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Characterization of Polyphenols, Flavonoids and Their Anti-microbial Activity in the Fruits of *Vangueria madagascariensis* J. F. Gmel

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Authors' contributions

This work was carried out in collaboration among all authors. The research idea was conceived by authors PKN and SMM. Author SMM sourced for the research funds. Authors PKN and SMM designed the experimental plan. Author PKN identified and collected the plant samples. Author TZ undertook experimental work under the guidance of author SMM. All authors were involved in the drafting of the manuscript. All the authors read and approved the final manuscript.

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ABSTRACT

Aim: The study aimed to characterize phenolic acids, flavonoids, and determine their antimicrobial activities in fruits of *Vangueria madagascariensis* (Tamarind of Indies).
Study Design: The design of the study included picking of *Vangueria madagascariensis* fruits from Jomo Kenyatta University of Agriculture and Technology (JKUAT) botanical garden and analysis for their antimicrobial activities at the Botany department research laboratory, JKUAT. Characterization of phenolic acids and flavonoids were conducted at MacEwan University Canada.
Place and Duration: JKUAT, Kenya and MacEwan University, Edmonton, Alberta Canada between June 2013 and June 2016.
Methodology: Phenolic acids and flavonoids from Tamarind of Indies were determined by high-performance liquid chromatography coupled with photodiode array detection and electrospray

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ionization tandem mass spectrometry (HPLC-DAD-ESI-MSN). The antimicrobial assay was determined using the disk diffusion method.
Results: Based on the retention time, the UV spectrum, and the tandem MS behavior, the results revealed a profile composed of 25 phenolic compounds. Some of the identified phenolic compounds included: 3-caffeoylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 4-feruloyl quinic acid, quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin, 3,4-di-caffeoylquinic acid, 4, 5-di-caffeoylquinic acid, kaempferol, diosmetin, caffeic acid, epicatechin, kaempferol 3-O-glucoside. The fruit extracts had a probable presence of quercetin 3-O-6'-malonylglucoside, ikarisoside C, epimedin C, unknown epigallocatechin-3-gallate and quercetin conjugate derivatives. Furthermore, the fruit extracts from *Vangueria madagascariensis* showed appreciable antimicrobial properties against human pathogen strains. Strong antimicrobial activity was observed for *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans.* The *Vangueria madagascariensis* was found to be highly potent against *Escherichia coli and Bacillus subtilis* even at low concentrations of 0.1 mg/mL.
Conclusion: The research findings may suggest value of the use of *Vangueria madagascariensis* fruits as a rich source of antioxidants with therapeutic and nutraceutical value.

Keywords: Phenolic acids; flavonoids; antioxidants; microbial activity; Vangueria madagascariensis; mass spectrometry.

1. INTRODUCTION

Vangueria madagascariensis is a species that is native to Madagascar. It belongs to the plant family Rubiaceae. It is an evergreen multi-stemmed tree and may reach a height of 15 meters [1]. It has been naturalized in many African countries like Kenya, Angola, Tanzania, and Madagascar. The fruits are rounded, green and are often in bunches of 5-6. On ripening fruits converted to yellowish-brown in color. Flowering takes place in the rainy season, while fruit ripening occurs during the dry season. Fruit normally takes 6-8 months from flower fertilization to ripening, depending on locality. The ripe juicy fruits are collected from the tree, peeled and the pulp is eaten fresh. It has a mealy taste like Irish potatoes and is eaten as a snack. The fruit is also used as a flavoring agent in beer. The pleasant-smelling flowers of *Vangueria madagascariensis* attract bees and are therefore a suitable honey source. A decoction of roots is used as a remedy for various intestinal worms. An infusion of the bark can also be used for treating malaria.

Over the last decade, there has been continued research and public interest on natural antioxidants which are largely phenolic compounds. These molecular entities are secondary metabolites synthesized by the plants as protective and defense agents in response to different stress factors, *e.g.* UV rays, presence of xenobiotics pollutants or to protect themselves from parasitic weeds. Natural antioxidants occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots, and barks. Natural antioxidants can be classified loosely as phenolic acids, flavonoids and flavonoid polymers, with the latter being highly diverse subgroup generally present as glycosylated conjugates [2,3,4]. Flavonoids can be further classified into 7 classes as shown in Fig. 1.

On the other hand, numerous classes of phenolic acids have been studied, with chlorogenic acids being the most prevalent and widely distributed in plants. About 30 chlorogenic acids have been determined especially in coffee [5,6,7,8].

A few examples of representative chlorogenic structures are shown in Fig. 2. The public interest towards natural antioxidants (phenolic compounds) has largely been due to various scientific research reports on their values such as antiallergenic, antiartherogenic, anti-inflammatory, antimicrobial, antidiabetic, cancer chemopreventive agents (especially anthocyanidins), antiviral activities, antithrombotic, cardioprotective, antimutagenic, vasodilatory, insulin secretion ability, and neuroprotective effects. A classic example of anecdotal evidence of the benefits of phenolics, particularly the anthocyanins (main class of flavonoids in red wine) is the so-called "French Paradox", which refers to the lower incidents of coronary atherosclerosis in the French population compared to the Western populations, though the former consume a diet that contains more fat.

