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Dermatologic photoprotective potential of *Sutherlandia frutescens extracts*

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Abstract: This work explores the dermatologic photoprotective potential of the phenolic acids and antioxidant activity of the plant *Sutherlandia frutescens* subspecies *microphylla* commonly known as the cancer bush (CB). The medicinal value of CB and its reported role in the management of chronic ailments like HIV/AIDS generates interest for the identification and quantitation of the total phenolic acid content. The antioxidant properties of phenolic acids are known to reduce the risk of chronic diseases including cancer and heart sicknesses linked to oxidative stress. Phenolic acids were extracted from the leaves of the CB by Soxhlet (SXE) and ultrasonication (USE) extraction methods. These extracts were analysed by ultraviolet (UV) spectroscopy, high performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (RP-HPLC-PDA-ESI-MS). Six phenolic acids were identified and quantitated by RP-HPLC-PDA, under isocratic elution conditions with an external standard method. The identified phenolic acids were: gallic, *p*-hydroxybenzoic, vanillic, caffeic, syringic and *p*-coumaric acids. The concentration of p-coumaric acid was the highest in all the extracts. RP-HPLC-PDA-ESI-MS was used to characterise three novel phenolic acids: 5-hydroxy-2-vinylbenzoic acid, an isomer of *p*-coumaric acid (**C-1**); (Z)-3-(4-hydroxy-2-methoxyphenyl)acrylic acid (**C-2**); and (Z)-2-hydroxy-3-(4-methoxyphenyl)acrylic acid (**C-3**) ferulic acid isomers. The Folin-Ciocalteu protocol was used to determine the total phenolic content of the extracts. The ultrasonication-diethyl ether (USDE) fraction gave GAE = 0.1247 mg g^{-1} and ultrasonication-ethyl acetate (USEA) GAE = 0.0769 mg g^{-1} as the highest and lowest total phenolic content respectively. Antioxidant activity was investigated by the DPPH free radical scavenging assay and the FRAP assay. The USDE extract (EC_{50} = 30.38µg mL^{-1})and the Soxhlet-diethyl ether extract (SXDE) (EC_{50} = 48.63µg mL^{-1})exhibited the highest and lowest antioxidant activity by DPPH assay respectively. The FRAP assay showed higher activity for USDE (EC_1= 41.53 µg mL^{-1}) and lower value for SXDE extract (EC_1= 33.05 µg mL^{-1}). The CB extracts with higher phenolic content had higher antioxidant activity and are thus a suitable remedy for free radical mediated ailments. Also the UV-vis spectra of the CB extracts had significant absorption in the UV region, and hence are viable ingredients in sunscreen preparations.

Keywords: *Sutherlandia frutescens,* radicals, antioxidants, phenolic acids, UV-photoprotection.

1 Introduction

Qualitative and quantitative investigations of the phenolic acid content of plants are of great interest due to their antioxidant properties especially for reported medicinal plants. Several working groups have reported the anti-inflammatory, antiseptic, antibiotic, antitumour and antioxidant properties of phenolic acids (Tarnawski et al. 2006; Baublis et al. 2000; Arimboor et al. 2008). The antioxidant properties of phenolic compounds draw attention for research because of their effect in preventing diseases related to oxidative stress (Yashin et al. 2011). Antioxidants have also been shown to be inhibit the formation of ultraviolet B (UVB) induced cyclopyrimidine dimers in human HaCaT cells(Guahk et al. 2010; Thongrakard et al. 2013). These dimers are the precursor lesions to skin cancer. Antioxidants are also known to offer systemic protection by stimulating cellular defence mechanisms(Thongrakard et al. 2013), remaining active for days. A body is considered to be under oxidative stress when there are excess reactive oxygen species (ROS) or reactive nitrogen species (RNS) conditions relative to its endogenous antioxidant capacity. This excess leads to "oxidation" of a variety of biomacromolecules, such as enzymes, proteins, DNA and lipids (Dai and Mumper 2010; Marxen et al. 2007). The oxidation of these biomacromolecules is linked to health complications such as cancer, heart disease, rheumatoid arthritis, inflammatory bowel disease, ageing and cataracts (Tarnawski et al. 2006; Dai and Mumper 2010). Humans can be exposed to oxidative stresses by exposure to pollutants and UV radiation; by smoking cigarettes; by ingestion of oxidized or burnt foods; and from cellular metabolism (Tarnawski et al. 2006; Baublis et al. 2000). These are initiators of ROS such as the hydroxyl radical ($^{\bullet}OH$); superoxide anion ($^{\bullet}O_2^-$); and hydrogen peroxide (H_2O_2) (Tarnawski et al. 2006; Marxen et al. 2007).

To prevent an imbalance between reactive oxidising species and the body's natural antioxidant capacity

requires dietary antioxidant supplements (Baublis et al. 2000; Tarnawski et al. 2006; Paulo et al. 1999). The proposed mechanisms linked to the antioxidant properties of phenolic compounds include scavenging radical species, the suppression of ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production; and the protection of antioxidant defence (Dai and Mumper 2010). As antioxidants, phenolic acids enhance the protection against the above mentioned diseases by scavenging free radicals in the body (Baublis et al. 2000; Tarnawski et al. 2006; Cvetkovic and Markovic 2011). In general, phenolic compounds have been found to be more potent antioxidant s*in vitro* than vitamin C and E and carotenoids (Baublis et al. 2000). For example, caffeic acid has been found to inhibit intracellular free radical production, not achievable with vitamin C (Kadoma and Fujisawa 2008). Epidemiological data show that the presence of phenolic acids in the diet can act as a preventive measure for various diseases (Biglari et al. 2008; Ramos 2008).

Phenolic acids are aromatic carboxylic acids, containing a single benzene ring bearing hydroxyl or methoxyl substituents. They are generally classified into two groups: benzoic acid derivatives and cinnamic acid derivatives (Fig. 1). Structurally, they can be distinguished by the number and position of the hydroxyl or methoxyl substituents on the benzene ring of benzoic acid. They are plant secondary metabolites, for fighting external stresses including pathogens, predators, UV radiation, mechanical damage, and low temperature conditions (Stalikas 2007). A commonly known phenolic acid is salicylic acid (*m*-hydroxybenzoic acid), an active signal molecule in plants.

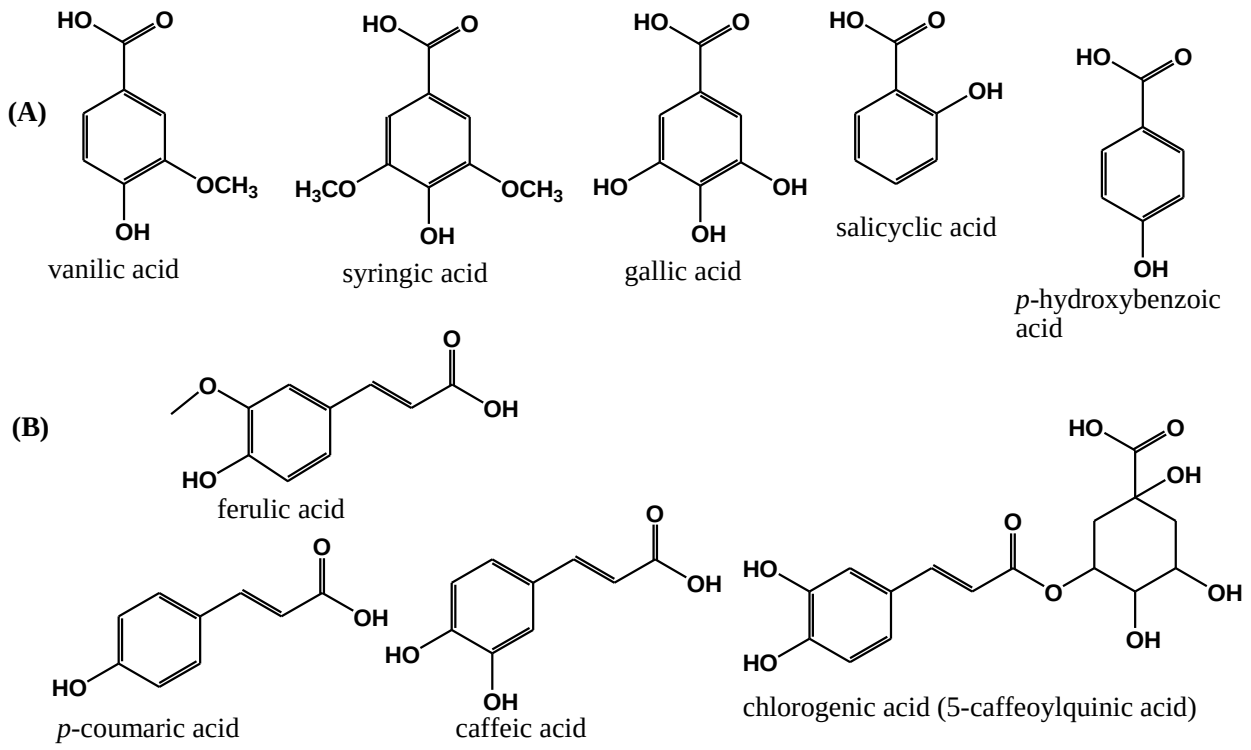


Figure 1: Structure of some phenolic acids: (A) benzoic acid derivatives; (B) cinnamic acid derivatives.

