Isolation of antioxidant constituents from *Combretum apiculatum* subsp. *apiculatum*

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Abstract

Species of the family Combretaceae are used extensively in traditional medicine against inflammation and infections, and although antibacterial activity has been reported in non-polar extracts, further rationale for the widespread use of the Combretaceae is expected to exist. Methanol extracts of leaves of ten different *Combretum* species were evaluated for antioxidant activity by spraying TLC chromatograms of each leaf extract with 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Compounds with antioxidant activity were detected by bleaching of the purple DPPH colour. Leaf extracts of *Combretum apiculatum* subsp. *apiculatum* had the most antioxidant compounds. This species was consequently selected for phytochemical investigation. A DPPH assay-directed fractionation of the leaf extracts of *C. apiculatum* led to the isolation of four antioxidant compounds from the ethyl acetate and butanol soluble fractions. The structures of the compounds were determined by spectroscopic analyses (^{1}H-NMR, ^{13}C-NMR and MS) and identified as: cardamonin (**1**), pinocembrin (**2**), quercetrin (**3**) and

kaempferol (**4**). In a quantitative antioxidant assay, the more polar fractions (ethyl acetate and butanol) obtained by solvent-solvent fractionation had the highest antioxidant activity among the solvent fractions obtained from *C. apiculatum*, with EC_{50} values of 3.91 ± 0.02 and 2.44 ± 0.02 µg/mL respectively. Of the four isolated compounds, quercetrin (**4**) and kaempferol (**3**) had the strongest antioxidant activity, with EC_{50} values of 11.81 ± 85 and 47.36 ± 0.03 µM respectively. Cardamonin (**1**) and pinocembrin (**2**) did not demonstrate strong activity. L-ascorbic acid was used as standard antioxidant agent (EC_{50} = 13.37 ± 0.20 µM or 2.35 µg/mL).

The cytotoxicity of cardamonin and pinocembrin was evaluated on Vero kidney cells using the MTT (3-(4, 5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide) assay with berberine as positive control. At concentrations higher than 50 µg/ml of cardamonin or pinocembrin, the cells were not viable. Cardamonin was more toxic (LC_{50} = 1.97 µg/ml) than pinocembrin (LC_{50} = 29.47 µg/ml) and even the positive control, berberine (LC_{50} = 12.35 µg/ml).

Keywords: Combretaceae, *Combretum apiculatum*, antioxidant, cytotoxicity

1. Introduction

Combretum species are used in many cultures in folk medicine for treatment of microbial infections and several inflammatory conditions such as abdominal pains, headache and toothache (Hutchings et al., 1996; Eloff et al., 2001). Antimicrobial activity of many *Combretum* species has been confirmed (Martini and Eloff, 1998; Eloff, 1999b; McGaw et al., 2001; Eloff and McGaw, 2006). Antimicrobial compounds reported from *C. apiculatum* include alpinetin, pinocembrin and flavokawain (Serage, 2003).

Traditional healers use mainly aqueous extracts, as availability of alternative solvents for preparing medicinal plant extracts is limited. In all species of the Combretaceae that we have

studied, extracts prepared using water generally showed little *in vitro* antibacterial activity (Martini and Eloff, 1998; Eloff, 1998b; Kotze and Eloff, 2002; Eloff et al., 2005). In several studies on the Combretaceae family, reviewed by Eloff et al. (2008), it was found that non-polar extractants generated extracts with the highest antibacterial activity. Aqueous plant extracts of many plant species usually have very little direct *in vitro* antimicrobial activity relative to extracts prepared using more non-polar solvents. As the nature of the solvent used to prepare plant extracts is crucial in determining the polarity and bioactivity of compounds extracted, it is important to use similar solvents to those used by traditional healers when setting out to validate the reasons for traditional use of the plants. Different *Combretum* species have been used to treat microbial infections by traditional healers. One explanation for the anomaly that aqueous extracts have such low *in vitro* antimicrobial activity could be that the antimicrobial effect is not exerted via killing the microorganisms, but rather by stimulating the immune system of the patient. As antioxidant compounds generally tend to be more polar, one would expect that aqueous extracts would have higher antioxidant activity and may stimulate the immune system of patients thereby combating the infection indirectly. This possibility prompted qualitative investigation of the antioxidant potential of polar extracts of ten *Combretum* species, namely *C. celastroides, C. orientale, C. erythrophyllum, C. taborense, C. zeyheri, C. apiculatum* subsp. *apiculatum, C. moggii, C. microphyllum, C. paniculatum,* and *C. mossambicense.* The most active species in this study, *C. apiculatum,* was selected for phytochemical investigation and isolation of bioactive constituents.