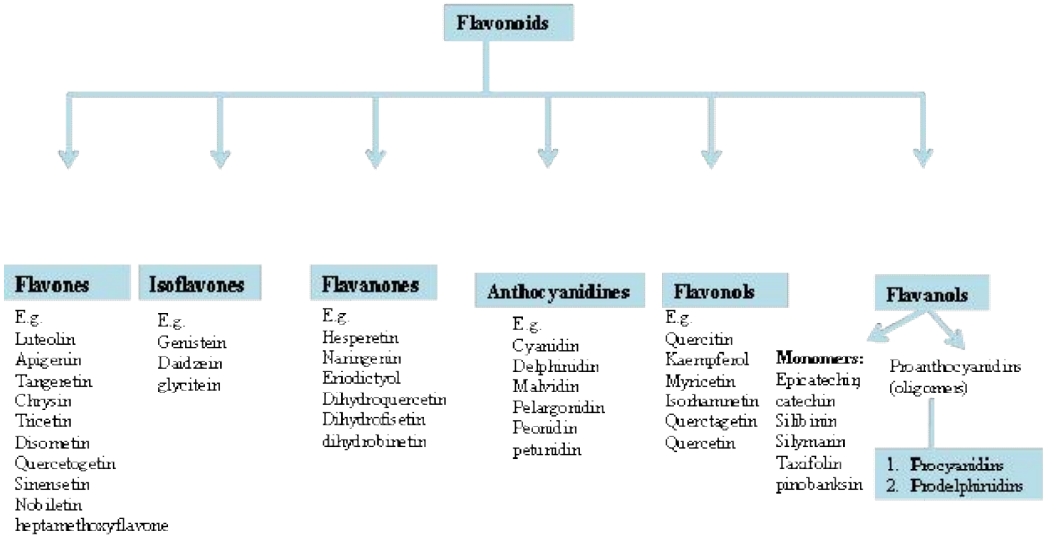

Fig. 1. Classification of general flavonoids

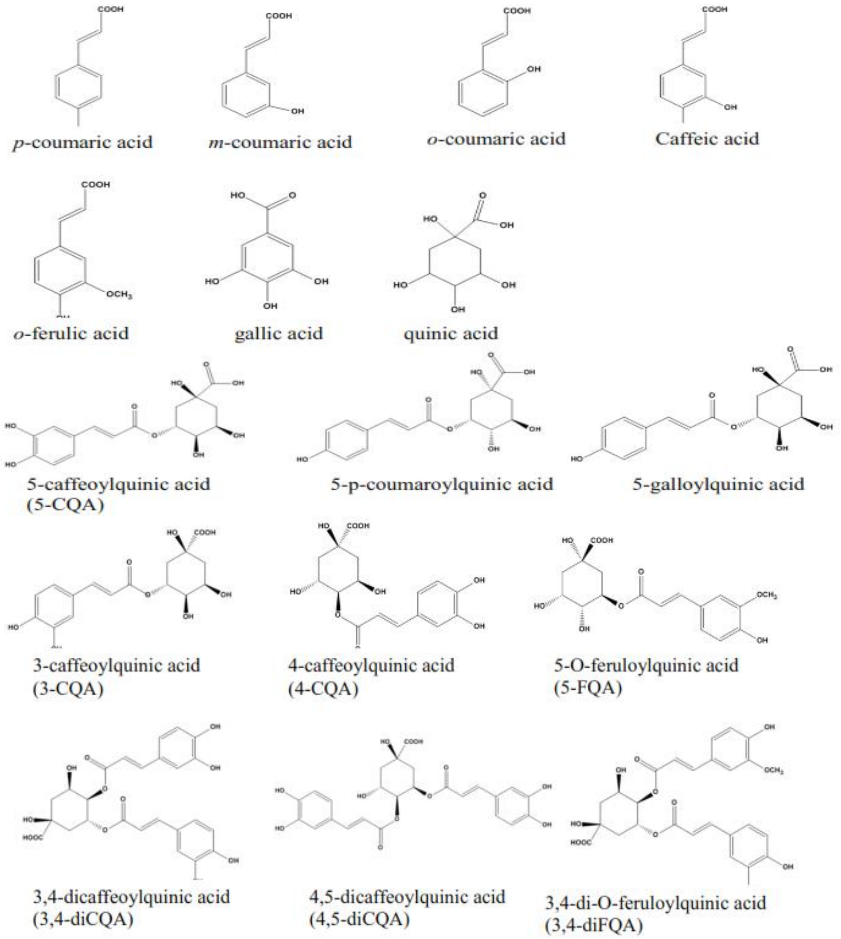

Fig. 2. A few examples of representative chlorogenic structures

The main characteristic of the antioxidants that largely explain their health benefits enumerated above is their inherent ability to scavenge highly reactive free radicals and reactive oxygen species (e.g. superoxides, peroxides, and hydroxyl radicals). These species have been identified as major contributors to the oxidation of cellular entities (nucleic acids, proteins, DNA, lipids, and biomembranes) leading to tissue damage and the initiation of many diseases. With the rapid increase in the number of these cases to an epidemic level, the public has accepted the promise of natural antioxidants. Research output on antioxidants has been enormous, but there remain many unanswered questions on bioavailability, viability, and in general validity of clinical and epidemiological benefits of ingestion of phenolic compounds.

