

Isolation and characterisation of antibacterial compounds from *Combretum apiculatum* subspecies *apiculatum* (Combretaceae) leaves

Authors:

David R. Katerere^{1,2}, Andrew Serage¹, Jacobus N. Eloff^{1*}

Affiliations:

¹Phytomedicine Programme, Faculty of Veterinary Science, University of Pretoria

²Current address: Department of Pharmaceutical Science, Faculty of Science, Tshwane University of Technology

Corresponding author:

JN Eloff
kobus.eloff@up.ac.za

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Aims: To isolate the antibacterial compounds and to investigate the activity of extracts of, and the antibacterial flavonoids isolated from leaves of *Combretum apiculatum* against four important community-acquired and nosocomial bacterial pathogens viz. *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Methods and results: Ten organic and aqueous extracts of the leaf powder of *Combretum apiculatum* Sond subsp. *apiculatum* Exell were initially tested against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* using a serial dilution microtitre plate and bioautography assays using *p*-iodonitrotetrazolium violet (INT) as indicator of growth. The acetone extract was the most potent and selected for further bioassay-guided fractionation of antibacterial compounds which resulted in the isolation of three known flavonoids viz. flavokawain, alpinetin and pinocembrin.

Conclusions: The flavonoids which were isolated from *C. apiculatum* for the first time were moderately active against *S. aureus* and *E. faecalis* with MICs of 40 µg/ml. Ethyl ether and ethyl acetate extracts were equally active against *E. faecalis* pointing to synergistic effects of phytochemical constituents in exerting antibacterial activity.

Significance and impact of the research: The Combretaceae taxa are important materia medica in Africa and Asia. The results confirm the validity of using *C. apiculatum* crude extracts against bacterial infections and the superiority of extracts over isolated individual compounds.

Keywords: Combretaceae; *Combretum apiculatum*; flavonoids; flavones; Nuclear Magnetic Resonance Spectroscopy (NMR); antibacterial; bioautography

Isolering en karakterisering van antibakteriese verbindings uit blare van *Combretum apiculatum* subsp. *apiculatum*:

Doel: Om antibakteriese verbindings te isoleer en die aktiwiteit van ekstrakte, fraksies en antibakteriese verbindings uit *Combretum apiculatum* subsp. *apiculatum* blare teen vier belangrike bakteriese patogene *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* en *Pseudomonas aeruginosa* te bepaal.

Metodes en Resultate: Die aktiwiteit van tien organiese en watererige ekstrakte van blare teen *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* en *Pseudomonas aeruginosa* is bepaal deur 'n reeksverdunnings in mikroplate en bio-outografie met *p*-jodonitrotetrazolium pers (INT) as aanduider van groei. Die asetoonekstrak het die hoogste aktiwiteit gehad en is gebruik om die antibakteriese verbindings te isoleer en te karakteriseer. Drie bekende flavonoiede- flavokawain, alpinetin en pinesembrien was verantwoordelik vir die aktiwiteit.

Gevolgtrekkings: Die flavonoiede wat vir die eerste keer uit hierdie spesie geïsoleer is, was redelik aktief teen die twee Gram-positiewe bakterieë *Staphylococcus aureus* en *Enterococcus faecalis* met minimum inhiberende konsentrasies (MIK) van 40 µg/ml. Die etieleter en etielasetaat ru-ekstrakte het dieselfde aktiwiteit teen *E. faecalis* gehad as die geïsoleerde verbindings wat daarop dui dat daar sinergistiese interaksies tussen verbindings in die ekstrak was.

Impak van die navorsing: Lede van die Combretaceae word wyd in Afrika en Asië gebruik vir medisinale doeleindes. Die navorsing ondersteun die tradisionele gebruik teen infeksies en dui ook daarop dat dit meer voordelig is om ekstrakte te gebruik as die geïsoleerde antibakteriese verbindings.

Introduction

The development of antimicrobial drugs represents one of the most important advances of modern medicine (Katzung 1998). However over-prescribing and irrational use of antibiotics have partly led to the emergence of resistant pathogenic microorganisms.