2. Material and methods

All solvents used were laboratory grade reagents and were distilled prior to use. All thin layer chromatography analyses were performed at room temperature using pre-coated plates (Merck, silica gel 60 F_{254} 0.2 thickness). Detection of spots was performed by viewing under ultraviolet light (254 and 366 nm). Column chromatography was carried out using silica gel 60 (Merck)

and Sephadex LH-20 (Sigma). Nuclear magnetic resonance (NMR) ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra were recorded on a Varian Unity Inova 300 MHz NMR spectrometer. Chemical shifts are expressed in parts per million (ppm). Mass spectrum of each of the isolated compounds was obtained using a VG70-SEQ mass spectrophotometer.

2.1. Plant collection

Leaves of 10 *Combretum* species, namely *C. celastroides, C. orientale, C. erythrophyllum, C. taborense, C. zeyheri, C. apiculatum* subsp. *apiculatum, C. moggii, C. microphyllum, C. paniculatum,* and *C. mossambicense* were collected from the Lowveld National Botanical Garden in Nelspruit. Trees were identified from the tree labels. Voucher specimens were deposited at the Herbarium of the Department of Paraclinical Sciences, and at the HGWJ Schweickerdt Herbarium, University of Pretoria, South Africa. The collected plant materials were air-dried at room temperature.

2.2. Preparation of plant material

Plant material was ground to a fine powder in a Macsalab 200 LABmill. The ground sample of each plant was stored in closed amber glass containers in the dark at room temperature until further processed.

2.3. Extraction procedure

Initially, 1.0 g of finely ground plant material of each *Combretum* species was extracted with 10 mL of methanol (MeOH) with occasional shaking for 24 hrs. Each extract was filtered through Whatman No 1 filter paper and concentrated using a rotary evaporator at 40˚C. This afforded crude extracts of each of the selected ten plants.

2.4. TLC screening for phytochemical analysis and antioxidant activity

Qualitative screening of the constituents in each of the crude extracts of the ten selected *Combretum* species for antioxidant activity was done using thin layer chromatography (TLC) analysis. Ten µL of each sample (10 mg/ml) were loaded on TLC plates in lines of about 1 cm wide. Separate TLC chromatograms were developed in the following solvent systems (Kotze and Eloff, 2002).

i. Ethyl acetate/ methanol/water (EMW) 10:1.35:1

ii. Chloroform/ethyl acetate/formic acid (CEF) 10:8:2

iii. Benzene/ ethanol/ ammonium (BEA) 18:2:0.2

For detection of antioxidant compounds in the extracts, DPPH (0.2% in MeOH) was used as spraying reagent (Figure 1).

2.5. Isolation of antioxidant compounds from ethyl acetate fraction of C. apiculatum

C. apiculatum powdered leaf (1 kg) was extracted with 10 L MeOH and the crude extract obtained was partitioned using solvent-solvent extraction. Solvent-solvent extraction is one of the most popular methods for partial purification (group separation according to polarity) of crude plant material (Eloff, 1998c). The crude extract of the *Combretum* species was suspended in distilled water in a separatory funnel (2 L) and successively partitioned in turn with hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH). This afforded four partitioned fractions of the plant extract. The ethyl acetate fraction obtained (30.0 g) was subjected to column chromatography on silica gel. The column was eluted with hexane, followed by an increasing gradient of dichloromethane (DCM) in hexane up to 100% DCM. This was followed with an increasing gradient of ethyl acetate (EtOAc) in dichloromethane up to 100%. EtOAc (100%) was similarly followed with 10% methanol (MeOH) up to 20%. Seven

fractions were subsequently obtained when analysed on TLC using CEF as solvent system. Each fraction on the TLC plate was subsequently sprayed with DPPH solution to determine the presence of antioxidant compounds.

