_2Cl_2/MeOH	30	1.2	48.0
	34	CH_2Cl_2/MeOH	30		
	35	MeOH	100		
	36	MeOH	100		
	37	MeOH	100		
	38	MeOH/H_2O	50		

Table 8. HPLC, LC-MS, and Flavonoid test analysis of *Cicer arietinum* leaf extracts

Fraction	Weight in (mg)	%age of extract	RT (min)	UV λ_{max} (nm)	$[M]^+$	Purity	NH_3test
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 26.0~26.4	236,265,347 244, 351	-	Impure	+ve
CAL 6	1.83	0.0026	29.1~29.5 29.8~30.2	232, 254,354 236, 265,347	-	Impure	+ve
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

Figure 16. TLC card VII; fractionation of *Vigna radiata* root by column chromatography

Table 10. HPLC, LC-MS, and flavonoid test analysis of *Vigna radiata* root extracts

Fraction	Weight in (mg)	% of distillate	RT (min)	UVλmax (nm)	Molecular mass [M]+	Purity	NH3test
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

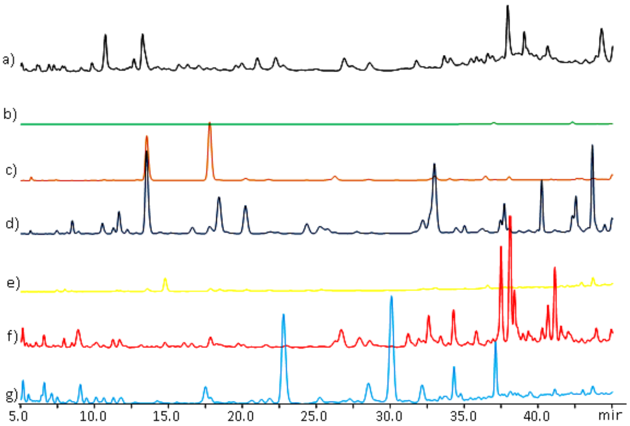

Figure 17. HPLC profiles of *Vigna radiata* root's fractions a) Crude sample; b) Fraction A; c) Fraction B; d) Fraction C; e) Fraction D; f) Fraction E;g) Fraction F

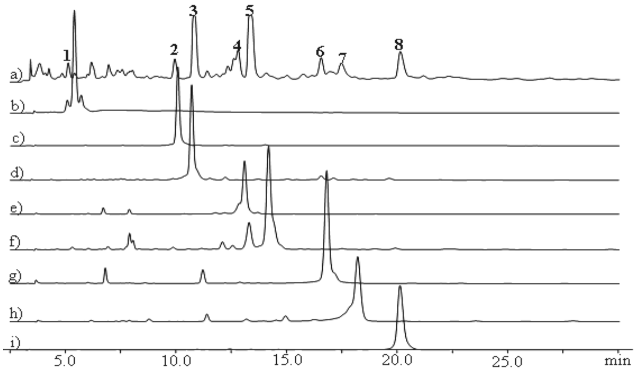

Figure 18. HPLC profile of segregated peaks from *Vigna radiata* roots' fraction F. a) Fraction F; b) VRR 1; c) VRR 2; d) VRR 3; e) VRR 4; f) VRR 5; g) VRR 6; h) VRR 7; i) VRR 8

Analysis utilizing the RP-HPLC showed all the eight fractions; VRR 1, VRR. 2, VRR 3, VRR 4, VRR 5, VRR 6, VRR 7, and VRR 8, and they were of substantial concentration and gave characteristic of single peaks at their respective retention times (Figure 18). It meant that they were all good enough to be described utilizing the LC-MS.

Though all these fractions showed negative results on the ammonium test, VRR 6 had flavonoid aglycon based on the database.