Sutherlandia frutescens (Fig. 2) is a medicinal plant, indigenous to dry parts of Southern Africa, and occurs mainly in the Western Cape up to Namibia and Botswana and in the western Karoo up to the Eastern Cape (Shaik et al. 2010). In South Africa it has various names such as *kankerbos* (Afrikaans), cancer bush (CB), and *unwele* (Zulu) (Shaik et al. 2010; Directorate of Plant Production 2009). The name cancer bush emanates from the ethnopharmacological belief that it cures cancer (Shaik et al. 2008). It serves different purposes including: washing of wounds and the treatment of colds, flu, rheumatism, bronchitis and dysentery. It is a reputed immune booster in the treatment of HIV/AIDS (Shaik et al. 2010; Shaik et al. 2008; Directorate of Plant Production 2009). The therapeutic effect of the cancer bush like in many other herbal medications is related to the presence of polyphenols. The role of polyphenols as antioxidants has been widely reported especially their ability to

modify immune cell functions. The antioxidant activity of the cancer bush has previously been demonstrated by Fernandes et al. (2004) but a comparison of the total phenolic content to antioxidant activity has not been exhaustively reported. Therefore, an investigation of the cancer bush phenolic acid content and the relationship with the antioxidant activity is relevant, given its popularity in Southern African traditional medicine. In this work, phenolic acids present in the leaves were extracted, identified, quantified and their antioxidant activity and photoprotection ability investigated.

Figure .2: Leaves, pods and flower of *Sutherlandia frutescens* (Directorate of Plant Production 2009).

2 Experimental

2.1Materials and Equipment

Leaves from the *Sutherlandia frutescens* (family: *Fabaceae*) plant were harvested and air-dried in the shade in the vicinity of Murraysburg in the Karoo, South Africa by W. Grobler. The plants were identified as *Sutherlandia frutescens* (L.) R. Br. Var. *microphylla* (Burch. Ex DC) Harv., by Professor B.-E. van Wyk of the Botany and Biotechnology Department of the University of Johannesburg [voucher specimen from W. Grobler: C. Albrecht s.n. sub. B.-E. van Wyk 4126 (JRAU)]. The phenolic acid standards supplied were: gallic acid (Hopkin and William), *p*-hydroxybenzoic acid (Aldrich Chemicals), vanillic acid (Merck kGaA), caffeic acid (Sigma-Aldrich), syringic acid (Sigma-Aldrich) and *p*-coumaric acid (Sigma-Aldrich) were all supplied at high purity (> 99%).2,2-Diphenylpicryl-1-hydrazyl (DPPH) was obtained from Aldrich, 2,4,6-tripyridyl-s-triazine (TPTZ) was purchased from Merck KGaA, ammonium ferrous sulphate was from BDH, ferric chloride from UniLAB, glacial acetic acid was from ACE, anhydrous sodium carbonate and sodium sulphate from BDH Chemicals Ltd, and Folin-Ciocalteu phenol reagent from Merck kGaA and acetic acid was from Sigma-Aldrich. The solvents used were deionised water obtained from a Millipore Milli-Q® water purification system (Millipore, Bedford, MA, USA), methanol (BDH Prolabo), ethanol (Sigma-Aldrich), diethyl ether (DE) (Sigma-Aldrich), ethyl acetate (EA) (SMM Instruments) and petroleum ether (Sigma-Aldrich).

2.2 Sample preparation, extraction and purification of phenolic acids

A sample of dried CB leaves (84 g) was ground to a fine powder by using a mechanical grinder. To obtain crude extracts two methods were employed: Soxhlet extraction (SXE) and an ultrasonication (USE) method. After the crude extraction in methanol and soaking the extract in water, diethyl ether (DE) and ethyl acetate (EA) were used to extract the phenolic acids from the aqueous phase. Both DE and EA have been used extensively in literature for the extraction of phenolic acids, giving similar extraction efficiencies (Stalikas 2007). In this work, both solvents were used in order to compare their effectiveness in isolating phenolic acids from the rest of the methanolic extract.

2.2.1 Soxhlet extraction

About 20 g of dry CB powder was extracted with approximately 100 mL of methanol by means of Soxhlet extraction. The extraction was carried out for 18 hours, and then the crude extract was filtered through Munktell grade (3hw) filter paper under gravity into a clean pre-weighed round bottomed flask. The methanol was removed from the crude extract by

means of rotary evaporator to dryness under vacuum at 56 °C to give a dark green tar-like residue. The extractible amount of the crude phenolic extract by this method was calculated on a dry weight basis (Table 1) by using equation 1.

$$\% \text{ yield } = \frac{\text{weight of dry extract}}{\text{weight taken for extraction}} \times 100 \qquad \text{equation 1}$$

2.2.2 Ultrasonic extraction

About 10 g of CB was placed in conical flask and 50 mL of methanol was added. The mixture was ultrasonicated for 30 min and then filtered under gravity through a Munktell grade (3hw) filter paper. The procedure was repeated with a further 10 g of CB. The filtrates from the two extractions were combined and the solvent was evaporated by means of a rotary evaporator under vacuum conditions at 56 °C. The percentage amount extracted by this method was similarly calculated from equation 1.

2.2.3 Liquid-liquid extraction

A volume of about 120 mL of boiling water was added to each the crude Soxhlet and ultrasonication extracts in a round-bottomed flask and left to stand for 16 hours to allow water extractible phenolics to dissolve. The solutions were filtered through What man No. 1 filter paper under gravity. The filtrates were then divided into two portions and re-extracted with 30 mL portions of petroleum ether (PE) six times in order to remove lipophilic components. After extraction with PE, half of the aqueous phase from SXE or USE was re-extracted with 6 × 30 mL diethyl ether (DE) and the other half with 6 x30 mL ethyl acetate (EA). The EA or DE layers were dried by adding some anhydrous sodium sulphate (Na_2SO_4). The DE or EA was removed from the extract by rotary evaporation under vacuum, at temperatures of about 30 ºC for DE and about 45 ºC for EA. Each residue was reconstituted in methanol to achieve a concentration of approximately 12.5 mg mL^{-1}.

2.3 HPLC separation and quantification of phenolic acids

Shimadzu LC-20 AD XR liquid chromatograph fitted with Zorbax Eclipse XDB C-18 column of dimensions 4.6 × 150 mm, 5 µm particle size, with a photodiode array (PDA) detection was used for identification and quantitation of the phenolic acids. The phenolic extracts were analysed by isocratic elution with a mobile phase consisting of 2 % (v/v) acetic acid in water-methanol 88:12 (v/v), and the flow rate was 1.00 mL min^{-1}. The column temperature was 25 °C. A 500 µL aliquot of each CB extract was diluted with an equal volume of mobile phase and a 10 µL volume of this resultant solution (now approximately 6.25 mg mL^{-1}) was injected into the chromatograph. The chromatograms were detected at 255, 260, 271, 274, 309 and 323 nm. All samples and standards were filtered through 0.45 µm Millipore Millex-LCR syringe filters before being injected into the chromatograph. The identification of phenolic acids was done by matching the retention time and UV spectra of the extract components with those of six phenolic acid standards. The quantitation of identified phenolic acids was done by an external calibration method. Stock solutions of each of the six acids containing approximately 10^3 mg dm^{-3} were prepared in methanol. Aliquots of these standard stock solutions were diluted with the mobile phase to obtain multi-standard solutions with approximate concentration of 10, 20, 80 and 100 mg dm^{-3} of each acid. These solutions were used to obtain the calibration curves for each standard acid. Method validation was done by spiking extracts with 10 µL of each of the six phenolic acid stock solutions.