The use of plant extracts (as botanical, herbal or phytomedicine) has regained its popularity over the last two decades. Several publications on members of the Combretaceae have shown that they have antimicrobial activity that could have therapeutic potential (Katerere et al. 2003; Martini et al. 2004; Eloff et al. 2008).

The Combretaceae family has 18 genera distributed in Africa, Asia and the Americas (Exell 1970). The two biggest genera are *Combretum* with 370 species and *Terminalia* with 200 species and both are widely used in traditional medical practice in Africa and Asia (Rogers and Verotta 1995).

In this study we investigated the antibacterial activity of extracts of *Combretum apiculatum* Sond subspecies *apiculatum* Exell. *C. apiculatum*, known as bushwillow (English), rooiblaar or rooiboswilg (Afrikaans), mohwelere (Pedi), umbondwe omnyana (Zulu), mugodo (Shona), umbondo (Ndebele), is widely distributed in southern Africa and is known to be used by traditional healers to treat snake bite, diarrhoea, conjunctivitis and abdominal disorders (Gelfand et al. 1993; Rogers and Verotta 1995; Hutchings et al. 1996). Its use in this way may be a pointer to possible antimicrobial/antiseptic activity which this study set out to investigate.

In the present study, powdered leaves of *C. apiculatum* subspecies *apiculatum* were extracted with ten solvents of varying polarity and tested for activity against four bacterial species using bioautographic and microtitre plate assays. The most active extracts were then further fractionated resulting in the isolation of three flavonoids.

Materials and methods

Chemicals

All chemicals with the exception of the extractants were of analytical grade and purchased from Merck (Darmstadt, Germany). *p*-iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Germany.

Collection of plant material

The leaves of *Combretum apiculatum* Sond subspecies *apiculatum* Exell were collected from a tree growing in the Lowveld National Botanical Gardens, Nelspruit, South Africa in April 2001. A voucher specimen was deposited in the herbarium of the University of Pretoria. The leaves were allowed to dry in the shade at room temperature for two months after which they were ground to a fine powder using a Junkel and Kunkel Model A10 mill. *Combretum erythrophyllum* leaves collected from the same area and kept in a dried condition in an herbarium for more than 90 years did not lose any antibacterial activity (Eloff 1999a).

Extraction and isolation

For initial screening, 0.5 g of powder was extracted with 5 ml solvent by vigorous shaking and then centrifuged at 3 000 x g for 5 min using methods previously described (Eloff, 1998a; Kotze and Eloff, 2002). The extraction process was repeated twice. Ten solvents of technical grade and purchased from Merck (Darmstadt) were used, i.e. hexane, isopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, methanol, ethanol and water.

The acetone extract had the best minimum inhibitory concentration (MIC) and the most antibacterial compounds determined by a microtitre plate assay and bioautography using *p*-iodonitrotetrazolium violet (INT) as indicator of growth. Subsequent large scale extraction using a 10:1 volume:mass ratio (Martini and Eloff 1998) was performed with acetone followed by solvent-solvent fractionation using the method of Suffness and Douros 1979; Martini and Eloff, 1998. The separation step was performed with equal volumes of solvents and repeated a few times to enable exhaustive partition. The extracts were filtered and combined and dried under vacuum using a vacuum rotary evaporator (Buchi, Germany).

Chromatography

The samples were all loaded onto 20 x 10 cm Silica Gel 60 TLC F254 Merck plates and developed in one of three solvent systems namely: chloroform:ethylacetate:formic acid (CEF) (5:4:1), benzene:ethanol:ammonia (BEA) (36:4:0.4) and ethylacetate:methanol:water (EMW) (40:5.4:4.0) (Kotze and Eloff 2002). Five µl of a 20 mg/ml extract solution (i.e. 100 µg) of the different extracts was applied in a line of c. 1 cm wide and developed to c. 9 cm in sealed TLC tanks. The separated components were visualised at 254 nm and 360 nm with a UV lamp. Chromatograms were subsequently sprayed with either vanillin-sulphuric acid or anisaldehyde-sulphuric acid spray reagent and then heated at 100 °C to optimal colour development.