Utilizing the LC-MS, only VRR 6 TIC scans showed a single peak and had flavonoid aglycone based on the Phenol-Explorer database (Anon 2012). It was the only one of the fractions distinguished in this research. The rest of the isolates' TIC scans showed multiple peaks; hence several molecular masses were examined for these fractions.

Though unidentified, VRR 3 showed 3 peaks on the TIC scans, in which one of the peaks had a molecular mass of 484, portraying characteristics of C-Glucosylation characteristics. Fragmentation patterns divulged by the MS technique (Figure 19) supplied structural information about inter glycosidic linkages and aglycone replacement (Andersen 2006). Usually, fragments 162 and 132 amu are gained when the sugar is connected to the aglycone through an O-link. But a direct connection to the aglycone shows characteristic fragmentation of 120 or 90 amu (Andersen 2006). The integrity of the aglycone structure is kept at the expense of the glycan for C-glucosides (Raymond et al. 2006). Two fragmentations were examined on this sample's glycan.

Identity of the mixtures

Some of the peak elution times were stable with that of a diglycoside accessible in the *icipe*'s (BCED) library. For this reason, MS scans were arranged up to 1415 mass/charge (*m/z*) because most diglycosides possess an *m/z* of 431 to 700, and a single moiety had 269 to 317 *m/z*. Parent peaks were distinguished 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 gained these peaks as well as several others in lesser quantities. These masses were compared with all combinations of known diet flavonoid and glycosyl moieties.

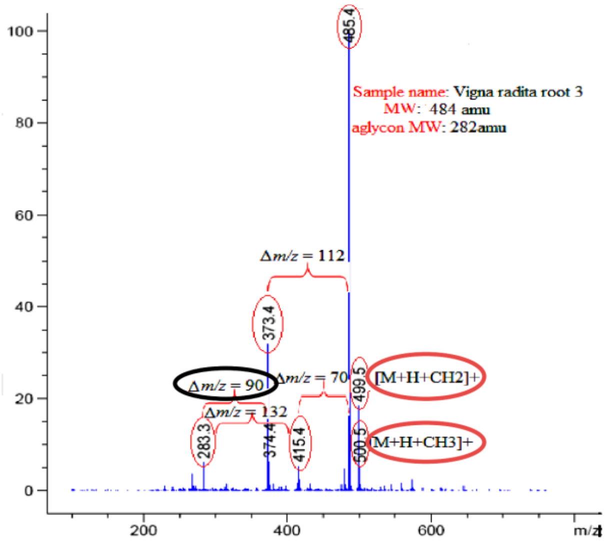

Figure 19. MS fragmentation of C-Glucosylated VRR 3

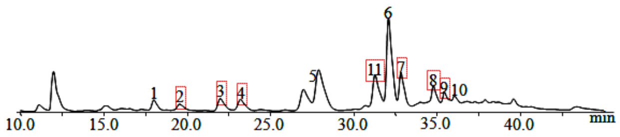

Figure 20.A. Segregated and distinguished mixtures from *Cicer arietinum* leaf

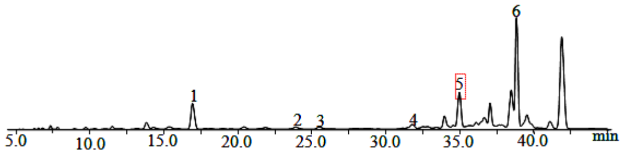

Figure 20.B. The segregated and recognized mixture from *Cicer arietinum* root

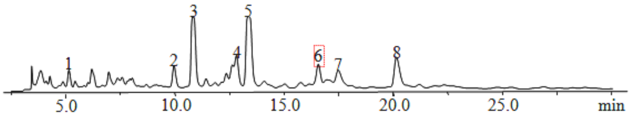

Figure 20.C. The segregated and distinguished mixture from *Vigna radiata* root

Though eleven fractions were gained from *C. arietinum* leaf (Figure 20.A), only the outlined seven mixtures were distinguished from the plant (CAL 2, CAL 3, CAL 4, CAL 7, CAL 8, CAL 9, and CAL 11) (Figure 20.A). Six fractions were segregated from *C. arietinum* root (Figure 20.B). But only (CAR5) outlined in Figure 20.B was recognized in this study.