2.4 Identification of novel phenolic acids by RP-HPLC-PDA-ESI-MS/MS

A reverse phase, Zorbax Eclipse-XDB C-18 column of dimensions 150 mm × 4.60 mm, 5 µm particle size protected with a 4 mm × 4 mm Zorbax Eclipse-XDB guard column under isocratic conditions of 12 % methanol; 88% water with 2 %acetic acid was used to achieve chromatographic separation at a flow rate of 1 mL min^{-1} and with 50 µL injection volumes. The HPLC system consisted of an Agilent 1100 series equipped with an Agilent 1100 series photo diode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A auto sampler, a G1322A degasser and a G1315B photodiode array detector controlled by ChemStation software (Agilent, v.08.04). The chromatograms were detected at 255, 260, 271, 274, 309 and 323 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.4.1). The nebulizing gas was nitrogen set at a pressure of 65 psi and a flow rate adjusted to 116 mL min^{-1}. A heated capillary and voltage was maintained at 350 °C and 4 kV respectively. The detector was programmed to scan masses in the range *m/z* 90 up to *m/z* 2000. All collision-induced fragmentation experiments were performed in the ion trap with helium as collision gas, with the voltage being ramped in cycles from 0.3 up to 2 V. MS^2 data were acquired in the negative ionization automatic smart mode to get MS^{n-1}; primary precursor

ion. MS^3 data were obtained by manual fragmentation, targeting the most abundant ions in the precursor ion MS spectra. Targeting much lower abundant mass values on MS^3 only yielded the primary precursor ion of the series. Frequent characteristic fragment ions shown in Table 11.2 were used to elucidate the structures of compounds C-1, C-2 and C-3.

2.5 Determination of total phenolic content

The determination of the total phenolic content of each extract was done by using the Folin-Ciocalteu assay. A 150 µL of extract, 2400 µL of Millipore water and 150 µL of 0.25 N Folin–Ciocalteu reagent were combined in a plastic vial and then mixed thoroughly. The mixture was allowed to react for 3 min and then 300 µL of 1 N Na_2CO_3 solution was added and mixed well. The solution was incubated at room temperature (25 °C) in the dark for 2 hr. The absorbance was measured at 765 nm with a Perkin Elmer Lambda 35 UV-Vis dual beam spectrophotometer and the results were expressed in gallic acid equivalents (GAE; mg g^{-1} dry mass) based on an external calibration of gallic acid standards ranging from 50 mg $dm^{-3}$to 500 mg dm^{-3}. The measurements for both gallic acid standards and the samples were done in triplicate.

2.6 DPPH scavenging assay

The free radical scavenging activity of the extracts was assessed by using the 2,2-diphenylpicryl-1-hydrazyl (DPPH) assay according to the method reported by Blois (1958). The reaction mixture contained 1.8 mL of 0.1 mM DPPH methanolic solution and 0.2 mL of each serial dilution of cancer bush extracts. Simultaneously a control was prepared without sample extract and both reaction mixture sets were incubated at room temperature for 1 hour in the dark. The antioxidant activity of each fraction was quantitated by the loss in colour at 522 nm on a Perkin Elmer Lambda 35 UV-Vis dual beam spectrophotometer. The percentage DPPH scavenged was calculated by using equation 11.2, where A_{control} is the absorbance of the solution containing only DPPH diluted with the solvent, and A_{sample} is the absorbance of the DPPH solution after incubation with different concentrations of the CB extracts.

$$\%\ \text{DPPH scavenged} = \frac{A_{control} - A_{sample}}{A_{control}} \text{x } 100 \qquad \text{equation 2}$$

The percentage DPPH scavenged and the absorbance due to the remaining DPPH were plotted against the volume of each extract. The EC_{50} value for each extract was obtained by reading off the linear section of the curve.

2.7 FRAP Antioxidant Assay

The FRAP assay was performed according to the protocol described byBenzie and Strain (1996). The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \bullet 3H_2O$ and 16 mL $C_2H_4O_2$) of pH 3.6, 10mM 2,4,6-tripyridyl-s-triazine (TPTZ)solution in 40 mM HCl, and 20 mM $FeCl_3 \bullet 6H_2O$ solution. A fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \bullet 6H_2O$ solution. The standards were then incubated for 4 and 30 minutes at 37 ºC in a water bath before analysis in a 1 cm pathlength glass cuvette with Perkin Elmer lambda 25 UV-vis spectrophotometer fitted with a Peltier temperature controller set at 37 ºC. The absorbance of the solutions were measured at 596.00 nm. Standard graphs were constructed using known concentrations of ammonium ferrous sulphate dissolved in 80 % (v/v) aqueous methanol. All tests were done in triplicate and mean values were used to calculate EC_1 values. EC_1 is defined as concentration of an antioxidant having a ferric reducing ability equivalent to that of mM ferrous salt (Sarla et al. 2011). An aliquot of 5 mg mL^{-1} solution of cancer bush extracts (150 µL) were allowed to react with 2850 µL of the FRAP solution for 4min and 30 min in the dark condition before absorbance measurements were taken.

2.4 Potential role of phenolic acid extracts in photoprotection

The potential role of the CB extracts in photoprotection was investigated by recording the UV-vis spectra of each extract. The UV-vis spectrum of a mixture of the six phenolic acids was also measured for comparison. All UV spectra were recorded on a Perkin Elmer Lambda 35 UV-vis dual beam spectrophotometer. For this experiment, the CB extracts and the phenolic acid standard solutions were diluted with methanol to achieve concentrations of 0.0625 mg mL^{-1} and 0.005 mg mL^{-1} respectively.

3 Results and Discussion

The extraction of phenolic acids from the CB leaves was carried out by both Soxhlet extraction (SXE) and ultrasonic extraction (USE), due to the sample matrix dependence of phenolic acids (Waksmundzka-Hajnos et al. 2007). An additional step was introduced to remove lipophilic components so as to avoid masking the HPLC determination of phenolic acids (Ćetković et al. 2004). The effect of pH on the extraction of phenolic acids by releasing ester bound phenolics (Ayaz et al. 2005) was investigated and compared with un-acidified samples (Table 11.1). A comparison of the

percentage yields of crude extracts indicated the USE yield to be higher than the SXE yield. The yields of purified extracts from the two solvents (Table 11.1) show that re-extraction with ethyl acetate (EA) has a higher yield of extract than re-extraction with diethyl ether (DE) for all extraction methods. Acidified extracts: UHDE, SHDE, and SHEA showed a yield increase effect for SXE with EA (SXEA; 0.91% and SHEA; 1.58%) and a decrease in yield for DE (SXDE; 0.63% and SHDE; 0.48%) (Table 1).

Table 1: Yield of crude extract and purified extract obtained from each extraction method.

Method	Solvent for LLE	Extract	Mass of dried CB powder used/g	Mass of crude (methanolic) extract/g	Mass of purified extract/g	% Yield of crude extract/g	% Yield of purified extract/g
USE	DE	USDE	10.23	2.68	0.0442	26.2	0.43
USE	EA	USEA	10.23	2.68	0.0742	26.2	0.73
USE	DE*	UHDE	10.24	3.02	0.0808	29.5	0.79
SXE	DE	SXDE	10.06	1.94	0.0629	19.3	0.63
SXE	EA	SXEA	10.06	1.94	0.0919	19.3	0.91
SXE	DE *	SHDE	10.16	1.60	0.0492	15.7	0.48
SXE	EA *	SHEA	10.16	1.60	0.1601	15.7	1.58

* pH was adjusted to 2.1 with HCl before purification by LLE.

RP-HPLC-PDA quantitation was based on ultraviolet (UV) spectra and retention times (RT) of the phenolic acid standards after optimising column conditions (Fig. 3). Each phenolic acid was identified and quantitated at its wavelength of maximum absorption. Diluting the standards and extract samples with the mobile phase gave better peak profiles with baseline resolution (Figure 11.4 and 5).