Silica gel column chromatography was used to isolate active compounds by bioassay guided fractionation. Columns were packed with silica gel 60 (0.040–0.063 mm, Merck) and eluted with hexane followed by a mixture of hexane with increasing portions of dichloromethane then dichloromethane with increasing amounts of methanol. Fractions of 50 ml each were collected, allowed to concentrate under a stream of air at room temperature and then analysed by TLC. Similar fractions were pooled together and the antibacterial activity was determined. Pooled fractions were then separated on another open column packed with silica gel and developed with hexane:chloroform:methanol gradients until pure antimicrobial compounds were isolated. The R_f values of antibacterial compounds determined by bioautography were used to isolate the antibacterial compounds.

Spectroscopic analysis

Nuclear Magnetic Resonance experiments were performed on a 300 MHz Varian instrument (Oxford Instruments) at Medunsa Campus of the University of Limpopo. Mass spectrometry was performed at Cape Technikon (Cape Town) on a VG70-SEQ (Micromass, UK) instrument.

Antimicrobial screening

Two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 292163) and *Enterococcus faecalis* (ATCC 29212) and two Gram-negative bacterial species, *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 25922) were used using a serial microplate dilution method with INT (Sigma) as indicator of growth (Eloff, 1998b).

A direct bioautographic method was used to determine the number of compounds active against *S. aureus* and *E. coli* (Masoko and Eloff 2006). Chromatograms were left in a steam of air to remove all the TLC eluents, sprayed with a dense culture of the relevant bacteria, incubated overnight at 37 °C and then sprayed with 0.2 mg/ml INT. For quantitative antibacterial activity, a serial dilution microplate assay of the extract, fraction or compound in acetone with INT as a growth indicator (Eloff 1998b) was used to determine minimum inhibitory concentrations (MIC). The positive control was gentamicin and the negative control was acetone.

Results

Extraction efficiency

Tetrahydrofuran and acetone extracted the highest quantity of the 10 extractants investigated with a yield of 14% and 13% respectively. This is in the same order as the value (16.2%) for acetone from this species obtained earlier (Eloff, 1999b). Water on the other hand only managed to extract 0.8% of the original mass of powdered leaf.

The different extracts were separated by TLC using one of three solvent systems described earlier (Kotze and Eloff 2002). CEF and EMW led to better chromatographic resolution compared to the least polar of the mobile phases. Based on the BEA chromatograms it appeared that the majority of compounds had an intermediate polarity.

Antimicrobial assays

Initially 10 extracts were evaluated for antimicrobial activity by bioautography using *S. aureus* and *E. coli*. (results not

shown). There were two clear zones of inhibition against *S. aureus* in isopropyl ether and ethyl ether and to a lesser extent methylene dichloride and ethyl acetate extracts and they had the same R_f values on TLC plates developed in CEF. There were at least three zones of inhibition against *E. coli* and all extracts except acetone and ethanol showed these zones which have similar R_f values.

The minimum inhibitory concentrations (MIC) were determined using the serial dilution microplate method described by Eloff (1998b). Ethyl ether and ethyl acetate extracts were most active against *E. faecalis* with MIC of 40 µg /ml (Table 1).

When evaluated for extraction efficiency and total antibacterial activity, acetone and tetrahydrofuran and ethyl acetate performed better than the other solvents. The active compounds therefore probably have intermediate polarity. The water extract had very low activity. It is noteworthy that the extracts were active against both Gram-positive and Gram-negative bacteria.

The total activity is a measure to compare different plants and takes into account not only the MIC but also the quantity extracted from one g of plant material. It is calculated by dividing the quantity in mg extracted from 1 g of plant material (mg) by the MIC (mg/ml) and gives an idea of potency of different plants (Eloff, 2000 and 2004). The value can also be used to compare the efficacy of different extractants. The average total activity indices for the four bacterial species varied from as low as 3 ml/g for water extracts to as high as 764 ml/g for acetone extracts (Table 2). The acetone extract was especially active against *E. coli* (1 433 ml/g) and the ethylacetate extract against *E. faecalis* (1 075 g/ml). This means that the quantity extracted from 1 g of plant material can be diluted to 1 433 ml and will still inhibit the growth of *E. coli*.