In Figure 21.A, a white solid CAR 5 possessed a retention time of 34.1-34.4 min on HPLC. It presented $[M + H]^+$ 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 permeation 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 indicated ions at *m/z* 269 representing a loss of 162 amu recommending the existence of a sugar molecule. In the Phenol-Explorer database (Anon 2012), Formononetin-7-*O*-glucoside owned a molecular mass of 430 amu. Based on these data, CAR 5 was temporarily recognized as Formononetin-7-*O*-glucoside.

In Figure 21.B, CAL 2, a yellow solid, possessed a retention time of 19.4~19.7 min on HPLC. It exhibited $[M+Na]^+$ at *m/z* 663 and an intense *m/z* 700 ions in the normal mass spectra, which disconnected to harvest an *m/z* 641 in the ms2 spectra. The resulting 59 amu neutral loss indicated the existence of an acetate $[M+H+CH_3COO^{\cdot}]^+$ radical ion (confirming *m/z* 641 as the molecular ion). Further disconnection of the *m/z* 641 ions yielded an intense *m/z* 479 in the ms3 spectra, which shows the existence of a terminal galactose sugar (because of the 162 amu neutral loss). An additional *m/z* 317 ion fragment was noticed in the ms3 ion spectra, showing the existence of another terminal glucose sugar (because of 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 displayed maximum permeation at 255 and 352 nm, a shoulder peak at 222 nm, and minimum permeation at 245 nm. These data showed that its first aglycon had a molecular mass of 316. From the precursor ion in the molecule, the existence of two sugar molecules of molecular weights 162 amu is shown. Rhamnetin-3-O-galactoside-4'-O-glucoside had a molecular mass of 640 amu in the Phenol-Explorer database (Anon 2012). Based on these data, the coalition of the various chromatographic and spectroscopic methods (Table 11), and comparison, CAL 2 was temporarily recognized as rhamnetin-3-O-galactoside-4'-O-glucoside.

Figure 21.C presented that yellow solid CAL 3 got 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, representing that its molecular mass was 626. Its UV spectrum displayed maximum permeation at 238, 258, and 353 nm, a shoulder peak at 225 nm, and minimum permeation at 246 nm.

The positive MS spectrum of *m/z* 627 gave fragmentation ions at *m/z* 465, 446, and 303. These data showed that CAL 3 was a diglucoside, and the aglycon had a molecular mass of 302. In the Phenol-Explorer database (Anon 2012), quercetin-3,7-O-diglucoside had a molecular mass of 626. Based on these data, the coalition of the various chromatographic and spectroscopic methods (Table 11), and comparison, CAL 3 was temporarily recognized as Quercetin-3,7-O-diglucoside.

Figure 21.D indicated that a yellow solid CAL 4 owned 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, representing that its molecular mass is 610. Its UV spectrum showed maximum permeation at 265, 325, and 337 nm, a shoulder peak at 230 nm, and minimum permeation at 245 nm.

The positive MS spectrum of *m/z* 611 gave fragmentation ions at *m/z* 449, 430, and 287. These data showed that CAL 4 possessed an aglycon of molecular mass of 286. From the precursor ion, the existence of two sugar molecules of molecular weights 162 amu were in the molecule. Sakuranetin-5,4'-O-diglucoside had a molecular mass of 610 on the Phenol-Explorer database (Anon 2012). Based on these data, the coalition of the various chromatographic and spectroscopic methods (Table 11) and comparison CAL 4 was temporarily recognized as Sakuranetin-5,4'-O-diglucoside.