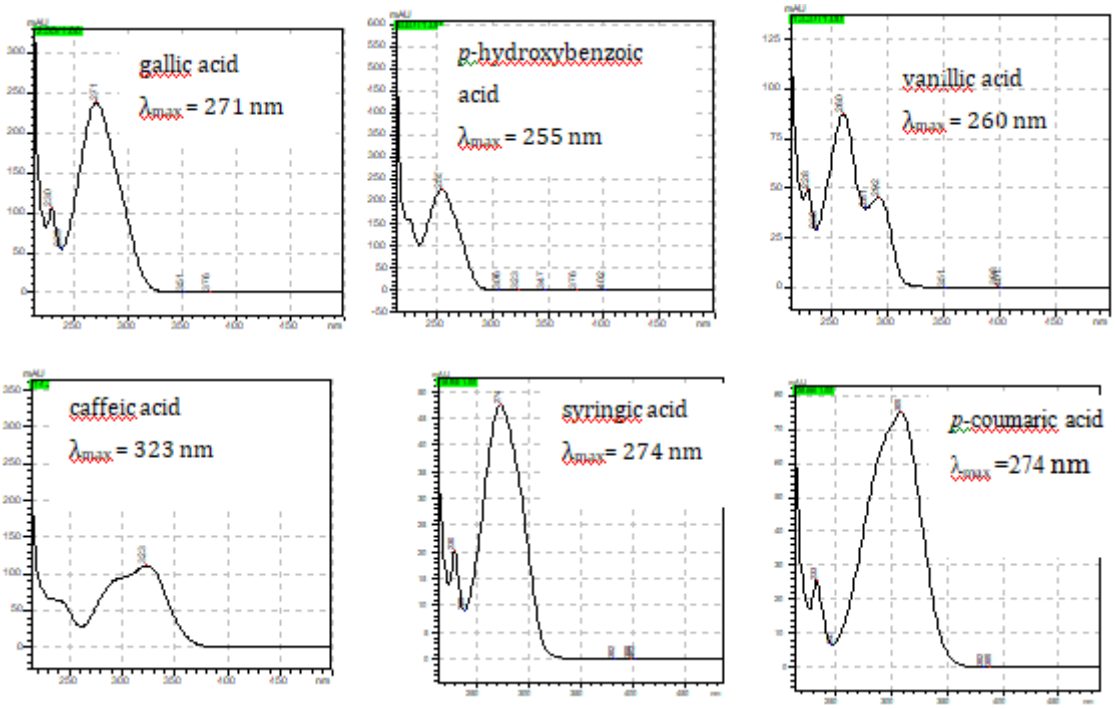


Figure 3: UV spectra of the six phenolic acid standards recorded by the PDA detector. The separation was effected on a reversed-phase, Zorbax Eclipse-XDB C-18 (150 mm × 4.60 mm, 5 µm particle size) column protected with a 4 mm × 4 mm Zorbax Eclipse-XDB guard column under isocratic conditions of 12 % methanol; 88 % water with 2 % acetic acid the flow rate was 1.00 mL $min^{-1}$and the injection volume was 10 µL.

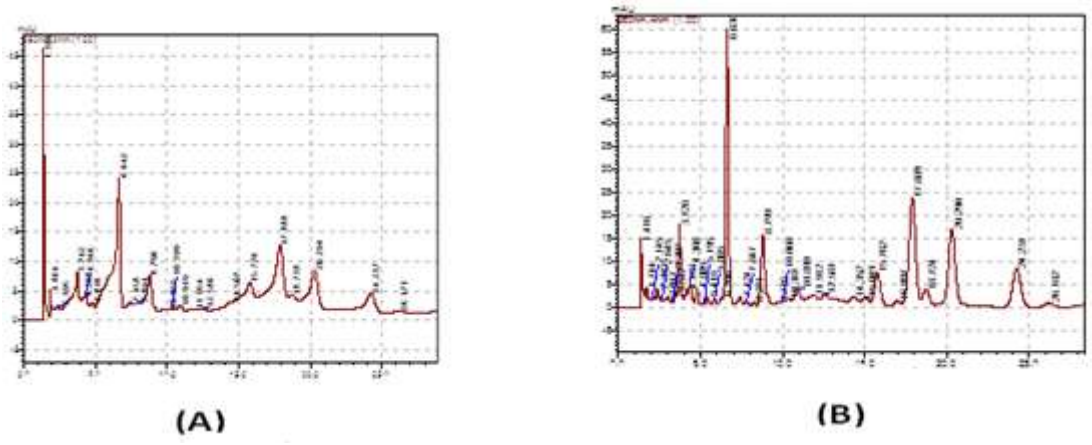


Figure 4: Comparison of partial HPLC chromatograms of the SXDE extract diluted in MeOH (A), and in the mobile phase (B). The separation was effected on a reversed-phase, Zorbax Eclipse-XDB C-18 (150 mm × 4.60 mm, 5 µm particle size) column protected with a 4 mm × 4 mm Zorbax Eclipse-XDB guard column under isocratic conditions of 12 % methanol; 88 % water with 2 %acetic acid, the flow rate was 1.00 mL $min^{-1}$and the injection volume was 10 µL.

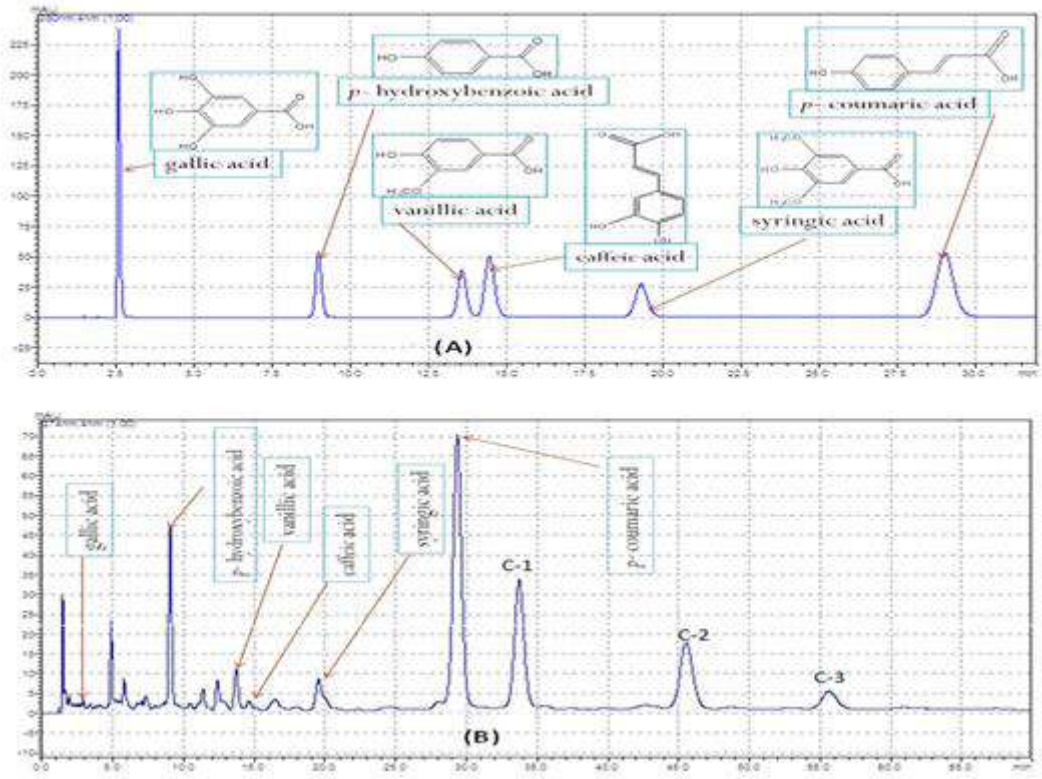


Figure 5: HPLC chromatograms of six phenolic acid standards monitored at 280 nm (A), and of the SXDE extract monitored at 274 nm (B). The labelled phenolic acids were identified by matching the retention times and UV spectra of the extract and of the phenolic acid standards. The separation was effected on a reversed-phase, Zorbax Eclipse-XDB C-18 (150 mm × 4.60 mm, 5 µm particle size) column protected with a 4 mm × 4 mm Zorbax Eclipse-XDB guard column under isocratic conditions of 12 % methanol; 88 % water with 2 %acetic acid, the flow rate was 1.00 mL $min^{-1}$and the injection volume was 10 µL.

The HPLC chromatogram of SXDE showed three other prominent peaks at 274 nm (Fig. 5) with unique UV spectra (Fig. 6). These three new compounds had retention times 33.2 min, 44.2 nm, and 53.3 nm. Characterisation targeting these peaks on HPLC-DAD-ESI-MS^n revealed the presence of a *p*-coumaric acid isomer (peak **C-1**) and two ferulic acid isomers (peaks **C-2** and **C-3**) (Figs. 5 and 6). Each peak had unique MS fragmentation pattern allowing for differentiation (Fig.7, 8 and 9) and structure speculation. An isomer of *p*-coumaric acid, 5-hydroxy-2-vinylbenzoic acid, (**C-1**); and (Z)-3-(4-hydroxy-2-methoxyphenyl)acrylic acid (**C-**

2), and; (Z)-2-hydroxy-3-(4-methoxyphenyl)acrylic acid (**C-3**), both isomers of ferulic acid were similarly elucidated by manual target ion fragmentation (Fig. 10). An MS^3 mode targeting smaller molecular weights did not yield tangible mass fractions hence the MS^2 precursor ion was used (Table 2). This could be attributed to low currents and, hence, the low field frequencies of MS^3 mode.