2.5.1. Column chromatography of fraction 4

Fraction 4 (2.1 g) was fractionated on a second silica gel column. The column was eluted with hexane, followed by an increasing gradient of EtOAc up to 100%. The fractions were collected in 25 mL test tubes and a total of 76 tubes were collected. Compound **2** (36 mg) crystallised out from tubes 2 -14, and the crystals were purified on another silica gel column eluting with hexane: DCM 4:1 to give white crystals. Compound **1** (59 mg) was obtained as spot from tubes 36 -76. Tubes 17 - 30 contained one major compound, and the fractions were pooled together and purified on a silica gel column using a mixture of hexane: ethyl acetate (5:1). This afforded a yellow powder, compound **3** (8 mg).

2.5.2. Column chromatography of fraction 6

This fraction (1.6 g) was fractionated on a Sephadex LH-20 column using toluene/ethanol (4:1). This was followed by an increasing gradient of ethanol in toluene up to 100%. The eluant was collected in 25 mL test tubes and a total of 50 tubes were collected. TLC analysis showed that tubes 5-18 contained one major component. Work-up of these fractions gave compound **4**, a yellow powder (20 mg).

2.6. Quantitative evaluation of antioxidant activity of the extracts and isolated compounds

Quantitative antioxidant activity was determined spectrophotometrically as described by Mensor et al. (2001) and modified by Aderogba et al. (2007). Briefly, reactions were carried out in 96-well microtitre plates and each of the solvent fractions and crude extract was tested at varying concentrations. Initial stock solutions of 300 µg/ml of the various extracts were prepared in

methanol. Final concentrations of 150.0, 75.0, 37.5, 18.75, 9.74, 4.69, 2.34 and 1.17 µg/ml were made from the stock solution. Purified and structurally determined compounds were also evaluated at different concentrations. From the initial stock solutions of 200 µM, final concentrations of 100.0, 50.0, 25.0, 12.5, 6.25, 3.13 and 1.56 µM were made. Twenty µl of 0.25 mM DPPH in methanol was added to 50 µl of each concentration of sample tested and allowed to react at room temperature in the dark for 30 minutes.

Blank solutions were prepared with sample solution (50 µl) and 20 µl of methanol only. The negative control was 20 µl DPPH solution and 50 µl methanol. Methanol served as blank for the microplate reader and the decrease in absorbance was measured at 515 nm. Percentage antioxidant activity (AA%) values were calculated from the absorbance values using the formula: $AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and

$Abs_{control}$ is the absorbance of the control). L-ascorbic acid (vitamin C) was used as a positive control.

The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the separate linear regression of plots of the mean percentage of the antioxidant activity against concentration of the test extracts (µg/ml) and compounds (µM) obtained from the three replicate assays.

2.7. Data handling

The results are expressed as mean ±SEM and the EC_{50} values obtained from the regression plots (Sigma Plots® 2001, SPSS Science).

2.8. Cytotoxicity assay

The tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) and modified by McGaw et al. (2007) was used to investigate cytotoxicity of two of the isolated compounds isolated in sufficient quantity. The compounds were tested for cytotoxicity against Vero African green monkey kidney cells. The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell suspensions were prepared from confluent monolayer cultures and plated at 0.5×10^3 cells per well (200 µl) into each well of a 96-well microtitre plate, except for columns 1 and 12 which contained MEM only. Plates were incubated overnight at 37 ºC in a 5% CO_2 incubator for the cells to attach to the plate wells. Stock solutions of the isolated compounds (20 mg/ml) were prepared by dissolving in DMSO. A range of dilutions of each isolated compound was prepared in growth medium. Berberine chloride (Sigma Chemical Company) was used as a positive control. The viable cell growth after 120 hours incubation with isolated compounds (200 µl in each well) was determined. After incubation, the wells were rinsed with 150 µl phosphate buffered saline (PBS) and fresh medium (200 µl) was placed in each well. Then, 30 µl of MTT (Sigma) dissolved in PBS (5 mg/ml stock solution) was added to each well and the plates were incubated at 37 ºC for 4 h. The medium was removed and the purple MTT formazan crystals dissolved in DMSO (50 µl per well). The absorbance was measured on a Versamax microplate reader at 570 nm wavelength. In the cytotoxicity assay, each concentration of each compound was tested in quadruplicate, and the assay in its entirety was repeated three times.

