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Biological activity and toxicity profile of 17 *Agathosma* (Rutaceae) species

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Abstract

The antimicrobial, anti-oxidant and cytotoxic activities of the extracts obtained from 17 indigenous *Agathosma* species (19 samples) were investigated in order to validate the historic and continued use of *Agathosma* species in traditional healing. The antimicrobial activity was evaluated using the minimum inhibitory concentration (MIC) method on four pathogens, i.e. *Staphylococcus aureus* (ATCC 12600), *Bacillus cereus* (ATCC 11778), *Klebsiella pneumoniae* (NCTC 9633) and *Candida albicans* (ATCC 10231). The anti-oxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, while the cytotoxic properties was determined using the MTT (3-[4,5-dimethyl-2-thiazol-yl]-2,5-diphenyl-2H-tetrazolium bromide) cellular viability assay. *Agathosma ovata* (round-leaf) displayed the best activity against *S. aureus* and *B. cereus* with MIC values of 0.16 mg/ml and 0.13 mg/ml, respectively. Most of the extracts had moderate to poor activity in the DPPH assay with the exception of *A. capensis* (Gamka) and *A. pubigera* which were the two most active species in the assay (IC_{50} values of 24.08±4.42 μg/ml and 35.61±0.86 μg/ml). The results obtained from the ABTS assay differed from that of the DPPH assay. All extracts showed greater activity in the ABTS assay with *A. namaquensis* and *A. capensis* (Besemfontein) being the most active species (IC_{50} values of 15.66±4.57 μg/ml and 19.84±0.09 μg/ml). *Agathosma lanata* (IC_{50} value of 26.17±9.58 μg/ml) and *A. ovata* (round-leaf) (IC_{50} value of 25.20±6.30 μg/ml) proved to be the most toxic in the MTT assay. *Agathosma arida*, *A. collina*, *A. hirsuta*, *A. pubigera*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* also displayed some degree of toxicity.

Keywords: *Agathosma*; Antimicrobial; Anti-oxidant; Buchu; Toxicity

1. Introduction

The Cape region of South Africa has veld-types with arguably the richest composition of indigenous aromatic plant species in the whole of South Africa. Amongst these aromatic plants is the genus *Agathosma* that is restricted to this region. These shrubs are typical of the fynbos vegetation and are found in mountainous areas in the Cape (Van Wyk and Gericke, 2000). The genus *Agathosma* (Rutaceae) consists of approximately 150 species indigenous to South Africa (Goldblatt and Manning, 2000). They are commonly known as 'Buchu' and are perennial shrubs with woody branches and small leaves that vary from flat and convex to ericoid and concave. 'Buchu' (especially *Agathosma betulina* and *A. crenulata*) is probably one of the best-known South African herbs both locally and internationally. The indigenous people first introduced 'Buchu' as a medicinal plant to the European settlers in the Cape. The use of 'Buchu' subsequently spread to Europe and America where it was extensively used as a medicine. 'Buchu' is an important part of the San and Khoi culture in the Cape and is still used as a general tonic and medicine throughout South Africa. These tribes used the word 'Buchu' for any Rutaceous fragrant plant that could be dried and powdered, so this name does not designate a single species (Schwegler, 2003). The San used the aromatic plants lubricated with fat, to keep their skin soft and moist in the desert climate, as an antibacterial and antifungal agent, insect repellant, deodorant and to promote the general well-being of the body through the uptake of aromatic substances through the skin (Simpson, 1998). The leaves were chewed or prepared in a tincture containing brandy to relieve stomach complaints. A mixture of 'Buchu' and vinegar is still used today to clean wounds (Van Wyk et al., 1997). The oil distilled from 'Buchu' leaves is known to have therapeutic

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properties and the major uses include the treatment of urinary tract infections, stomach ailments, fever, coughs, colds, flu and rheumatism (Simpson, 1998; Moolla, 2006).

