International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (4), pp. 001-007, April, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Bioactivity of *Hydnora africana* on selected bacterial pathogens: Preliminary phytochemical screening

Nethathe B. B.[1] and Ndip R. N.[1,2*]

[1]Microbial Pathogenicity and Molecular Epidemiology Research Group, Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Fort Hare, P/Bag X1314, Alice 5700, South Africa. [2]Department of Biochemistry and Microbiology, Faculty of Science, University of Buea, Box 63, Buea, Cameroon.

Accepted 23 February, 2019

Medicinal plants contain a variety of chemical substances with important therapeutic properties that can be utilized in the treatment of human diseases. *Hydonora africana* is used in folklore remedies for the treatment of diarrhoea, dysentery, kidney and bladder complaints among other ailments; hence we assessed the *in vitro* antimicrobial activity of this plant against three bacterial species (*Helicobacter pylori* ATCC 43526*, Helicobacter pylori* PE 252C*, Staphylococcus aureus* NCTC 6571 *and Aeromonas hydrophila* ATCC 35654). The agar well diffusion method was used to determine the susceptibility of bacterial strains to crude extracts of the plant. The minimum inhibitory concentration (MIC_{50}) and minimum bactericidal concentration (MBC) of the active crude extracts were determined by the microdilution test. Ciprofloxacin (0.0125 mg/mL) was used as positive control. The presence of phytochemicals was also assessed using standard methods. Results were analyzed statistically by the one-way ANOVA test. *Hydnora africana* demonstrated antimicrobial activity against all the organisms with a mean zone diameter of inhibition ranging from 0 to 22 mm. The MIC_{50} of the extracts ranged from 0.078 to 2.5 mg/mL and MBC ranged from 0.78 to 25 mg/mL. Phytochemical assay revealed the presence of alkaloids, tannins, flavonoids, saponins and steroids in the extracts. It is concluded that *H. africana* may contain compounds with therapeutic activity.

Key words: *Hydnora africana,* medicinal plant, minimum inhibitory concentration, minimum bactericidal concentration, phytochemicals.

INTRODUCTION

Plants have a great potential for producing new phytochemicals with profound antimicrobial activity against human pathogens. According to the world health organization (WHO), more than 80% of the world's population depends on traditional medicine for their primary health care needs (Duraipandiyan et al., 2006). There is an upsurge of resistant microbial strains to conventional antimicrobials necessitating the need for a search and development of new drugs to circumvent the problem (Panda et al., 2009).

Hydnora africana is a parasitic plant which is predominant in the dry and semi-arid parts of the Succulent Karoo, Eastern Cape Karoo and the dry coastal thickets between the Eastern Cape and KwaZulu-Natal Provinces of South Africa (Asfaw et al., 1999). The plant belongs to the family *Hydnoraceae* (Musselman, 1991). It is renowned for its healing properties, and has been used by traditional medicine practitioners to treat aliments such as diarrhea, dysentery, kidney and bladder complaints (Van and Gericke, 2000). For example, infusions are used as face wash to treat acne by the Xhosa people of South Africa (Van and Gericke, 2000). More than 12 *Hydnora* species have been described with antimicrobial properties (Bolin and Musselman, 2009). However, there is paucity of data in South Africa on its antimicrobial activity as well as its chemical constituents, hence the present investigation against different Gram-positive and negative bacterial pathogens. *Helicobacter pylorus is* a Gram-negative, microaerophilic bacterium that causes

*Corresponding author. E-mail: rndip@ufh.ac.za; ndip3@yahoo.com. Tel: +27 782696191. Fax: +27 866224759.