3. Results

The results of the qualitative antioxidant assay of the crude methanol extracts are shown in Figure 1 while those of the quantitative antioxidant activity of the crude extracts, solvent fractions and isolated compounds are shown in Table 1. The structures of the compounds were determined by spectroscopic analyses (^{1}H-NMR, ^{13}C-NMR and MS) and identified as:

Cardamonin (4′, 6′-dihydroxyl-2′-methoxychalcone) **-** Compound **1** (59 mg) was isolated as yellow crystals. The mass spectrum showed the molecular ion [M^{+}] peak at m/z = 270.08 corresponding to the molecular formula $C_{16}H_{14}O_4$. The ^{1}H NMR (DMSO-d_6) showed a singlet peak at δ 3.87 (3H, s, 2′-OCH_3). Meta coupling related two doublets at δ 5.93 (1H, d, J=1.8 Hz) and δ 6.02 (1H, d, J=2.1 Hz). Another two sets of doublets at δ 7.67(1H, d, J=15.3 Hz, H-7) and δ 7.85 (1H, d, J=15.9 Hz, H-8) represented a trans olefinic group. A multiplet appeared between δ 7.43 – 7.72 (5H, multiplet) due to the monosubstituted ring B of a chalcone and a singlet peak at δ 13.71 (1H, s, H-6′-OH). The ^{13}C NMR spectra data are in agreement with the literature (Itokawa et al., 1981; Krishna and Chaganty, 1973). The compound was identified as 4′, 6′-dihydroxyl-2′-methoxychalcone (cardamonin) and reported for the first time from this species (Figure 2).

Pinocembrin (5, 7-dihydroxy-2-phenyl flavanone) **-** Compound **2** (36 mg) was obtained as powder. The mass spectrum gave a molecular ion peak [M^{+}] at m/z = 256.07 corresponding to the molecular formula $C_{15}H_{12}O_4$. The ^{1}HNMR (acetone-d_6) spectrum and ^{13}C NMR spectral data are in agreement with the literature (Serage, 2003, Harborne and Mabry, 1982). The compound was identified as 5, 7-dihydroxy-2-phenyl flavanone (pinocembrin, Figure 2).

Kaempferol (3, 4', 5, 7-tetrahydroxy flavones) - **Compound 3** (8 mg) mass spectrum had a molecular ion peak at m/z = 286.03 [M^{+}, 100%] base peak, corresponding to the molecular formula $C_{15}H_{10}O_6$. The ^{1}H NMR (acetone-d_6) spectrum showed AA'BB' system due to ring B

ortho related protons at δ 7.01 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.14 (2H, d, J=9.0, H-2', H-6'). The presence of a free 5-OH group was confirmed by a peak at δ 12.13. Also there was the presence of two set of doublets at 6.25 and 6.50 (1H each, J = 1.8 and 2.1 Hz respectively H-6, H-8 that coupled to each other). ^{13}C NMR spectra data are in agreement with the literature (Markham, 1982). Kaempferol is widely distributed in the plant kingdom but is reported from this species for the first time (Figure 2).

Quercetrin (quercetin-3-O-rhamnoside) - **Compound 4** (20 mg) mass spectrum had a molecular ion peak [M^+] at m/z = 447.89 corresponding to the molecular formula $C_{21}H_{20}O_{11}$. Another prominent peak appeared at m/z = 300.95 corresponding to $C_{15}H_9O_7$, [M^+- rhamnosyl]. The ^{1}H NMR (300 MHz, acetone-d_6): δ 0.91 (3H, d, rhamnose-CH_3), δ 3.370 - 4.21 multiplet (rhamnosyl Hs), δ 5.5 (1H, rhamnosyl H-1), meta coupling related protons at δ 6.25 (1H, d, J = 2.1 Hz, H-6) and 6.35 (1H, d, J = 1.5 Hz, H-8) depicting substitution pattern of ring A. Ring B protons appeared at 6.99 (1H, d, J=8.4 Hz, H-5'), 7.49 (2H, d, J = 2.1 Hz, H-2' and 6'). The presence of 5-OH was evident from the peak at δ12.71. ^{13}C NMR spectra data are in agreement with the literature (Harborne and Mabry, 1982). The compound was identified as quercetin-3-O-rhamnoside (quercetrin), and is reported from this species for the first time (Figure 2).