It is almost ironic that of the ca. 150 *Agathosma* species indigenous to South Africa, only two species, *A. betulina* and *A. crenulata* have been extensively investigated. Although the essential oil of some species have been thoroughly studied (Fluck et al., 1961; Kaiser et al., 1975; Blommaert and Bartel, 1976; Collins et al., 1996; Posthumus et al., 1996; Viljoen et al., 2006) the phytochemistry and especially the medicinal properties of the non-volatile fraction remains virtually unexplored. In view of the ethnobotanical uses of *Agathosma* species as described and the fact that they have been known since antiquity to possess biological activities, the aims of this study were to investigate the antimicrobial, anti-oxidant and toxic properties of the extracts of selected *Agathosma* species.

2. Materials and methods

2.1. Plant material

The aerial parts of 17 *Agathosma* species (19 samples) were collected from various localities in the southern Cape (Table 1). Voucher specimens were prepared and the identification confirmed by staff at the Bolus Herbarium (University of Cape Town) and the National Herbarium (SANBI, Pretoria). Duplicate specimens are housed in the herbaria and in the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

2.2. Preparation of solvent extracts

Known quantities of fresh plant material of each species were dried, ground and thereafter extracted for 24 h with methanol and dichloromethane (1:1). The extraction procedure was performed three times. The extracts were filtered and dried using a rotavaporator.

2.3. Determination of different pharmacological properties

2.3.1. Antimicrobial activity

The minimum inhibitory concentration (MIC) values were determined by a micro-titre plate dilution method (Eloff, 1998). Stock solutions were prepared by dissolving the extracts in acetone to obtain a concentration of 64 mg/ml. Sterile water (100 µl) was pipetted into all wells of the micro-titre plate before transferring 100 µl of stock solution into the plate. Eight serial dilutions were prepared using acetone to obtain concentrations ranging from 16 mg/ml to 0.125 mg/ml. A fixed bacterial culture (100 µl) of an approximate inoculum size of 1×10^6 CFU/ml was added to all wells and incubated at 37 °C for 24 h. After incubation, a 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) solution (50 µl) (Sigma) was added to each well. Plates were examined after 6 h to observe the concentration that inhibited bacterial growth completely (the first clear well). This was taken as the MIC value. A starting concentration of 0.01 mg/ml ciprofloxacin (Sigma) and amphotericin B (Sigma) were used as positive bacterial and yeast controls respectively. The last row of each plate containing sterile water and bacterial

Table 1
Biological activity and toxicity of *Agathosma* extracts

Species	Locality/source	Voucher	Antimicrobial				Anti-oxidant		Toxicity
			MIC (mg/ml), $n=2$				IC_{50} (µg/ml), $n=3$		IC_{50} (µg/ml), $n=3$
			B. cereus ATCC 11778	*S. aureus* ATCC 12600	*K. pneumoniae* NCTC 9633	*C. albicans* ATCC 10231	DPPH	ABTS	MTT
A. arida	Rooiberg	TTS 241	3.00	0.75	12.00	0.38	40.86±7.84	27.32±0.66	46.99±7.44
A. bathii	Kleinplaas	AV 1013	4.00	4.00	32.00	4.00	>100	29.25±0.59	>100
A. betulina	Landmeterskop	AV 852	4.00	4.00	4.00	2.00	>100	37.75±0.54	>100
A. capensis (B)	Besemfontein	TTS 348	3.00	3.00	3.00	4.00	30.79±0.43	19.84±0.09	94.63±6.41
A. capensis (G)	Gamka Mts	JEV 164	2.00	6.50	4.00	2.00	24.08±4.42	29.93±1.04	>100
A. collina	De Hoop	TTS 328	4.00	8.00	2.00	16.00	54.65±6.34	39.98±0.36	46.40±3.77
A. crenulata	Welbedacht	AV 853	2.00	2.00	4.00	2.00	>100	33.32±0.33	>100
A. hirsuta	Landdrostkop	TTS 310	0.75	0.25	2.00	3.00	>100	38.64±0.25	47.62±8.88
A. lanata	Rooiberg	TTS 242	4.00	1.50	6.00	4.00	>100	26.30±0.25	26.17±9.58
A. namaquensis	Khamiesberg	TTS 289	1.25	0.50	2.50	3.00	47.25±7.47	15.66±4.57	>100
A. ovalifolia	Droëkloof Mts	TTS 240	0.50	3.00	4.00	8.00	52.84±2.47	26.25±0.21	74.09±2.18
A. ovata (hook-leaf)	Gamka Mts	TTS 246	1.06	1. .00	7.00	6.00	51.45±4.13	24.71±0.19	81.15±5.93
A. ovata (round-leaf)	Anysberg	TTS 263	0.13	0.16	8.00	4.00	>100	46.81±1.54	25.20±6.30
A. parva	Die Galg	TTS 298	2.00	1.00	1.50	1.50	72.37±3.06	25.45±0.33	68.83±9.31
A. pubigera	Pakhuis	TTS 357	2.00	0.80	2.50	3.00	35.61±0.86	29.94±0.39	54.68±4.95
A. pungens	Kammanassieberg	TTS 253	1.00	0.75	4.00	6.00	94.65±1.65	31.57±0.82	66.07±6.78
A. roodebergensis	Rooiberg	TTS 237	0.50	1.00	12.00	4.00	56.71±4.76	29.63±0.32	38.05±7.29
A. stipitata	Die Galg	TTS 301	2.00	2.00	3.00	3.00	>100	28.20±0.34	40.96±8.24
A. zwartbergense	Swartberg	TTS 257	4.00	1.50	4.00	3.00	>100	31.73±0.36	38.12±3.08
Controls			3.1×10^{-3}	3.1×10^{-3}	6.3×10^{-3}	3.1×10^{-3}	2.47±0.178	2.96±0.001	136.06±4.06