Figure 21.E indicated that a yellow solid CAL 7 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 ensured to be 610. Its UV spectrum showed maximum permeation at 255 and 352 nm, a shoulder peak at 238 nm, and minimum permeation at 245 nm, recommending that it was an isomer of CAL 2.

The positive MS spectrum of *m/z* 611 represented fragment ions at *m/z*, 479, 461, 317, and 302; sequential losses of 132 amu, 132+H_2O amu, and 162 amu showed the existence of a pentosyl and a glycosyl moiety respectively. The sugar molecules were most likely enclosed in the aglycon, whose molecular mass is 316 amu. Based on these and data from the Phenol-Explorer database (Anon 2012), CAL 7 was temporarily recognized as Isorhamnetin-3-O-xyloside-7-O-glucoside.

In Figure 21.F, CAL 8, a yellow solid, had a retention time of 34.6~35.0 min on HPLC. It indicated $[M +Na]^+$ at *m/z* 471 and $[M+H]^+$ at *m/z* 449 in the positive ion mass spectrum. Therefore, its molecular mass was ensured to be 448.

Its UV spectrum showed maximum permeation at 265 and 337 nm, a shoulder peak at 230 nm, and minimum permeation at 246 nm, recommending an isomer of CAL 4. The positive MS spectrum of *m/z* 449 exhibited fragment ion at *m/z* 287; the loss of 162 amu pointed to a glucosyl enclosed to the aglycon, which molecular mass is 286. Based on these data from the Phenol-Explorer database (Anon 2012), CAL 8 was temporarily recognized as Isosakuranetin-5-O-diglucoside.

In Figure 21.G, CAL 9, a yellow solid, bore a retention time of 36.6~36.9 min on HPLC. It exhibited $[M +H+3CO]^+$ 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 mixture was assumed to be 448. Its UV spectrum showed maximum permeation at 244, 264, and 348 nm, a shoulder peak at 220 nm, and minimum permeation at 245 nm, recommending that CAL 9 is neither an isomer of CAL 4 nor of CAL 8.

The positive MS spectrum of *m/z* 449 represented fragment ions at *m/z* 287. The loss of 162 amu pointed to the existence of a glucosyl fragment and recommended that the glucosyl be enclosed to the aglycon, which molecular mass was 286. In the Phenol-Explorer database on polyphenol content in foods (Anon 2012), luteolin, kaempferol, scutellarein, and fisetin owned a molecular mass of 286. This mixture was neither luteolin nor scutellarein because it was an isomer or the same mixture as CAL 11. Based on these data, CAL 9 was temporarily recognized as; either fisetin-3-O-glucoside, fisetin-7-O-glucoside, fisetin-4'-O-glucoside kaempferol-3-O-glucoside or Kaempferol-7-O-glucoside.

CAL 11 in Figure 21.H, a yellow solid, owned a retention time of 29.4~29.8 min on HPLC. It exhibited $[M +Na]^+$ at *m/z* 633 and $[M+H]^+$ at *m/z* 611 in the positive ionization mode. Therefore, its molecular mass was assumed to be 610.

Its UV spectrum showed maximum permeation at 244 and 348 nm, a shoulder peak at 218 nm, and minimum permeation at 238 nm recommending that it was an isomer of CAL 9 but not an isomer of CAL 4. The positive MS spectrum of *m/z* 449 indicated fragment ions at *m/z* 449 and 287; sequential losses of 162 amu, and 2 (162) amu, and showed the existence of two sugar molecules recommending the two glucosyls adhered to the aglycon whose molecular mass is 286. Compared with published data, O-diglucosides of scutellarein is yet to be reported in the diet. Based on these data from the Phenol-Explorer database (Anon 2012), CAL 11 was temporarily recognized as either Fisetin 3,7-O-diglucoside or Kaempferol 3,7-O-diglucoside.

Out of eight fractions segregated from *V. radiata* root (Figure 20.C), only VRR 6 is outlined in Figure 20.C was recognized in this study.