The structures of the isolated antioxidant compounds from *C. apiculatum* are presented in Figure 2. In this study, cardamonin had poor quantitative antioxidant activity and was much more toxic to the cells (LC_{50} = 1.97 µg/ml) than pinocembrin with an LC_{50} of 29.47µg/ml. Berberine, the positive control, had an LC_{50} of 12.35 µg/ml.

4. Discussion and conclusion

The use of *Combretum* species in many cultures in folk medicine for treatment of several inflammatory conditions (Hutchings et al., 1996; Eloff et al., 2001) and their established antimicrobial activities (Martini and Eloff, 1998; Eloff, 1999a; Eloff 1999b; McGaw et al., 2001, Eloff and McGaw 2006) prompted us to investigate the antioxidant potential of this plant genus. There are several possible mechanisms to explain the use of plant extracts to treat microbial infections. A direct effect involves the action of active agents (antimicrobial) and the indirect effect may involve the stimulation of the host immune system to overcome the effects of microorganisms via the host immune system. Traditional healers use mainly aqueous extracts and in all species of the Combretaceae that we have studied, extracts prepared using water had hardly any *in vitro* antibacterial activity (Kotze and Eloff, 2002; Eloff et al., 2008). One may speculate that aqueous extracts have high antioxidant activity and stimulate the immune system of patients thereby combating the infection indirectly.

Leaves of ten *Combretum* species were extracted with methanol, a relatively polar solvent, for screening because most antioxidant constituents are polar phenolic compounds, especially flavonoids. Extracts of all the species examined contained antioxidant compounds based on TLC chromatograms sprayed with DPPH (2, 2-diphenyl-1-picrylhydrazyl). However the number of constituents with antioxidant activity varied amongst the C*ombretum* species. The highest number of antioxidant constituents was present in *Combretum apiculatum* subsp. *apiculatum,* followed by *Combretum moggii* (Figure 1). These results are in line with those of Masoko and Eloff (2007) who investigated the qualitative antioxidant activity of 30 members of the Combretaceae.

Combretum apiculatum subsp. *apiculatum* was therefore selected for fractionation and isolation of active constituents. Four solvent fractions were obtained from the crude methanol extract using solvent-solvent fractionation. All four fractions had antioxidant compounds but the highest

numbers were present in the ethyl acetate fraction based on spraying chromatograms with DPPH. Therefore ethyl acetate was selected as the extracting solvent to use for further fractionation and purification leading to the isolation of the antioxidants.

Antioxidant activity directed fractionation of the ethyl acetate soluble fraction of the leaf extract of *C. apiculatum* subsp. *apiculatum* led to the isolation of four antioxidant compounds. The structures of the compounds were determined by spectral analysis (^{1}H-NMR, ^{13}C-NMR and MS) and identified as 2', 4'- dihydroxy-6'-methoxy chalcone, cardamonin (**1**), 5, 7–dihydroxy-2-phenyl flavanone or pinocembrin (**2**), kaempferol (**3**) and quercetrin (**4**). Kaempferol and quercetrin are widespread in the plant kingdom, but these two compounds, together with cardamonin, are reported from *C. apiculatum* subsp. *apiculatum* for the first time. Antimicrobial activity of pinocembrin has previously been reported from this species- *C. apiculatum* from our laboratory (Serage, 2003).

All of the isolated compounds bleached the purple DPPH colour to pale yellow when the TLC plate on which they were loaded was sprayed with 0.2% DPPH in methanol. In the quantitative DPPH antioxidant assay, the more polar fractions (ethyl acetate and butanol) had the highest activity among the extracts with EC_{50} values of 3.9 ± 0.02 and 2.4 ± 0.02 µg/ml respectively. This compared well with the value of ascorbic acid, the positive control, with an EC_{50} value of 2.4 ± 0.04 µg/ml.