culture represented the blank control incorporated into the assay to ensure consistent microbial growth. The MIC values were determined at least in duplicate.

2.3.2. Anti-oxidant activity

Two assays namely, the DPPH and ABTS assays were performed.

2.3.2.1. The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. A 96 μM DPPH (Fluka) solution was prepared in HPLC grade methanol and kept at 4 °C in the dark. Test extracts were dissolved in dimethyl sulfoxide (DMSO) (Saarchem) to obtain a stock solution of 10 mg/ml. For the first dilution, 50 μl of the stock solution was added to 950 μl DMSO to obtain a concentration of 500 μg/ml, with a final concentration of 100 μg/ml in the well. Serial dilutions (1:1) were thereafter performed using DMSO. Using a 96-well micro-titre plate, 50 μl of the initial stock solution and serial dilutions were plated out in triplicate from rows B to G. DMSO (50 μl), for control purposes was plated out in rows A and H. HPLC grade methanol (200 μl) was added to columns 2, 4, 6, 8, 10 and 12; while an equal volume of DPPH solution was plated out in columns 1, 3, 5, 7, 9, and 11. The micro-titre plate was shaken for 2 min and left to stand in the dark at room temperature for 30 min. The absorbance was read at 550 nm using a UV-VIS spectrophotometer (Labsystems Multiskan RC) linked to the computer equipped with GENESIS® software. The assay was performed in triplicate. The percentage decolourisation (free radical scavenging activity) of the test compound was calculated using the equation: [% decolourisation = 100 × (absorbance of control − average test absorbance + average absorbance of methanol) / absorbance of control] and the IC_{50} values were calculated using Enzfitter® version 1.05 software. Vitamin C (ascorbic acid) was used as the positive standard.

2.3.2.2. The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Stock solutions (10 mg/ml) of each of the extracts were prepared in DMSO. Working solutions were prepared at nine different concentrations. A stock solution of Trolox™ was prepared in ethanol and this was diluted to obtain working solutions. A 7 mM ABTS (Sigma-Aldrich) stock solution was prepared in double distilled water. The $ABTS^+$ was produced by reacting 5 ml of ABTS with 88 μl of 140 mM potassium persulphate ($K_2S_2O_8$) (Fluka) and the mixture was allowed to stand in the dark for 12–16 h in order to stabilize. The radical solution was stable for 2–3 days in the dark. The day of the assay, the $ABTS^+$ solution was diluted with cold ethanol to obtain an absorbance ranging between 0.68 and 0.72 at 732 nm in a 1 cm cuvette. Ethanol was used as a negative control. The radical scavenging activity was quantified by reacting 1 ml of $ABTS^+$ solution with 50 μl of sample. The mixture was thereafter heated for 4 min, after which the absorbance was read at 734 nm on a Specord 40 spectrophotometer. The assay was done in triplicate. The percentage inhibition was plotted as a function of the concentration, from which the equation of the straight line was calculated. The concentration that produced 50% decolourisation (IC_{50}) was determined as well as the standard deviation. Trolox™ was used as the positive anti-oxidant control.