Figure 21.I represented that VRR 6, a red solid, held a retention time of 15.1~15.4 min on HPLC. It pointed $[M +Na]^+$ at *m/z* 617 and $[M +H]^+$ at *m/z* 595 in the positive ion mass spectrum. Therefore, its molecular mass was assumed to be 594.

Its UV spectrum represented maximum permeation at 256, 282, and 319 nm, a shoulder peak at 219 nm, and minimum permeation at 245 nm. The positive MS spectrum of *m/z* 432 represented fragment ions at *m/z* 271, sequential losses of 162 amu. It showed that two glucosyls adhered to the Aglycon molecular mass of 270.

Based on these data and the Phenol-Explorer database (Anon 2012), Apigenin, Baicalein, Genistein, and Galangin possess a molecular mass of 270. But a comparison of the UV results of the mixture with Apigenin and Genistein standards indicated that the aglycone of VRR 6 cannot be Apigenin or Genistein. Also, from the database, glucosides of Galangin are yet to be reported. Therefore, VRR 6 was temporarily recognized as Baicalein-7-O-diglucoside.

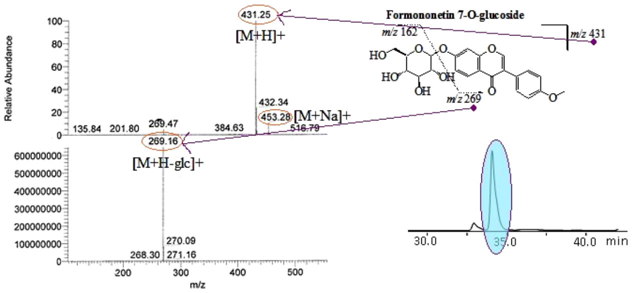

Figure 21.A. HPLC profile, LC-MS fragmentation, and likely structure of CAL 5

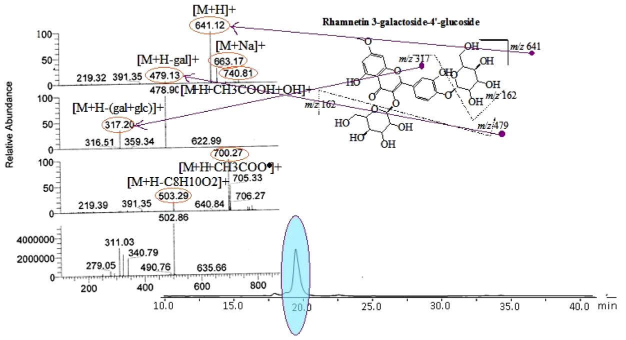

Figure 21.B. HPLC profile, LC-MS fragmentation, and likely structure of CAL 2

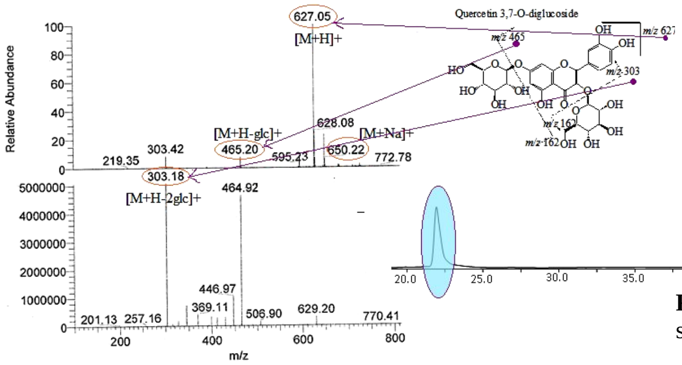

Figure 21.C. HPLC profile, LC-MS fragmentation, and likely structure of CAL 3

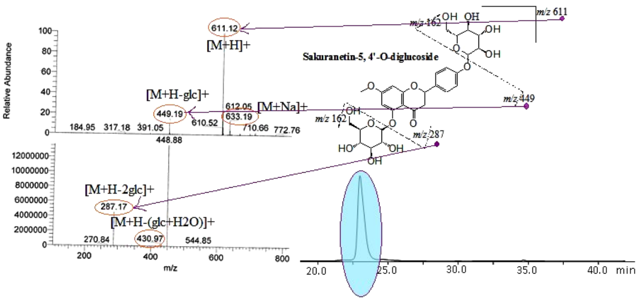

Figure 21.D. HPLC profile, LC-MS fragmentation, and likely structure of CAL 4

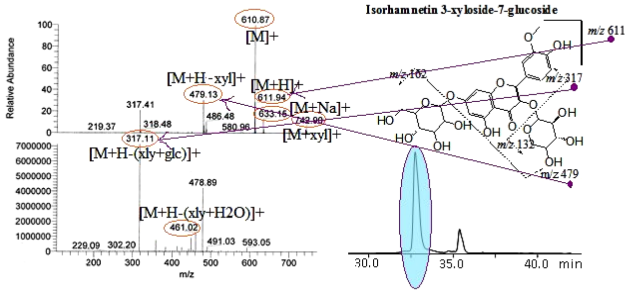

Figure 21.E. HPLC profile, LC-MS fragmentation, and likely structure of CAL 7