Although there is no doubt about the importance of antioxidants, there is still a need to profile the presence of these phytochemicals in plants. As a result, a huge research opportunity in the antioxidant profiling of plants from developing countries, especially in Africa, has arisen. Therefore, this work was developed to determine phenolics in the fruits of *Vangueria madagascariensis*, by high-performance liquid chromatography (HPLC) coupled with diode array (DAD) and electrospray ionization mass spectrometry with tandem MS (HPLC-DAD-ESI-MS/MS).

The extract from the *Vangueria madagascariensis* fruits was also evaluated for antibacterial properties against five human pathogen strains including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans*.

2. MATERIALS AND METHODS

2.1 Materials

Acetonitrile, methanol (HPLC grade), formic acid, acetic acid, caffeic acid, ferulic acid, vanillic acid, protocatechuic acid, p-coumaric acid, gallic acid, and chlorogenic acid were purchased from Sigma-Aldrich, Oakville Ontario Canada. C-18 SPE cartridges were obtained from Fisher Scientific, Edmonton, Canada, and 0.22 µm hydrophobic filters were obtained from VWR Edmonton, Canada.

2.2 Methods

Ripe fruits of Tamarind of Indies were picked from trees growing at Jomo Kenyatta University of Agriculture and Technology (Juja, Kenya) compound. The fruits were crushed and the macerated material extracted with hexane (5x100 mL, each extraction for 5 hours) at room temperature. All the extracts from the five extractions were pooled, evaporated and preserved for analysis. To extract the phenolics, the fruit residues were extracted (5x100 mL, each extraction for 5 hours) with 80% aqueous acetone (v: v). Acetone was evaporated and the remaining aqueous phase was further extracted by 5x30 mL of ethyl acetate to extract the flavonoids (labeled VAF) while leaving phenolics in the aqueous phase. The pH of the aqueous phase was adjusted to pH 1.5 and then extracted by 5x30 mL of ethyl acetate to extract the phenolic acids (labeled VAP).

2.2.1 Phenolic acid standards

Caffeic acid, ferulic acid, vanillic acid, protocatechuic acid, p-coumaric acid, gallic acid, and chlorogenic acid were used to prepare a 0.1mg/mL standard mixture. UV spectra and MS/MS data were recorded as a reference, which was then used to compare with the plant extract chromatograms and spectra. A mango peel extract that has been extensively characterized by Schieber, *et al* [9] was used as a standard to help in the structural identification of the phenolic constituents in *Vangueria madagascariensis* fruit extract.

2.2.2 Coffee extract preparation

Since it was not possible to obtain all the required pure standards for complete identification of phenolic compounds present in *Vangueria madagascariensis.,* the coffee extract was used as a standard. The coffee extract has been extensively characterized by several authors and its wide availability makes it an inexpensive pseudo standard [10,11,12].

500 mg of ground green robusta coffee beans were bought from a local store and extracted (4 x25 mL, 25 min each) with 70% methanol/30% water (v:v). The extract was then pre-concentrated by the removal of methanol using a rotary evaporator (rotavap). The aqueous fraction was filtered through a hydrophilic syringe filter and analyzed by LC-MS.

Table 1. HPLC gradient setting for the separation of phenolic acids and flavonoids

No.	Time	C%	D%	Flow rate (µL/min)
0	0.00	100.0	0.0	500.0
1	15.00	70.0	30.0	500.0
2	30.00	60.0	40.0	500.0
3	70.00	40.0	60.0	500.0
4	85.00	20.0	80.0	500.0
5	95.00	0.0	100.0	500.0
6	100.00	0.0	100.0	500.0
7	103.00	0.0	100.0	500.0
8	105.00	100.0	0.0	500.0
9	107.00	100.0	0.0	500.0

Table 2. ESI-MS setting for the detection of phenolic acids and flavonoids

Polarity	Negative
Spray voltage (kV)	5.0
Sheath gas flow rate	50
Auxiliary gas flow rate	30
Sweep gas flow rate	0
Capillary temperature	270.0
Capillary voltage (V)	-19.0
Tube lens (V)	-125.0
MS/MS isolation width	3.0
MS/MS collision energy (%)	35.0

2.3 Apparatus

2.3.1 HPLC-DAD-ESI-MS separation of coffee extract, mango peel extract, standard mixture, *Vangueria m.* (phenolic acids and flavonoids extract portions)

The characterization of phenolic acids and flavonoids in coffee bean extract, mango peel extract, phenolic acid standard mixture, *Vangueria madagascariensis* phenolics, and flavonoids extracts (VAF) was subjected to a Thermo LCQ Fleet HPLC-DAD-ESI-MS system. The system consisted of a Surveyor binary pump, a Surveyor autosampler with tray temperature setting at 6ºC, a column oven, a six-port injection valve, a Surveyor DAD detector, electrospray ionization (ESI) source, and an ion trap MS detector. A Varian C18 PAH RPLC column (250 x 3.0 mm I.D., 5 m) (Agilent, Santa Clara, CA, USA) operating at 20ºC with a flow rate of 500.0 µL/min was used to separate phenolic acids and flavonoids. The diode array detector (DAD) had a scan range from 190 nm to 400 nm, with three detection channels at 280, 320, and 380 nm. 2% acetic acid in water (C) and 0.5% acetic acid in water/acetonitrile (50/50, v/v) (D) were used as mobile phases in gradient separation. Table 1 shows the gradient setting during a 107-min run.