Of the isolated compounds, kaempferol (**3**) and quercetrin (**4**) had good antioxidant activity with EC_{50} values of 47.36 ± 0.03 and 11.81 ± 0.32 µM respectively compared to that of ascorbic acid (13.37 ± 0.20 µM). Based on structural activity studies, good antioxidant activity requires the presence of a catechol group (3′, 4′ -OH) on ring B, the presence of 2, 3 unsaturation along with 3-OH and a keto group in position 4 (Op de Beck et al., 2003 and Saskia et al., 1996). Only quercetrin **(4)** fulfilled these entire requirements and as expected had the highest activity

comparable to L-ascorbic acid. Cardamonin (**1**) and pinocembrin (**2**) did not have as strong activity as these compounds could not scavenge 50% of the DPPH free radicals at the highest concentration (200 μM) tested. The lower antioxidant activity confirms the relationship between structure and antioxidant activity.

The presence of these antioxidant compounds in the leaf extract of *C. apiculatum* provides a rationale for the ethnomedicinal use of this plant for the treatment of inflammatory conditions and microbial infections in traditional medicine.

The cytotoxicity of cardamonin and pinocembrin was evaluated using the MTT assay with berberine as positive control and DMSO as negative control. There was not enough material available to determine the cytotoxicity of kaempferol and quercetrin. There are differing reports in the literature concerning the cytotoxicity of quercetrin and kaempferol to various cell lines. Ibrahim et al (2009) reported that cytotoxicity of four kaempferol derivatives against Vero cells was not observed up to a concentration of 100 μg/mL. At concentrations higher than 50 μg/ml of cardamonin or pinocembrin, the cells were not viable. Cardamonin was more toxic to the cells with an LC_{50} of 2.0 μg/ml than both pinocembrin (LC_{50} = 29.5 μg/ml) and the positive control, berberine (LC_{50} = 12.4 μg/ml).

The chalcone cardamonin has anti-inflammatory activity by means of targeting the NF-kappaB pathway (Israf et al., 2007), and it has also been shown to suppress nitric oxide production by blocking the IFN-γ/STAT pathway in endotoxin-challenged peritoneal mouse macrophages (Takahashi et al., 2011).

The high toxicity of cardamonin in C. *apiculatum* subsp. *apiculatum* indicates that the safety of the extracts could be of concern. It may be worthwhile to evaluate the antioxidant activity and cardamonin content of aqueous extracts intended for use in animals and man.

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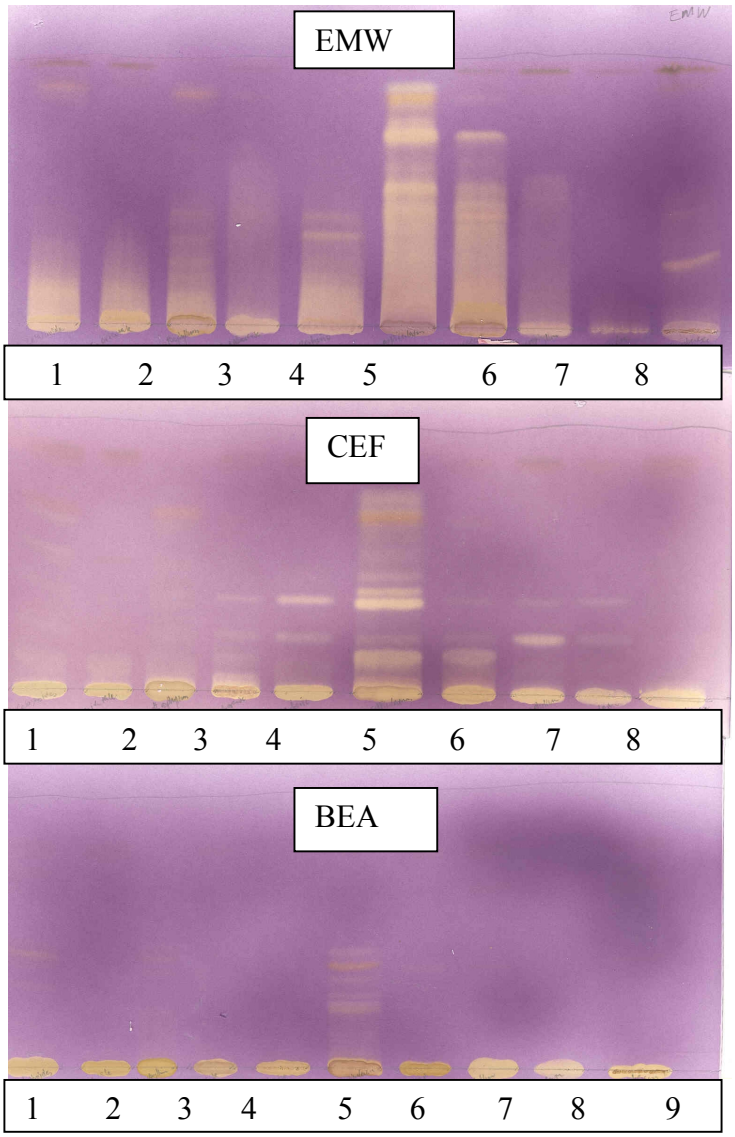
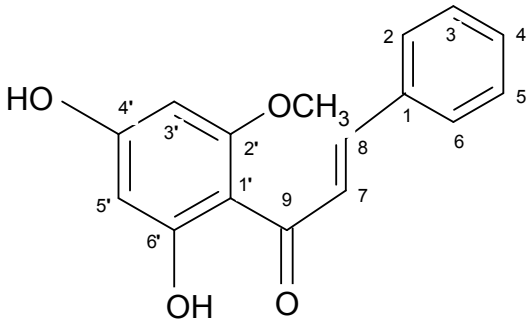
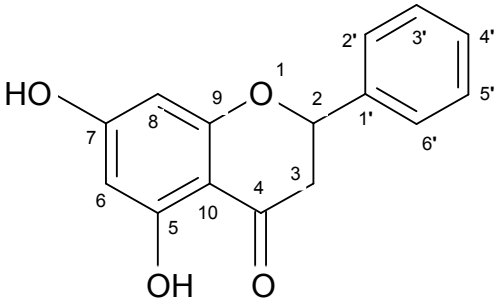