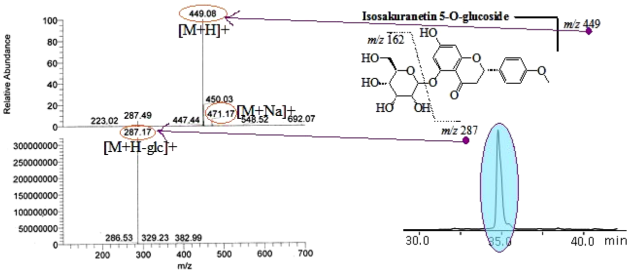

Figure 21.F. HPLC profile, LC-MS fragmentation, and likely structure of CAL 8

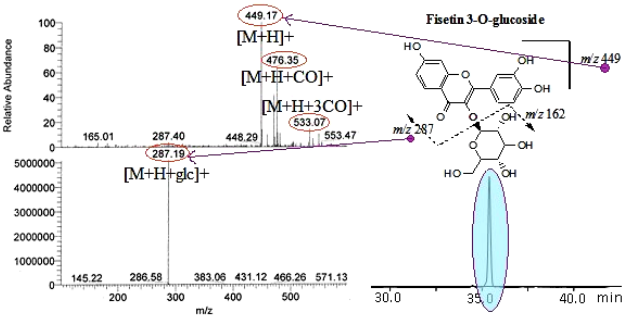

Figure 21.G. HPLC profile, LC-MS fragmentation, and likely structure of CAL 9

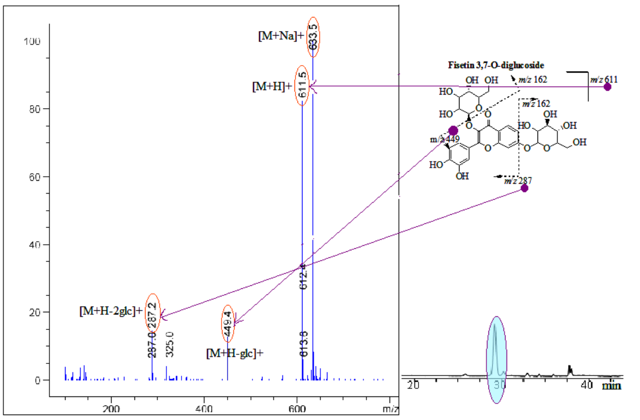

Figure 21.H. HPLC profile, LC-MS fragmentation, and likely structure of CAL 11

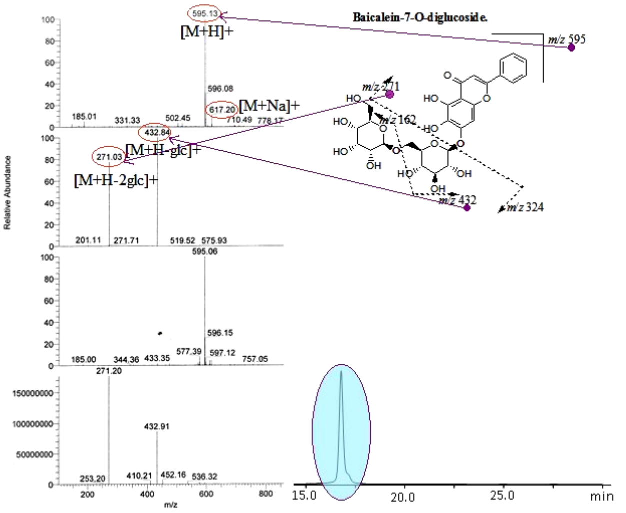

Figure 21.I. HPLC profile, LC-MS fragmentation, and likely structure of VRR 6

Summary of identified mixtures

Finally, the coalition of the various chromatographic and spectroscopic methods and comparison with published data (Anon 2012) allowed the temporary identification of several mixtures, as summarized in Table 11.

In conclusion, the secondary metabolites segregated from the leaf of *C. arietinum* L. were eleven, and those from the root tissues were six. Description of these metabolites utilizing HPLC and LC-ESI-MS represented that they are all O-glucosylated. It had no inhibitory chemistry and proposed biosynthesis by C-glucosylation (Pickett 2011). The plant gathered several unidentified secondary metabolites in its leaf and root since all the mixtures described and recognized in this research have never been reported before. The ones noticed in the leaf were not replicated in the root or vice versa. Eight secondary metabolites were segregated from the root of *V. radiata* L. Out of these, HPLC and LC-ESI-MS described four mixtures. One mixture, VRR 6, was recognized as Baicelein-7-O-diglucoside. The compound of VRR 3 indicated the characteristic of C-glucoside in MS fragmentation though it was not recognized in this research. This signified that *V. radiata* bore inhibitory chemistry and proposed biosynthesis by C-glucosylation (Pickett 2011).