The effluent from DAD detector was then introduced to the ESI interface in the negative mode for sample ionization and MS^n detection. Table 2 shows the ESI-MS settings. High purity nitrogen was used as the sheath and auxiliary gas. Structure information of phenolic acids and flavonoids was obtained by tandem MS spectrometry (MS^n) (n = up to 3) through collision-induced dissociation (CID), where argon gas was used as the collision gas. MS^n was performed with a relative collision energy setting of 35%. Data acquisition was obtained using Xcalibur software version 2.0 (Thermo, San Jose, CA, USA).

2.3.2 Antimicrobial assay test Organisms

An equal ratio of VAP and VAF dry extracts were combined, reconstituted in distilled water to make extract solutions of 100, 10, 1, and 0.1 mg/mL. The extract's microbial inhibition activity was tested using the disc diffusion method [13]. The following microorganisms were used as test organisms: *Staphylococcus aureus* (ATCC 25923), a facultative anaerobic Gram - positive coccal bacterium frequently found as part of the normal skin flora on the skin and nasal passages, *Bacillus subtilis (ATCC 6633),* a Gram - positive obligate aerobe commonly found in soil), *Escherichia coli* (ATCC 25922), a

Gram - negative, rod - shaped bacterium commonly found in the lower intestine of endotherms, *Pseudomonas aeruginosa* (ATCC 2785), a Gram - negative, aerobic rod - shaped bacteria, and *Candida albicans* (ATCC 90028), a diploid fungus that grows both as yeast and filamentous cells.

All the bacteria were obtained from the Botany department laboratory of Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja, Kenya, and standard isolates were used. The bacteria were maintained at 4 ^{0}C on nutrient agar plates. Base plates were prepared by pouring 20 mL Mueller-Hinton agar into sterile Petri dishes and allowed to air dry. Molten Mueller-Hinton agar held at 48 ^{0}C was inoculated with a broth culture of the test organism and poured over the base plates forming a homogenous top layer. Filter paper discs (Whatman No. 3, 6 mm diameter) were sterilized by autoclaving. Ten µl of each extract (100, 10, 1 and 0.1 mg/mL) were applied per filter paper disc. The discs were air-dried and placed onto the seeded top layer of the Mueller-Hinton agar plates. Each extract was tested in triplicate. Air-dried Dimethylsulfoxide saturated discs were used as negative controls. Gentamicin antibiotics (30 µg/ml) in distilled water was used as a positive control. The plates were evaluated after incubation at 37°C for 24 hr after which the zones of inhibition were measured. The size of the inhibition zone in (mm) produced by the plant extract and the inhibition zone around the gentamicin reference (mm) was used to express antibacterial activity. Antifungal activity was performed on *Candida albicans. In vitro* activity was performed as described for bacterial assays except that Sabourands dextrose agar was used for antifungal bioactivity testing and incubation was done at 30°C for 24 hr.

3. RESULTS AND DISCUSSION

Using LC-ESI-MS/MS, the profile of phenolic compounds in the fruits of *Vangueria madagascariensis.* was characterized and most of the major compounds present were tentatively identified. The identification was based on the comparison of mass spectrometry data with that of authentic standards, coffee extracts, and mango peel extracts. Some of the compounds were also tentatively identified from the published MS^n data in the literature, which will be explained in detail.

3.1 Profiling of Phenolic Acids and Flavonoids from Standard Mixture, Coffee Extract and Mango Peel Extract

3.1.1 Standard mixture

The ESI-MS^n results obtained from authentic standards are summarized in Table 3. The MS and tandem MS data were used as the reference for the identification of phenolic acids and flavonoids in VAF and VAP extracts.

3.1.2 Coffee extract

Coffee beans have been extensively studied and have been found to contain a huge amount of phenolic acids especially chlorogenic acid derivatives. Analysis of coffee extract by HPLC-DAD, the UV chromatogram at 320 nm is shown in Fig. 3a. The chromatogram peaks were identified by elucidation of diagnostic daughter ions emanating from HPLC-MS^n (n = up to 3) and comparison with literature. As shown in Table 4, numerous chlorogenic acid derivatives were identified, including caffeoylquinic acid (CQA), feruloyl quinic acid (FCQA), and dicaffeoyl quinic acid (diCQA). The isomerization of CQA has been reported with 3 isomers of the quinic acid in three different positions on the caffeoyl backbone: (3-CQA), (4-CQA), and (5-CQA). To identify the three possible CQA isomers, MS/MS fragmentation and detection were conducted. 4-CQA was verified by the MS peak at *m/z* 173, while other CQA isomers did not have this peak. 3- and 5-caffeoyl quinic acid were identified based on the parent ion peak at m/z 191 and MS^2 ions at *m/z* 179. As reported in the literature the intensity of *m/z* 179 was higher in 3-CQA than that in 5-CQA [10,12].