Isolation of antibacterial compounds

Because the acetone extract had the highest average total activity of all the extracts and for other reasons, such as low toxicity to test organisms, previously discussed by Eloff

TABLE 1. MIC values (µg/ml) of 10 extracts of *C. apiculatum* leaves tested against four bacterial species.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA	CA-1*	CA-2*	CA-3*
<i>E. coli</i>	600	100	100	100	100	100	100	100	100	600	300	130	300
<i>P. aeruginosa</i>	300	200	200	200	100	600	600	200	100	250	400	40	40
<i>E. faecalis</i>	300	100	40	200	40	200	200	300	300	500	600	250	130
<i>S. aureus</i>	600	200	200	200	200	200	200	200	100	130	40	40	80

IE-isopropyl ether, EE-ethyl ether, MD-methylene dichloride, EA-ethyl acetate, TH-tetrahydrofuran, AC-acetone, ET-ethanol, ME-methanol, HE-hexane, WA-water.

CA-1 – flavokawain-A, CA-2 – alpinetin, CA-3 – pinocembrin

* MIC values of compounds isolated in this study.

Gentamicin the positive control and had MICs between 0.02 and 0.6 µg/ml.

TABLE 2: Total activity in ml/g of *C. apiculatum* leaves, extracted with ten different extracts. IE-isopropyl ether, EE-ethyl ether, MD-methylene dichloride, EA-ethylacetate, TH-tetrahydrofuran, AC-acetone, ET-ethylacetate, ME-methanol, HE-hexane, WA-water.

Solvents	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
<i>E. coli</i>	19	307	358	588	537	163	1433	486	256	6
<i>P. aeruginosa</i>	38	153	179	294	537	204	179	243	255	2
<i>E. faecalis</i>	38	307	716	294	1075	819	717	121	64	1
<i>S. aureus</i>	19	153	179	294	268	820	717	243	256	3
Average	29	230	358	368	604	502	762	273	208	3