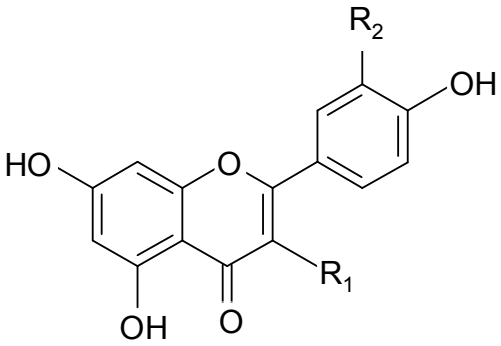
Figure 1: TLC of crude methanol extracts of ten *Combretum* species developed in different solvent systems of varying polarities and sprayed with DPPH: *C. celastroides* (1), *C. orientale* (2), *C. erythrophyllum* (3), *C. taborense* (4), *C. zeyheri* (5), *C. apiculatum* subsp. *apiculatum* (6), *C. moggii* (7), *C. microphyllum* (8), *C. paniculatum* (9) and *C. mossambicense* (10). EMW (Ethyl acetate/ methanol/water, 10:1.35:1),CEF (Chloroform/ethyl acetate/formic acid , 10:8:2), BEA (Benzene/ ethanol/ ammonium, 18:2:0.2)



Cardamonin (**1**)



Pinocembrin (**2**)



	R_1	R_2
Kaempferol (**3**)	OH	H
Quercetrin (**4**)	O-rhamnosyl	OH

Figure 2: Structures of antioxidant compounds from *C. apiculatum*

Table 1: Antioxidant activity (EC_{50} values) of the extract, fractions and isolated compounds from *C. apiculatum*

Sample	EC_{50} ± SEM
Crude extract	14.5 ± 0.12 µg/ml
Hexane fraction	17.3 ± 0.50 µg/ml
Dichloromethane fraction	20.3 ± 1.56 µg/ml
Ethyl acetate fraction	3.9 ± 0.02 µg/ml
Butanol fraction	2.4 ± 0.02 µg/ml
Cardamonin	>200 µM
Pinocembrin	>200 µM
Kaempferol	47.4 ± 0.03 µM
Quercetrin	11.8 ± 0.32 µM
L- ascorbic acid	13.4 ± 0.20 µM i.e (2.4 ± 0.04 µg/ml)

Note: Cardamonin and pinocembrin did not inhibit 50% of DPPH free radical at 200 µM, the highest concentration tested. AA% at this concentration was 11.7% and 6.2% respectively.