2.3.3. Toxicity

Graham cells (transformed human kidney epithelium cells) were maintained continuously in culture at 37 °C in 5% CO_2. The MTT (3-[4,5-dimethyl-2-thiazol-yl]-2,5-diphenyl-2H-tetrazolium bromide) cellular viability assay was used to determine the toxicity profile of the extracts (Mosmann, 1983). The trypsinised cell suspension (0.25 million cells/ml) was plated out and incubated under humidified conditions for 6 h before the addition of the extracts (Van Zyl and Viljoen, 2002). The extracts were dissolved in DMSO (Saarchem) to prepare a stock solution of 10 mg/ml, from which seven 1:10 dilutions were prepared in Ham F10 culture medium. After 44 h of incubation at 37 °C, 2 mM MTT (USB™) was added to the plates and incubated at 37 °C for a further 4 h. DMSO was added to stop the reaction and dissolve the formazan crystals. The absorbance was read at a test wavelength of 540 nm and a reference wavelength of 690 nm from which the percentage cellular viability was calculated with the appropriate controls taken into account. Analysis was done in triplicate. Quinine was used as a standard control.

3. Results and discussion

3.1. Antimicrobial activity

The results obtained from the MIC assay are depicted in Table 1. The data reveals that all of the extracts were active against the four pathogens tested with the exception of *Agathosma bathii* which showed poor activity against *Klebsiella pneumoniae*. The MIC values ranged between 0.13 mg/ml and 32 mg/ml. The extracts exhibited greater activity towards the Gram-positive bacteria than the Gram-negative bacterium with *Agathosma ovata* (round-leaf) displaying the best activity against *Staphylococcus aureus* and *Bacillus cereus* (MIC values of 0.16 mg/ml and 0.13 mg/ml respectively). *Agathosma arida* displayed the best activity against *Candida albicans* with an MIC value of 0.38 mg/ml.

Fabry et al. (1998) reported that MIC values ≤8 mg/ml indicate good antibacterial activity for extracts. Based on this it is concluded that most of the extracts exhibited good antimicrobial activity against the tested pathogens.

In 1976, Finkelstein and Rivett (Finkelstein and Rivett, 1976) discovered a new prenyloxy-coumarin, namely puberulin in *Agathosma pubigera*, and the presence of coumarins have also been reported in other *Agathosma* species (Campbell et al., 1986). Several other coumarins have since been identified in *Agathosma* species (Campbell and Cragg, 1979; Campbell et al., 1982; Gray, 1981; Khalid and Waterman, 1983). Coumarins are phenolic substances consisting of fused benzene and α-pyrone rings and several have been found to have antimicrobial properties (Cowan, 1999). As a group, they have been found to stimulate macrophages, thereby indirectly eliminating an infection.

3.2. Anti-oxidant activity

Table 1 summarizes the radical scavenging activity of *Agathosma* species in the DPPH and ABTS anti-oxidant assays. The oxidative activity of the DPPH radical was inhibited by 11 of the extracts. Most possessed moderate to poor activity in the DPPH assay, with the exception of *Agathosma capensis* (Gamka) and *A. pubigera* which were two of the most active species (IC_{50} values of 24.08±4.42 μg/ml and 35.61±0.86 μg/ml). *Agathosma bathii*, *A. betulina*, *A. crenulata*, *A. hirsuta*, *A. lanata*, *A. ovata* (round-leaf), *A. stipitata* and *A. zwartbergense* showed weak anti-oxidant activity, all having IC_{50} values >100 μg/ml. The remaining species showed moderate to poor activity with IC_{50} values ranging between 30.79 μg/ml and 94.65 μg/ml. None of the extracts were found to exhibit radical scavenging activity equivalent to that of the standard, ascorbic acid (IC_{50} value of 2.47±0.178 μg/ml).