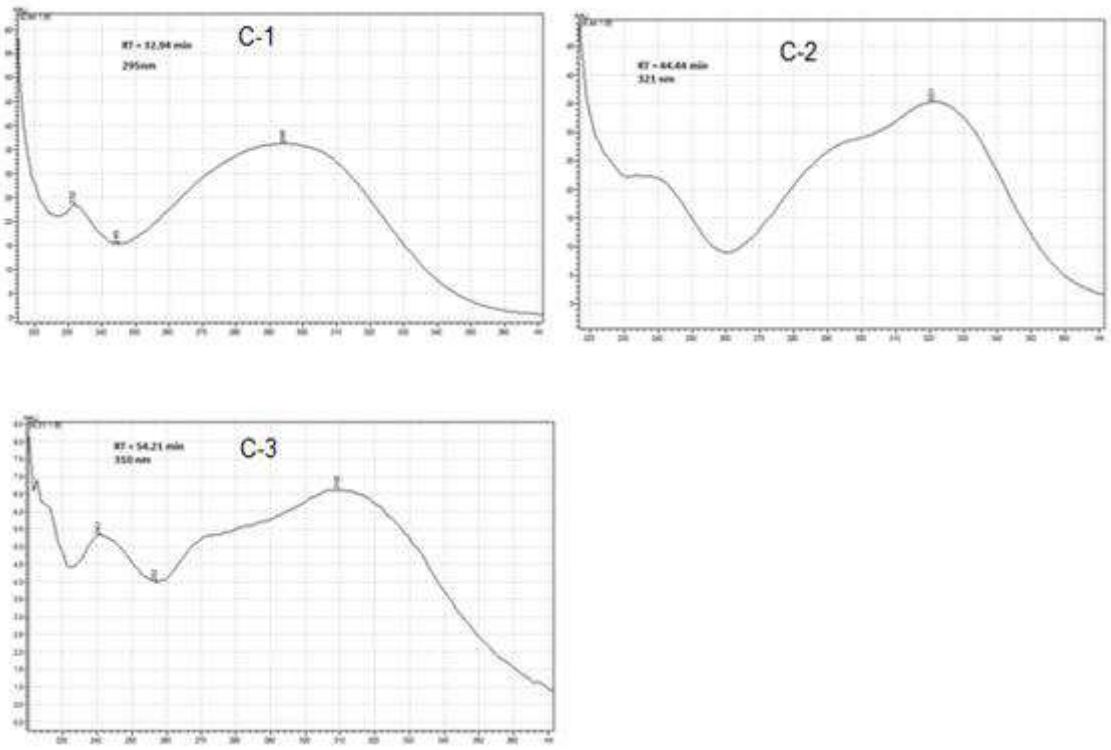


Figure 6: The UV spectra ofpredicted compounds C-1, C-2 and C-3 of the SXDE extracts. The separation was effected on a reverse phase, Zorbax Eclipse-XDB C-18 (150 mm × 4.60 mm, 5 µm particle size) column protected with a 4 mm × 4 mm Zorbax Eclipse-XDB guard column under isocratic conditions of 12 % methanol; 88 % water with 2 %acetic acid, the flow rate was 1.00 mL $min^{-1}$and the injection volume was 10 µL.

Table 2: MS^n fragmentation pattern of three phenolic acids.

Compound	RT/min	MS^2 $[M-H]^-$	MS^3 $[(M-H)^- \rightarrow (M-H-X)^-]$	MS^3 $[(M-H)^- \rightarrow (M-H-Y)^-]$	MS^3 $[(M-H)^- \rightarrow (M-H-Z)^-]$
C-1	33.2	164	119	134	75.2
C-2	44.2	194	137	117	75.2
C-3	53.3	194	149	117	75.2

*Masses that were not structurally helpful are not considered.

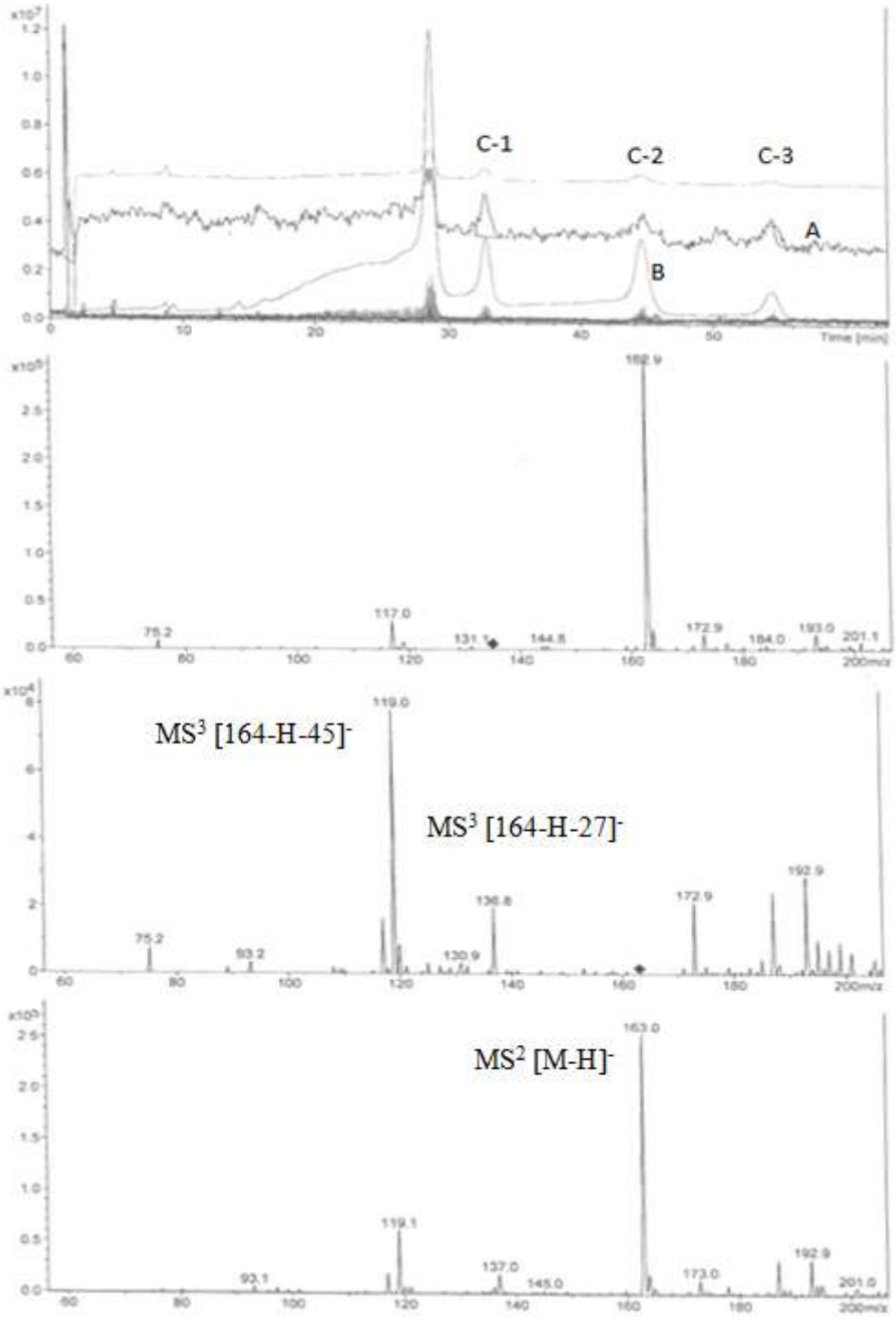


Figure 7: MS^n analysis of 5-hydroxy-2-vinylbenzoic acid (C-1) in the negative mode. MS^2 $[M-H]^-$; MS^3 $[M-H]^-$ → MS^3 $[164-H-27]^-$; MS^3 $[164-H-45]^-$. A is the and total ion mass spectrum and B is theHPLC chromatogram monitored at 309 nm respectively