Table 11. Summary of the temporarily recognized mixtures

Compound/ possible identification	Retention time (min)	UVMaximum absorbance (λmax) (nm)	ESI-MS fragment ions (% proportional intensity)	[M]+
CAR 5 Formononetin 7-O-glucoside	34.1~34.4	236, 254	m/z 453 [M+Na]+ (10) and431[M+H]+ (100).m/z269 [M+H-162]+ (<5)	430
CAL 2 Rhamnetin 3-O-galactoside-4'-O-glucoside	19.4~19.	222,238,255, 352	m/z740[M+CH_3COOH+OH]+ (<5)663[M+Na]+ (30)and641[M+H]+ (100).m/z623 [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/z650[M+Na]+ (<10)and627[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) and611[M+H]+ (100).m/z449 [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/z742[M+132]+ (10),633[M+Na]+ (15),611[M+H]+ (30) and 610[M]+ (100). m/z479[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/z471[M+Na]+ (<10)and449[M+H]+ (100). m/z 287 [M+H-162]+ (5)	448
CAL 9 Fisetin 3-O-glucoside	36.6~36.9	220, 244,264, 348	m/z533[M+H+3CO]+ (15), 476[M+H+CO]+ (65) and 449[M+H]+ (100). m/z287 [M+H-162]+ (<5)	448
CAL 11 Fisetin 3,7-O-diglucoside or kaempherol 3,7-O-diglucoside	29.4~29.8	218, 244,348	m/z633[M+Na]+ (100)and 611 [M+H]+ (84). m/z449 [M+H-162]+ (13)and287 [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)and595[M+H]+ (100).m/z432 [M+H-163]+ (<5)and271[M+H-2 (162)]+ (<5)	594

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