A series of dicaffeoyl quinic acid (diCQA) isomers were also identified based on MS/MS data. A MS^2 peak at *m/z* 173 was detected based on the MS parent peak at *m/z* 515, which illustrated the presence of 3, 4-di CQA or 4, 5-di CQA. This *m/z* 173 peak is 4-CQA, thus 3,4-di CQA loses the caffeoyl moiety at position 3, while 4,5-di CQA loses the caffeoyl moiety at position 5. These two dicaffeoyl quinic acid isomers were further distinguished by the peak intensity at *m/z* 335, which is more intense in 3,4-di CQA than that in 4,5-di CQA [11,12].

Table 3. ESI-MS^n results of authentic standard mixture

[M-H]$^-$ (*m/z*)	MS/MS (*m/z*)	Identity
169	125/81/79	Gallic acid
153	109	Protocatechuic acid
353	191	3-caffeoylquinic acid
167	152/123/108	Vanillic acid
179	135	Caffeic acid
163	119/93	*p*-Coumaric acid
193	178/149/134	Ferulic acid
289	245/137	Catechin
289	245/206	Epicatechin
311	179/149	Caftaric acid

Table 4. Identification of phenolic acids in the coffee extract

	[M-H]$^-$ (*m/z*)	MS/MS (*m/z*)	Identity
1	353	191	3-Caffeoylqunic acid
2	353	191	5-Caffeoylqunic acid
3	353	173	4-Caffeoylqunic acid
4	367	193/173	3-Feruloylquinic acid
5	367	191	5-Feruloylquinic acid
6	367	191/149	4-Feruloylquinic acid
7	515	353/191	3,4-Dicaffeoylqunic acid
8	515	353/173	4,5-Dicaffeoylqunic acid

Table 5. Identification of flavonoids in mango peels extract

Peak number	[M-H]$^-$ (*m/z*)	MS/MS (*m/z*)	Identity
1	463	301	Quercetin 3-O-galactoside
2	463	301/179	Quercetin 3-O-glucoside
3	433	301	Quercetin 3-O-xyloside
4	433	301/300	Quercetin 3-O-arabinopyranoside
5	433	301	Quercetin 3-O-arabinoforanoside
6	447	301	Quercetin 3-O-rhamnoside
7	447	285/284/255	Kaempferol 3-O-glucoside

Similarly, three isomers of feruloyl quinic acid were distinguished by tandem MS. The parent ion of FQA at *m/z* 367 was fragmented and the MS/MS data were compared. Only 3-FQA can form a daughter ion at *m/z* 193. 4-FQA and 5-FQA were then further identified by the MS^2 fragments [12,14].

Similarly, the mango peel extract (chromatogram not shown) was analyzed by HPLC-DAD-ESI-MS with the summary of identified phenolics based on MS and MS^2 data shown in Table 5. Similar identifications have been made by other researchers including [9,15,16,17,18].

3.2 Identification of Polyphenols and Flavonoids in *Vangueria m.*

Table 6 and Table 7 show the identification of phenolic acid fraction (VAP) and flavonoids fraction (VAF) in *Vangueria madagascariensis* together with the parent and daughter ions by ESI-MS and tandem MS. Our data show that both the flavonoids and phenolic acids fraction generated very similar phenolic compounds with an exemption of a few, with the HPLC-DAD chromatograms shown in Figs. 3b and 3c respectively.

When comparing the identified phenolic acids in both plant fractions, some phenolic compounds were confirmed by the use of both authentic phenolic standards and previously identified mango peel and coffee extract fractions. These included: 3-, 5- and 4-CQA, 4-FQA, quercetin-3-O-galactoside, and quercetin 3-O-glucoside. By keenly evaluating the fragmentation patterns and comparing the results with those in the literature, a methoxylated flavonoid, diosmetin, was identified in both VAF and VAP. It has a [M-H]$^-$ peak at *m/z* 299, and can be fragmented to [M-H-CH_3]$^-$ at *m/z* 284 and [M-H-CH_3-CO]$^-$ at *m/z* 256 [19]. Kaempferol was also present in both fractions. It has a [M-H]$^-$ ion at *m/z* 285 and

resulted in a daughter ion at m/z 242 by the loss of CO_2 to form ($[M-H-CO_2]^-$). Kaempferol 3-O-glucoside was identified in the phenolic acid fraction as $[M-H]^-$ at *m/z* 477. With tandem MS, it loses a hexose moiety (-162) and a daughter ion at m/z 285 was detected [20]. 4,5-diCQA was present in VAP and was not detected in VAF. Caffeic acid was detected in VAP, not in VAF. On the other hand, epicatechin and quercerin were identified only in VAP. Epicatechin has a $[M-H]^-$ ion at m/z 289 and lost 44 amu (CO_2) to form a daughter ion at *m/z* 245 [21,22].