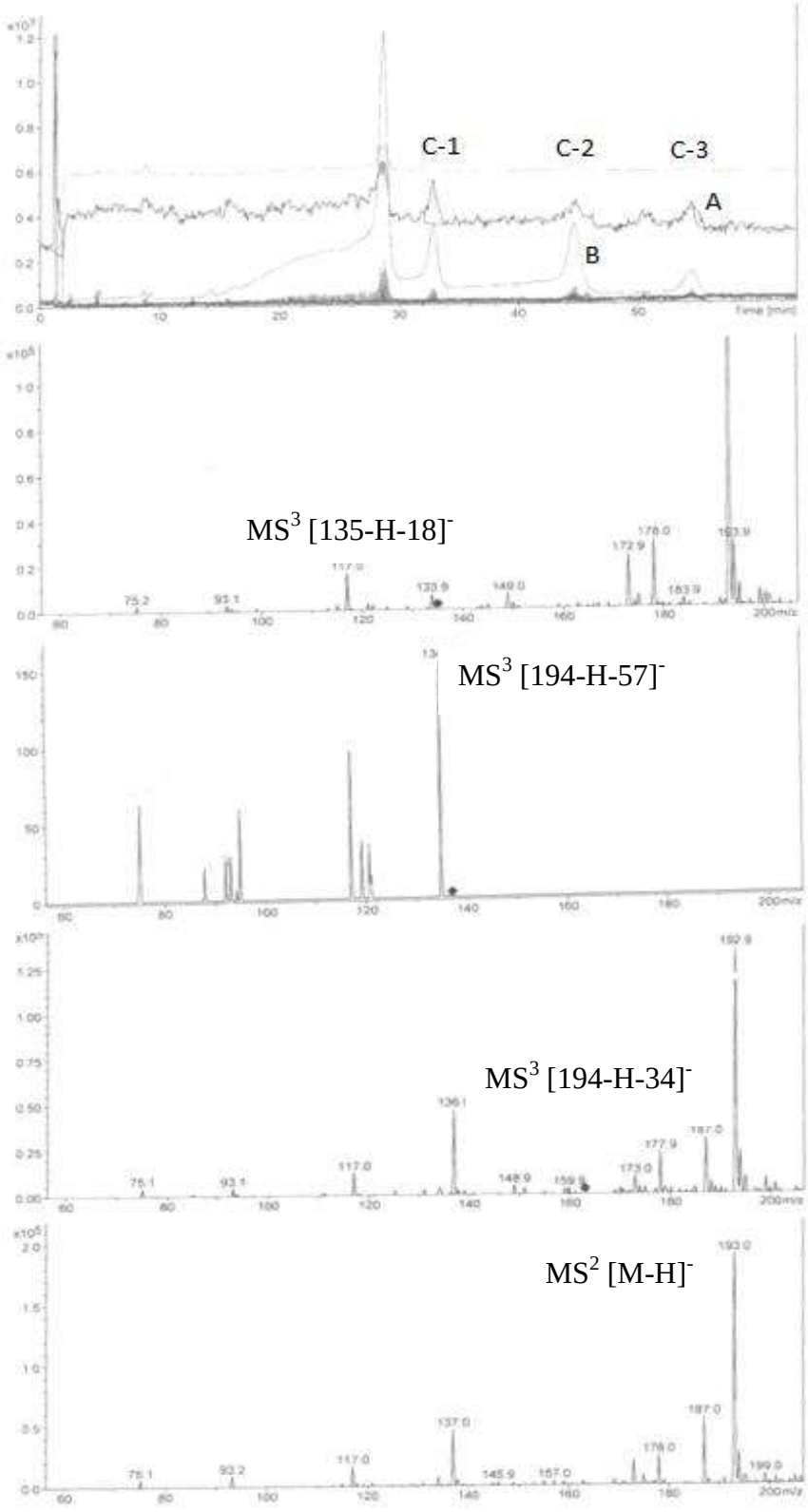


Figure 8: MS^n analysis of (Z)-3-(4-hydroxy-2-methoxyphenyl)acrylic acid (C-2) in the negative mode. MS^2 $[M-H]^-$; MS^3 $[M-H]^-$ →MS^3 $[194-H-31]^-$; MS^3 $[194-H-57]^-$; MS^3 $[135-H-18]^-$. A is the total ion mass spectrum and B is theHPLC chromatogram monitored at 309 nm respectively

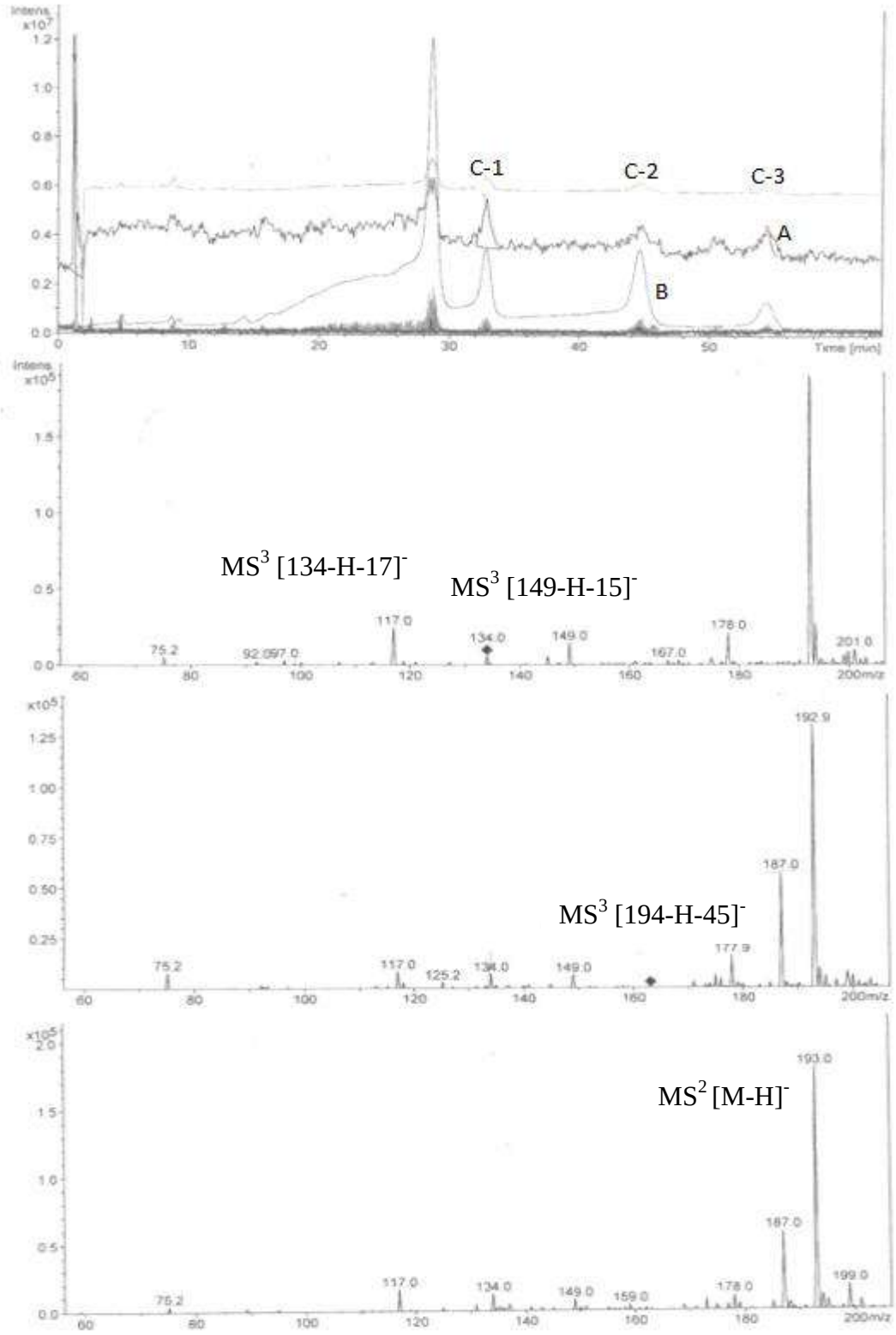


Figure 9: MS^n analysis of (Z)-2-hydroxy-3-(4-methoxyphenyl)acrylic acid (C-3) in the negative mode. MS^2 $[M-H]^-$; MS^3 $[M-H]^-$ →MS^3 $[194-H-45]^-$; MS^3 $[149-H-15]^-$; MS^3 $[134-H-17]^-$. A is thetotal ion mass spectrum and B is theHPLC chromatogram monitored at 309 nm respectively.

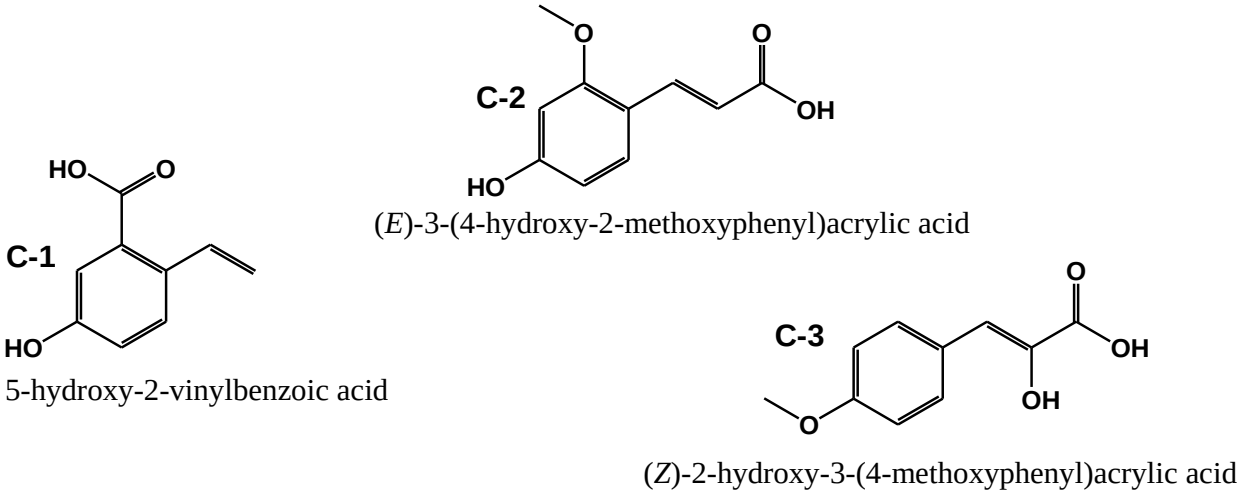


Figure 10: Predicted structures of compounds C-1, C-2 and C-3 from the chromatogram of the SXDE extract monitored at 274 nm, based on LC-MS precursor ion identification in MS^2 mode.