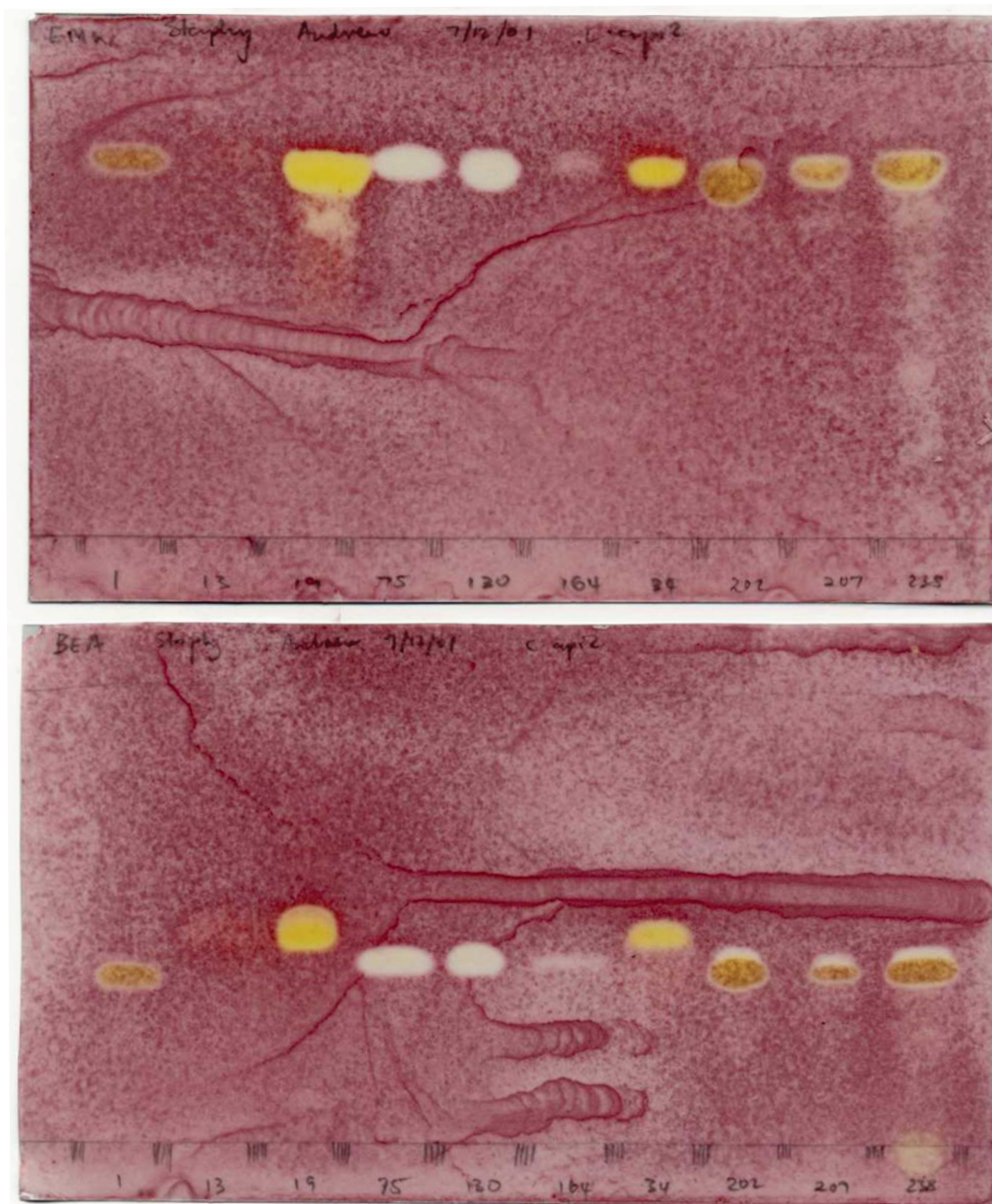


FIGURE 1: Bioautography of pooled fractions indicating the R_f values of antibacterial compounds where the development of red colour was inhibited against *S. aureus* separated EMW and BEA

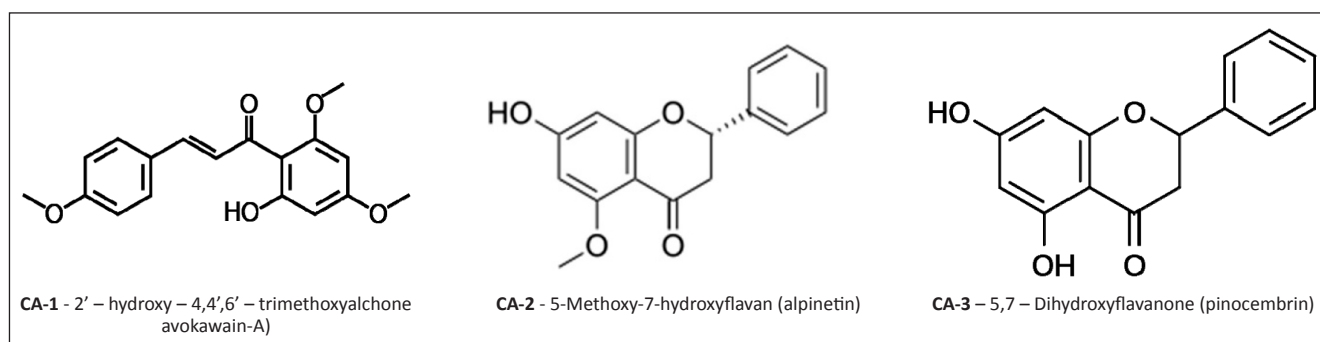


FIGURE 2: Structures of the three antibacterial flavonoids isolated from the leaves of *Combretum apiculatum* subsp. *apiculatum*

(1998a), acetone was chosen as the solvent of choice for further scale-up work. The dried acetone extract was then subjected to liquid-liquid partitioning and bioassay-guided isolation.

Repeated column chromatography of the chloroform sub-fraction resulted in the isolation of three compounds coded **CA-1**, **CA-2** and **CA-3** whose chemical structures were elucidated by NMR and MS (Figure 1). The NMR spectra of the compounds were similar and typical of flavonoids with a heterocyclic ring that had three carbons, C-2 resonating between 71–80 ppm, aliphatic methylene, C-3 resonating between 39–47 ppm and the carbonyl, C-4 between 186–199 ppm (Agrawal 1989). Based on the NMR spectra and HREIMS data and comparison with the literature, the compounds were established to be 4'-Hydroxy-2',6'-dimethoxychalcone (flavokawain-A) (**CA-1**) (Wollenweber and Sieglar 1982; Nascimento and Mors 1972), 5-Methoxy-7-hydroxyflavanone (alpinetin) (**CA-2**) (Jiang et al 2001) and 5,7 - Dihydroxyflavanone (pinocembrin) (**CA-3**) (Itokawa 1981).