There was a $[M-H]^-$ ion at *m/z* 549 detected in both fractions. By performing tandem MS, the parent ion resulted in a loss of CO_2 to give a fragment ion at m/z 505. MS^3 of the *m/z* 505 daughter ion resulted in fragment ions at *m/z* 463, 301, 271 and 255 with *m/z* 301 as the predominant peak, which could be caused by the loss of CH_2CO (42 amu), acetyl-glucose moiety (204 amu) and hexose moiety (162 amu). This fragmentation behavior was somewhat following literature data for quercetin 3-O-6"-malonylglucoside [23].

Also, the $[M-H]^-$ ion at *m/z* 821 was detected in both fractions. Fragmentation of this ion resulted in daughter ions at *m/z* 457 and 623. Further fragmentation of the base peak at *m/z* 457 resulted in fragmentation ions at *m/z* 429, 369, and 225 in the MS^3 spectrum. From the tandem MS spectra, the following mass fragmentation was observed: 232 amu (either malonyldeoxyhexose or succinyl pentose), 144 amu (loss of dideoxy-OCH3-hexose), 198, 364, 28, 108, 204 amu (acetylhexose), 60. The loss between m/z 821 to 369 could be due to the loss of two rhamnosyl (284 amu) moiety and one hexose moiety (162 amu). From the LIPIDS MAPS database, it would seem that fragmentation pattern to be following Epimedin C and the ion was tentatively identified as such [24,25].

Another ion in both the two fractions could not be positively identified as the $[M-H]^-$ ion at *m/z* 823. The collisionally induced dissociation (CID) on the parent ion resulted in *m/z* 763 in the MS^2 spectrum indicating a loss of 60 amu. Further CID of the *m/z* 763 ion resulted in fragment ions at *m/z* 719, 617, 573, and 455. The confirmed loss included 44 amu (763-719, CO_2), 102 amu (719-617), 146 amu (763-617) deoxyhexose also called rhamnose. Other fragmentations observed included 162 amu (617-455, hexose moiety), 308 amu (763-455, either dehydrated rutinose or coumaroyl glucoside) and 118 amu (573-455). The fragmentation seemed in accordance of that of ikarisoside C.

Several peaks in the flavonoids fraction (VAF) could not be identified. These were compounds with MS peaks at *m/z* 499 (peak 12), 1219 (peak 13), 463 (peak 15), and 504 (peak 16). As shown in Fig. 3b, *t*he peaks at *m/z* 1219 and 463 were also detected in VAP, which will be discussed in the VAP section. The *m/z* 499 peak bore a loss of 36 amu due to the loss of two water molecules, resulting in a MS^2 fragment ion at *m/z* 463 (possibly a quercetin derivative) and MS^3 ion of *m/z* 445 (loss of H_2O) and *m/z* of 433. On the other hand the *m/z* 504 peak lost 19, 163 and 169 amu to give MS^2 fragment ions at *m/z* of 485, 441 and 435.

For the phenolic acids fraction (VAP), Fig. 3c, the following compounds could not be identified: *m/z* 765 (peak 7), 381 (peak 10), 779 (peak 14), 1135 (peak 16), 1177 (peak 19), and 1219 (peak 20), 544 (peak 22) and 463 (peak 24), where *m/z* 1219 and *m/z* 463 were also detected in VAF. Briefly, *m/z* 765 (peak 7) ion lost 36 amu to give a MS^2 peak at *m/z* 729, and further lost 286 amu and 350 amu to give MS^3 peaks at *m/z* 443 and 373.

The compound with $[M-H]^-$ ion at *m/z* 779 (peak 14) lost 322 amu to form a daughter ion at *m/z* 457 (possibly indicative of an epigallocatechin gallate), and further lost 71 amu, 288 amu and 232 amu (could be due to malonyldeoxyhexose or succiylpentose) to give MS^3 peaks at m/z 386, 225 and 169 (gallate).The *m/z* 1135 ion (peak 16) resulted from a loss of 36 amu (loss of two water molecules) to give a MS^2 ion at *m/z* 1099 and further by losing 518 amu and 286 amu, to give MS^3 ions at *m/z* 813, 581 and 457 (possibly indicative of an epigallocatechin gallate).

The *m/z* 1177 peak (peak 19) on the other hand showed a loss of 36 amu (loss of two H_2O molecules) to form a daughter ion at *m/z* 1141, then further gave two MS^3 peaks at m/z 855 and 772 by losing 286 amu and 369 amu. The *m/z* 1219 ion (peak 20) showed a loss of 36 amu (loss of two H_2O molecules) to form a MS^2 peak at *m/z* 1183, and MS^3 peaks with *m/z* 855, 813 (predominant), 785, 753, 499 and 457. It is all apparent that peaks 14, 16, and 20 are compounds related in structure with fairly similar characteristic daughter ions especially, *m/z* 457 and m/z 813. The *m/z* 457 could be associated with epigallocatechin-3- gallate and as such the 3 peaks could be derivatives of epigallocatechin gallate.