It is well known that plant polyphenolics act as free radical scavengers and as anti-oxidants (Yen and Hsieh, 1998). Polyphenolic compounds have more than one mechanism of action for suppressing free radical reactions and act as anti-oxidants by virtue of the hydrogen-donating capacity of their phenolic groups. HPLC analysis has revealed that *Agathosma* species are rich in flavonoids (Moolla, 2006) of which the anti-oxidant activities have been extensively reported (Yao et al., 2004). The results obtained from the ABTS assay differed from that of the DPPH assay. All extracts showed greater activity in the ABTS assay with *A. namaquensis* and *A. capensis* (Besemfontein) being the most active species (IC_{50} values of 15.66±4.57 μg/ml and 19.84±0.09 μg/ml), although not as active as Trolox™ (IC_{50} value of 2.96±0.001 μg/ml). The remaining species showed good activity with the IC_{50} values ranging between 24.71 μg/ml and 46.81 μg/ml. In general, the ranges of the free radical scavenging activities of the extracts were dissimilar in both the methods and no correlation (r^2=0.233) was found between the two (Table 1). It has previously been reported that results obtained from the ABTS assay do not necessarily have to correlate with the anti-oxidant activity from the DPPH assay, due to the differing mechanisms by which these reagents act and the probability that they exercise their protective properties at different stages of the oxidative process. Furthermore, the complex composition of the extracts, as well as the reaction products, could be responsible for certain interactions (synergistic, additive or antagonistic effects) between their components or the medium which may contribute to the overall activity (Parejo et al., 2002; Arts et al., 2003).

3.3. Toxicity

The toxicity of the *Agathosma* species was evaluated using the MTT assay and the results obtained are presented in Table 1. *Agathosma lanata* (IC_{50} value of 26.17±9.58 μg/ml) and *A. ovata* (round-leaf) (IC_{50} value of 25.20±6.30 μg/ml) proved to be the most toxic. *Agathosma arida*, *A. collina*, *A. hirsuta*, *A. pubigera*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* also proved to be toxic with IC_{50} values ranging between 38.05 and 54.68 μg/ml. It was also observed that *Agathosma bathii*, *A. betulina*, *A. capensis* (Gamka), *A. crenulata* and *A. namaquensis* were not toxic at concentrations up to 100 μg/ml. Serial dilutions displayed different degrees of cellular inhibition and the samples proved to be toxic in a dose-dependant manner. The toxicity profiles of *A. arida*, *A. collina*, *A. hirsuta*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* were similar to one another.

The aerial parts of selected *Agathosma* species were screened for alkaloids in a study performed by Campbell et al. (1987, 1990). Positive results were obtained for five species including *A. capensis*, and the compounds halfordamine and skimmianine were identified. Quinoline alkaloids were also detected in *A. barosmaefolia* (Campbell and Bean, 1996). Seven alkaloids including skimmianine were tested against the A2780 human ovarian cancer cell line, and all proved to have weak cytotoxic activity (Chaturvedula Prakash et al., 2003). A study performed by Cheng et al. (1990) found skimmianine to have a selective inhibitory effect on the 5-hydroxytryptamine-induced vaso-pressor responses in rats and at higher concentrations produced a non-specific blockade of cardiovascular function. It was also found to have a significant inhibitory effect on spontaneous motor activity, exploratory behaviour, cataleptogenic activity, conditioned avoidance response and long-term isolation-induced fighting of animals (Cheng, 1986).

'Buchu' has been an important part of the San and Khoi healing culture in the Cape and is still used throughout South Africa. It has been used as a cough remedy, for the treatment of colds and flu, kidney and urinary tract infections, as well as for the treatment of cholera and other stomach ailments. These data provide a scientific basis for the use of *Agathosma* species in treating various infections. This study confirms that these species have anti-oxidant compounds which may (in part) contribute to their health benefit properties and hence promote the general well being of the user.

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