The limit of detection (LOD) and limit of quantitation (LOQ) for each phenolic acid was calculated by using an external standard method (Thomsen et al. 2003; Bunhu 2006). The LOD and LOQ were calculated as $3S_{y/x}$/band 3.3LOD respectively where $S_{y/x}$ is the standard error of the slope and b is the slope (Miller and Miller 1984). Among the six phenolic acids analysed, caffeic acid (4.33 μgmL^{-1}) and gallic acid (1.31 μgmL^{-1}) had the highest and lowest LOD (Table 11.3) respectively.

Table 3: Summary of results from the linear regression of the calibration curves of phenolic acids.

Phenolic acid	Conc. range/µg mL^{-1}	RT/min	Absorb λ_{max}/nm	slope/ 10^4/mL μg^{-1}	$S_b/10^2$	R^2	LOD/µg mL^{-1}	LOQ/µg mL^{-1}
Gallic acid	13.10 - 131.0	2.17	271	2.94	1.29	0.9992	1.31	4.37
p-hydrobenzoic acid	13.21 - 132.1	6.64	255	6.13	3.14	0.9979	1.54	5.12
Vanilic acid	12.68 - 126.8	8.81	260	3.64	2.47	0.9964	2.04	6.78
Caffeic acid	11.39 - 113.9	9.10	323	5.38	7.76	0.9877	4.33	14.42
Syringic acid	11.22 - 112.2	10.93	274	3.26	3.41	0.9945	3.13	10.43
p-coumaric acid	10.76 - 107.6	17.9	309	7.71	8.35	0.9922	3.25	10.81

λ_{max} = wavelength of maximum absorption, S_b = standard error of slope

The concentration of *p*-coumaric acid ranging from 2860 µg g^{-1} to 14520 µg g^{-1} was highest in all the extracts, followed by *p*-hydroxybenzoic acid; 106 µg g^{-1} to 500.5 µg g^{-1} (Table 11.4, Fig.11.11). Notably the concentrations of vanilic acid (48 µg g^{-1} to 193.5 µg g^{-1}) and gallic acid (80 µg g^{-1} to 180 µg g^{-1}) were much lower compared to the other four phenolic acids. Syringic acid was present in all extracts (360 µg g^{-1} to 1730 µg g^{-1}) (Table 4).

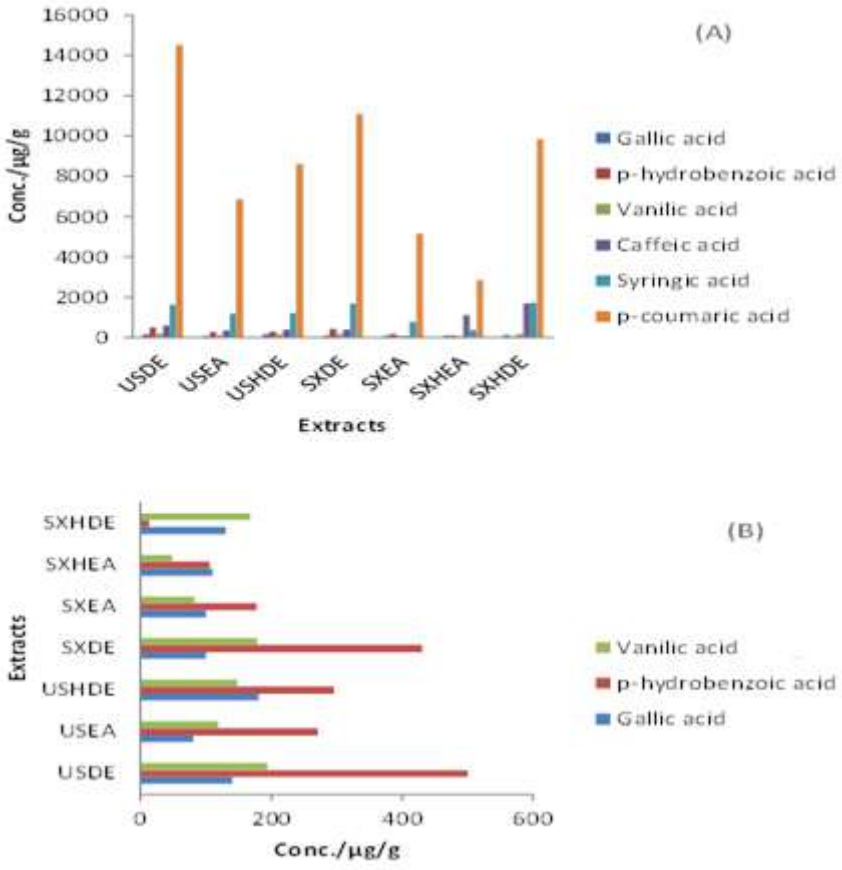
The total phenolic acids of the USDE (17584 µg g^{-1}) extract had the highest concentration, followed by SXDE (13859 µg g^{-1}); SXHDE (13667 µg g^{-1}); USHDE (10834 µg g^{-1}); USEA (8840 µg g^{-1}); SXEA (6349 µg g^{-1}); and SXHEA (4604 µg g^{-1}) extracts in decreasing order (Table 11.4). Total phenolic content of the eight CB extracts was determined by using the Folin-Ciocalteu (F-C) assay. The total phenolic content of each CB extract ranged from SXDE, 7.69 mg g^{-1} GAE to USDE, 12.12 mg g^{-1} GAE (Table 5). However, the total phenolic content may not correlate to phenolic acids content determined by HPLC as other phenolic compounds could be present in the extracts that may reduce the F-C reagent. A comparison of the SXE and USE shows that in general there are more phenolic compounds in the USE extract than the SXE extract. The effect of acidifying the aqueous phase before extraction with DE or EA did not show any significant trend.

Beside the determination of total phenolic content, the F-C assay is also an indicator of antioxidant capacity of the extract. This is because the hexavalent phosphomolybdic/ phosphotungstic acid complexes of the F-C reagent can be reduced to W_8O_{23} and Mo_8O_{23} by phenolic compounds (Kasavel 2008). Therefore, USDE extract is likely to have the highest antioxidant capacity, while the SXHEA extract may show lowest antioxidant capacity because it had the lowest total phenolic content (Table 11.4).

Table .4: Concentrations of phenolic acids in cancer bush extracts (n = 3).

Extracts	Gallic acid/µgg^{-1}	*p*-hydrobenzoic acid/µg g^{-1}	Vanilic acid/µg g^{-1}	Caffeic acid/µg g^{-1}	Syringic acid/µg g^{-1}	*p*-coumaric acid/µg g^{-1}	ΣPA/ µg g^{-1}
USDE	140 ± 0.02	500.5 ± 0.03	193.5 ± 0.01	605 ± 0.03	1625 ± 0.02	14520 ± 0.20	17584
USEA	80 ± 0.01	271 ± 0.01	119 ± 0.08	340 ± 0.02	1180 ± 0.01	6850 ± 0.60	8840
USHDE	180 ± 0.04	296 ± 0.01	148 ± 0.21	380 ± 0.01	1200 ± 0.13	8630 ± 0.01	10834
SXDE	100 ± 0.01	431 ± 0.01	178 ± 0.01	380 ± 0.01	1680 ± 0.01	11090 ± 0.01	13859
SXEA	100 ± 0.10	177 ± 0.02	82 ± 0.04	70 ± 0.03	750 ± 0.01	5170 ± 0.01	6349
SXHEA	110 ± 0.11	106 ± 0.02	48 ± 0.03	1120 ± 0.03	360 ± 0.10	2860 ± 0.01	4604
SXHDE	130 ± 0.10	130 ± 0.01	167 ± 0.02	1670 ± 0.02	1730 ± 0.03	9840 ± 0.19	13667