Table 6. Identification of phenolic acids and flavonoids in VAF on C18 column

Peak no.	$[M-H]^-$ (m/z)	MS^2(*m/z*)	MS^3 (*m/z*)	UV λ_{max}(nm)	Identity
1	353	191		242, 322	3-caffeoylquinic acid
2	353	191		242, 322	5-caffeoylquinic acid
3	353	191/173		242, 322	4-caffeoylquinic acid
4	367			308	4-feruloyl quinic acid
5	463	301		254, 344	Quercetin 3-o-galactoside
6	463	301/179		254, 344	Quercetin 3-o-glucoside
7	549	505	463/301/271/255	254, 346	Could be quercetin 3-O-6”-malonylglucoside
8	515	353	191	248, 326	3,4-di-caffeoylquinic acid
9	515	353/173		248, 326	4,5-di-caffeoylquinic acid
10	821	623/457	429/369/225	243	Could be Epimedin C
11	285	241/199		256, 344	Kaempferol
12	499	463	445/433	250, 344	Unknown
13	1219	1183	855/813/785/753/499/457	242, 331	Unknown but suggested to be an epigllocatechin-3-gallate derivative
14	299	284	256/151	252, 266sh, 346	Diosmetin
15	927/499/463			252, 342	Unknown
16	504	485/441/435		274, 330	Unknown
17	823	763	719/617/455	243, 271, small hump at 326	ikarisoside C

Table 7. Identification of phenolic acids and flavonoids in VAP on C18 column

Peak no.	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	MS^3 (*m/z*)	UV λ_{max}(nm)	Identity
1	353	191		242, 321	3-caffeoylquinic acid
2	353	191		242, 321	5-caffeoylquinic acid
3	353	191/173		242, 321	4-caffeoylquinic acid
4	179	135		328	Caffeic acid
5	289	245/206		242, 273	Epicatechin
6	463	301		308	4-feruloyl quinic acid
7	765	729	443/373	243, 321	Unknown
8	463	301		254, 344	Quercetin 3-O-galactoside
9	463	301/179		254, 344	Quercetin 3-O-glucoside
10	381			243, 340sh	unknown
11	549	505	463/301/255	244, 328	Could be quercetin 3-O-6”-malonylglucoside
12	447	285		244, 345sh	Kaempferol 3-O-glucoside
13	No MS peak detected			244, 334sh	Unknown
14	779	457	386/225/169	247	Unknown but suggested to be an epigllocatechin-3-gallate derivative
15	515	353/173		248, 326	4,5-di-caffeoylquinic acid
16	1135	1099	813/581/457	248	Unknown but suggested to be an epigllocatechin-3-gallate derivative
17	821	623/457	429/369/225	244	Could be Epimedin C
18	285	242	N/A	256, 344	Kaempferol
19	1177	1141	855/772	244, small hump at 334	Unknown
20	1219	1183	855/813/785/753/499/457	242, 331	Unknown but suggested to be an epigllocatechin-3-gallate derivative
21	301	179/151		242, 309	Quercetin
22	544			242, a 331 hump	Unknown
23	299	284	256	243, 266sh, 343	Diosmetin
24	927/499/463			252, 268, 342 max	unknown quercetin conjugate
25	823	763	719/617/573/455	243, 271, small hump at 326	Ikarisodide C