The antibacterial activity of the isolated compounds determined by the serial dilution microplate method are presented in Table 1. *S. aureus* was the most sensitive being inhibited at 40 µg/ml by **CA-1** and **CA-2**. *E. faecalis* was inhibited at 40 µg/ml by **CA-2** and **CA-3**.

Discussion

Plants produce inducible phytoalexins or constitutive phytoanticipins to protect themselves from fungal and bacterial aggressors (González-Lamothe et al. 2009). This gives rationale to the continued interest in bioprospecting for new antibiotic lead compounds from higher plants.

E. coli was the most sensitive with extracts generally active at 100 µg/ml, while *S. aureus* was the most resistant. Using the total activity index, acetone was the most potent across all test species followed by ethylacetate. The extreme solvents i.e. hexane (least polar) and water (most polar) were the least active. This is not an unusual finding (Martini et al. 2004). The acetone extract was chosen for further fractionation because of the overall superior activity.

The activity of the isolated flavonoids compares favourably with that reported on other flavonoids isolated from *C. erythrophyllum* by Martini et al (2004a). Pinocembrin is one of the compounds responsible for the antibacterial activity of honey (Katerere et al. 2003). The antibacterial activity of flavonoids is now widely accepted (Cushnie and Lamb 2005). There have been discrepancies reported regarding the anti-microbial activity (or lack of activity) of flavonoids and this has been due to the use of different assay types e.g. agar diffusion, hole-plate diffusion and broth macrodilution (Cushnie and Lamb 2005). Some of these assays need the

compounds to diffuse into the agar before activity can be seen. The advantage of the method used in this assay is that the compound is already solubilised in the well and no diffusion is required.

Structure Activity Relationships (SAR) are important for antibacterial activity in phenolic compounds. The 2', 4'-or 2', 6'-dihydroxylation of 5,7-dihydroxylated flavanones e.g. **CA-2** was important for anti-MRSA activity. Chalcones have also been shown to be more active compared to flavones and flavonones. In this study flavokawain, a chalcone, had better antistaphylococcal activity than the flavones alpinetin and pinocembrin, confirming the conclusion of Cushnie and Lamb (2005).

Although no new compounds were isolated in this study, the demonstration of some moderate antibacterial activity by constituents of this species provides important information which is of interest. It was surprising that the crude ethyl acetate and ethyl ether extracts had an activity of 40 µg/ml against *E. faecalis*, while the three isolated compounds had much lower activities, i.e. activities of 130, 250 and 600 µg/ml (Table 1). The main compound active against *E. coli* based on bioautography had an R_i value of 0.8 in the EMW, the same as the three isolated compounds. This indicates that the compounds isolated are not artefacts due to the isolation procedure. It may be that the extract is more active than the individual compounds due to synergistic interactions. Similar conclusions were drawn after investigating two other *Combretum* spp. (Martini et al. 2004; Angeh et al. 2007). Synergy between flavonoids, between flavonoids and antibiotic agents or other phytoconstituents has been previously reported (Sato et al. 2004; Lin et al. 2005). Some phytochemical compounds have been defined as "antibiotic potentiators" acting by inhibiting bacterial multidrug resistance (MDR) efflux pumps or increasing hypersensitisation of the bacteria to high ionic strength and low osmotic pressure (González-Lamothe et al. 2009).

The results confirm the validity of using *C. apiculatum* crude extracts against bacterial infections rather than using isolated individual compounds. Future work should focus on establishing possible synergy between the active crude extracts and the isolated flavonoids with commonly used antibiotics to see if there is a potential for reversal of resistance. This would have possible application in low-cost settings in the long term once drug-herb interactions and pharmacokinetic studies have been done.

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