ΣPA is the sum of the six phenolic acid concentrations



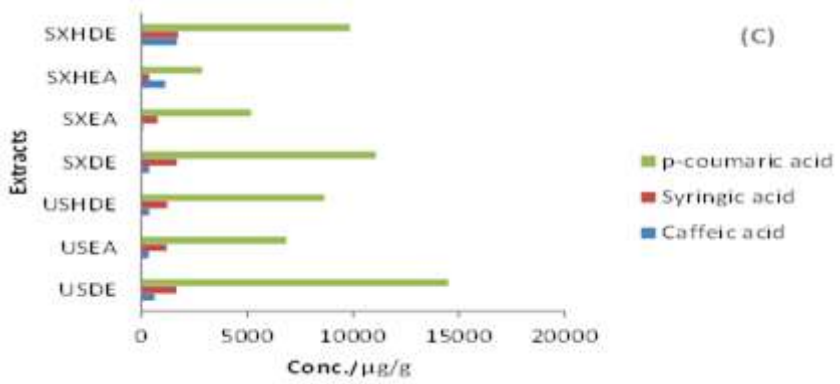


Figure 3: A comparison of (A) all the phenolic acids concentration in all extracts, (B) the minor phenolic acids: vanilic acid, *p*-hydrobenzoic acid and gallic acid, and (C) the major phenolic acids: *p*-coumaric acid, syringic acid and caffeic acid in all the cancer bush extracts.

The antioxidant activity of the extract was assessed by the 1,1-diphenylpicryl-2-hydrazyl (DPPH) assay. This assay is based on the scavenging of DPPH by an antioxidant through a hydrogen atom transfer (HAT) mechanism. In this study percentage DPPH scavenged extracts ranged from USDE (30.43 µgmL^{-1}) to SXDE (48.65 µgmL^{-1}) (Table 11.5).

This model was compared to the ferric reducing ability of plasma (FRAP), a single electron transfer (SET) antioxidant model. Electron donating species can be taken as antioxidant and the resulting deactivation of the species results in a redox reaction. Hence, total antioxidant power can be analogously referred to as total reducing power (Sarla et al. 2011). In this study all the fractions exhibited a total reducing capacity in the range of SXDE, 33.05 µg mL^{-1} to USDE, 41.53 µg mL^{-1} see Table 11.5

Table 2: Comparison of the total phenol (F-C), FRAP values and DPPH, IC_{50} values of the extracts (n = 3).

Extract	GAE/mg g^{-1}	DPPH/EC_{50}/µg mL^{-1}	FRAP value/µg mL^{-1}
USDE	12.12 ± 1.2	30.43 ± 0.92	41.53 ± 3.77
USEA	7.85 ± 0.3	42.92 ± 0.15	36.95 ± 3.09
SXEA	7.94 ± 0.03	38.75 ± 0.50	36.26 ± 2.59
SXDE	7.69 ± 2.8	48.65 ± 0.36	33.05 ± 6.03

(n = 3)

A low EC_{50} value for DPPH indicates that the antioxidant extract has a high free radical scavenging capacity which would mean a higher FRAP value. In the present work, the USDE extract had the highest free radical scavenging capacity (EC_{50} = 30.43 ± 0.92µg ml^{-1}), and the SXDE extract showed the lowest free radical scavenging capacity (EC_{50} = 48.65 ± 0.36µg ml^{-1}). Their corresponding FRAP results were 41.53 ± 3.77 µg mL^{-1} and 33.05 ± 6.03 µg mL^{-1}respectively (Table 11.5). Thus there is a good correlation between the models and the total phenolic content in the extracts in line with findings by Arora and Chandra (2010)studied the total phenolic content from *Aspergillus sp* isolate. These authors argued that the higher the total phenolic content the higher the antioxidant activity. By the same argument, the marked antioxidant activity of the CB extracts, should imply that these extracts can be effective remedies for free radical mediated ailments.

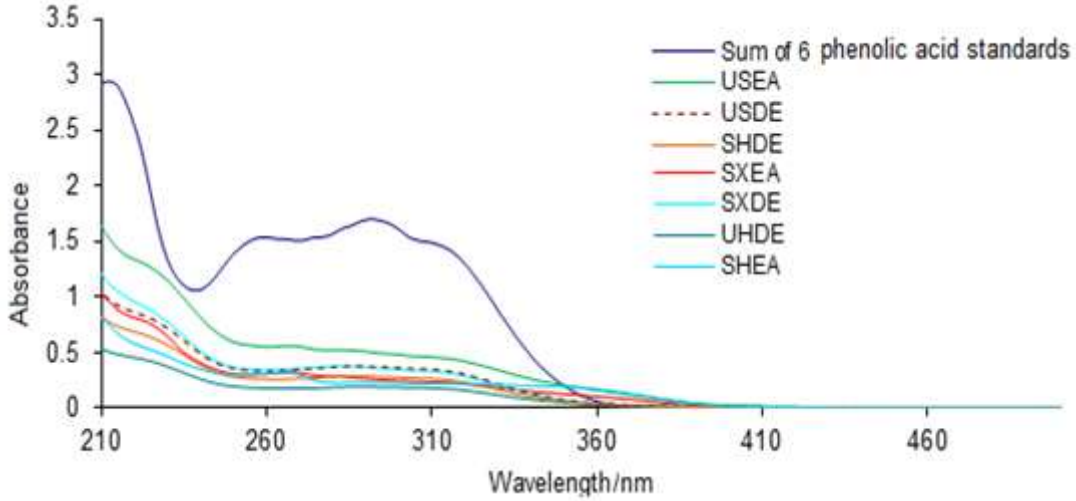


Figure 12: UV-vis spectra of the CB extracts and the sum of the six phenolic acids. The spectra are recorded on Perkin Elmer lambda 35 UV-vis dual beam spectrophotometer in a 1 cm pathlength quartz glass cuvette.

UV-vis spectra of 0.0625 mg mL^{-1} solutions of each of the extracts were recorded (Figure 12). These spectra show that all the extracts have significant absorption throughout the UVB (280-315 nm) region and part of the UVA (315-400) region. The combined absorbance of a solution of the six phenolic acids is similar to that of the extracts indicating the potential of using these extracts as photoprotectors against UVB and UVA radiation in sunscreen preparations. This corroborates the findings ofShapiro et al. (2009) who showed that caffeic acid, gallic acid and chlorogenic acid provided UV photoprotection to Beet armyworms at much lower concentrations of up to 0.005 M.

Another recent work by Oresajo et al. (2008) on the photoabsorption potential of phenolic compounds demonstrated that a mixture of vitamin C, ferulic acid and phloretin gave sufficient UV protection at a concentration of 100 ppm. This group observed that the thymine dimers were substantially inhibited an indication of UVA damage photoprotection afforded to the DNA. Though in their work, limited UV absorption was shown in the 320-400 nm bands, our work shows appreciable absorption in the region 280-360 nm (Fig. 12). Because of the intrinsic existence of conjugated double bonds and a benzene moiety, every phenolic acid exhibits some degree of photo absorption in the ultraviolet (UV) and/or ultraviolet/visible (UV-vis) region. This structural property may present proof for sufficient sun protection factor (SPF) afforded by these phenolic compounds. It is probable that phenolic acids may offer photoprotection by both absorption of UV radiation and scavenging of ROS. Thus, phenolic acids impart two important biological benefits if incorporated in sunscreen preparations and other cosmetic products.

11.4 Conclusions

The cancer bush extracts were extracted by two extraction procedures, Soxhlet extraction and an ultrasonic extraction method. Six known phenolic acids, namely gallic acid, caffeic acid, vanilic acid, syringic acid, ferulic acid and p-coumaric acid were identified and quantified. The acid with the highest concentration was *p*-coumaric. In addition, three other acids were identified. These were 5-hydroxy-2-vinylbenzoic acid, (Z)-3-(4-hydroxy-2-methoxyphenyl) acrylic acid and (Z)-2-hydroxy-3-(4-methoxyphenyl) acrylic acid. The extracts showed remarkable antioxidant activity proportional to the total phenolic content. The two antioxidant assays investigated in this work showed very good correlation implying both hydrogen atom transfer and single electron transfer can conveniently be used to describe the antioxidant activity of these plant extracts. The phenolic acid standards and the cancer bush extracts showed similar photoabsorption characteristics in the UV region. We speculate that the absorption potential demonstrated by the cancer bush extracts is mainly due to the phenolic acid content. The characteristic spectra of the three identified compounds in the extracts also show good absorption in the UVB and UVA region. We conclude that these extracts have high potential for use in the sun protection preparations as absorbers of UV

light. Combining the UV absorption and antioxidant activity of the cancer bush we propose that cancer bush extracts can be useful ingredients in sunscreens and other cosmetic preparations.

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