Bold: Most intense ion

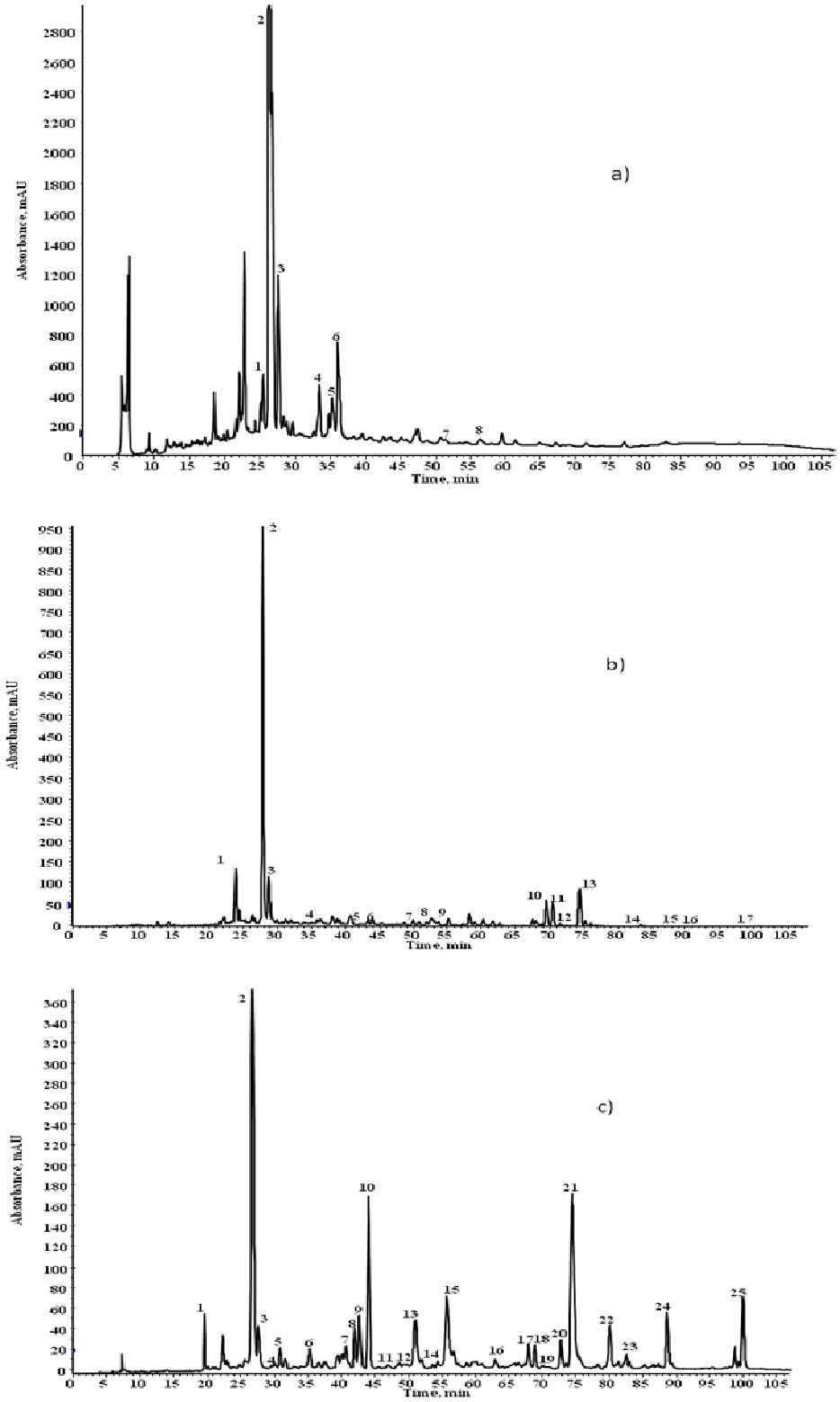

Fig. 3. HPLC UV chromatograms for a) Coffee extract; b) *Vangueria madagascariensis* flavonoid fraction (VAF); c) *Vangueria madagascariensis* phenolic acids fraction (VAP)

Table 8. Antimicrobial activity of *Vangueria madascariensis* fruit extract at different concentrations expressed as inhibition zone calculated in mean diameter around the disc in mm. Any diameter above 6 mm indicates some degree of inhibition

Extract concentration (mg/mL)	c.a	p.a	s.a	E.c	B.s	Gentamicin
100	10.5 ± 0.7	12±0.7	10 ±0.8	11.5 ±0.7	12 ± 0.9	B.s = 20.6 ± 0.4
10	9 ± 0.6	10.5±0.7	7.5 ± 0.3	9 ± 0.6	10.5 ± 0.7	E.c=22.0 ± 0.3
1	7 ± 0.0	9.5±0.6	7.5 ± 0.4	10 ± 0.7	12.5 ± 0.9	s.a =21.0 ± 0.6
0.1	6 ± 0.0	7.5 ± 0.3	7.5 ± 0.4	10.5 ± 0.8	12.5 ± 0.9	p.a = 18.7 ± 0.3
						c.a =21.7 ± 0.3

c.a- Candida albicans, p.a- Pseudomonas aerugenosa, s.a- Staphylococcus aureus, E.c- Escherichia coli, B.s - Baccilus subtilis

Finally, peak 24 showed MS^1 of 927, 499, and 463 with the latter being predominant, which would probably be dehydrated (loss of 2- H_2O molecules) derivative of m/z 499. It seems 926, could be a dimer of 463.

3.3 Antimicrobial Susceptibility Test Results against the Test Organisms

The results of antimicrobial activity of the combined VAF and VAP extracts at different concentrations are shown in Table 8. Notably, the *Vangueria madagascariensis* shows very significant inhibition for all the microorganisms tested at high extract concentration. The inhibition while still strong was least for *Candida albicans,* and decreased with the dilution of the plant extract. At 0.1 mg/mL the extract barely had any inhibitory properties against *Candida albicans.* A similar decrease with dilution was observed with *Staphylococcus aureus.* The inhibition against *Pseudomonas aerugenosa* was very considerable at 100 mg/mL and slowly decreased with dilution, even at 0.1 mg /mL some inhibition of 7.5 ± 0.3 was observed. For both *Escherichia coli and Bacillus subtilis*, the extract maintained appreciably high inhibitions even at the lowest concentrations of 0.1 mg/ml. This could attest to the potency of the extract against these microorganisms. The strong microbial inhibitory effects were not unexpected especially after considering the chemical composition of the VAF and VAP elucidated using LC-MS/MS. It is evident in the literature [26,27] flavonoids have significant microbial activity.

4. CONCLUSION

Phenolic acids and flavonoids from *Vangueria madagascariensis* were separated and identified using HPLC-DAD-ESI-MS. Numerous phenolic compounds were identified, but several remained still unidentified. The fruit extracts demonstrated significant antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans.* The high phenolic content and antimicrobial potency make the fruit a potential functional food. To fully identify all the compounds, it would be necessary to collect the fractions of the extracts after HPLC analysis and carry out NMR studies to elucidate the structures of the unidentified species. The antioxidant activity of the extracts can be further evaluated using well-known chemical assays and facile electroanalytical techniques.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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