duodenal ulceration, chronic gastritis and gastric cancer. (Ndip et al., 2008). An increasing number of infected individuals are found to harbour antibiotic-resistant strains (Ndip et al., 2008; Tanih et al., 2010). The emerging resistance to antibiotics, especially metro-nidazole and amoxicillin limits their use in the treatment of infections (Smith et al., 2001; Tanih et al., 2010). *Aeromonas hydrophila* is a heterotrophic, Gram-negative, rod shaped bacterium, mainly found in areas with a warm climate. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated water (Villari et al., 2003). It causes gastroenteritis, cellulitis, myonecrosis and eczema. Its increasing resistance to agents such as chloramphenicol, florenicol, tetracycline, sulfonamide, nitrofuran derivatives, and ciprofloxacin used to eliminate and control impending infection requires attention (Wang et al., 2011). *Staphylococcus aureus* is a facultative anaerobic, Gram-positive coccus and the most common cause of staphylococcal infections (Cosgrove et al., 2009). It causes a range of illnesses from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endo-carditis, toxic shock syndrome, bacteremia and sepsis (Kluytmans et al., 1997). The treatment of choice for *S. aureus* infection is penicillin; but in most countries, penicillin-resistance as well as MRSA is extremely common (Neely and Maley, 2000; Nkwelang et al., 2009). This study therefore documents the antimicrobial activity of *H. africana* against the selected bacterial pathogens, and a preliminary assessment of the possible phyto-chemicals responsible for its action.

MATERIALS AND METHODS

Bacterial strains

The following standard strains of bacteria *Aeromonas hydrophila* ATCC 35654, *S. aureus* NCTC 6571, *Helicobacter pylori* ATCC 43526 and a local metronidazole-resistant strain of *H. pylori* PE 252C isolated in our laboratory (Tanih et al., 2010) were used. Cultures of the other organisms were resuscitated and maintained on nutrient agar slants at 4°C (Cheesbrough, 1982; Cowan and Steel, 2004), while *H. pylori* was suspended in 20% glycerol and stored at -80°C until used.

Preparation of plant extracts

Hydnora africana was selected based on ethnobotanical information and preliminary data obtained in our laboratory. It was identified in collaboration with botanists at the University of Venda, Limpopo Province, South Africa where voucher specimens (BP04) have been deposited.

The method described by Ndip et al. (2008) to prepare extracts was employed with modifications. The plant was harvested, air dried for 2 weeks and ground to fine powder using a blender (ATO MSE mix, 702732, England). Organic solvents including methanol, ethanol, acetone, ethyl acetate (100%) and water were used for extraction. Briefly, the dried plant material (2.8 kg) was macerated in five fold excess of the solvent in extraction pots such that the level of the solvents was above that of the plant material. The slurry was put in a shaker incubator (Edison, N.J., USA) regulated at room temperature (RT) for 48 h then centrifuged at 300 rpm for 5 min (Model TJ-6 Beckman, USA) and filtered using filter papers of pore size 60^{Å}.The process was repeated twice for a total of three extractions (Okeleye et al., 2010). The combined extracts was concentrated in a rotavapor (BUCHI R461, Switzerland) and transferred to labelled vials and allowed to stand at RT to permit evaporation of residual solvents. A 3 gram sample of each plant extract was used for the preliminary bioassay, and 3 g kept in the extract bank for subsequent use. Stock solutions were prepared by dissolving the extracts in 10% Dimethyl Sulphoxide (DMSO).

Antibacterial susceptibility test

The agar well diffusion technique was employed as previously described by Dastouri et al. (2008). For *H. pylori*, Columbia base agar was prepared following the manufacturer's instructions, supplemented with 7% defibrinated horse blood and Skirrow's supplement (Oxoid, UK) while for *A. Hydrophila* and *S. aureus*, Muller-Hilton agar (Oxoid, UK) was prepared following the manufacturer`s instructions. A 0.5 McFarland standard was prepared by the method of Koneman et al. (1992), and 5 mL put into a sterile test tube. An inoculum of each microorganism was prepared from subcultures of the bacterial suspension and estimated to contain 10^8 colony forming units. Four to five colonies of the same morphological type were picked, emulsified in 0.9% physiological saline and used to evenly inoculate specific agar plates depending on the microorganisms. Wells were cut in each agar plate with a 6 mm cork borer. About 100 µL of the different concentrations (200, 100, 50 mg/mL) of the extract were put separately into each well, in each plate. Ciprofloxacin (0.0125 mg/mL) was used as a positive control. The plates were incubated at 37ºC for 24 h for *A. hydrophila* and *S. aureus* and 3 to 5 days for *H. pylori* under microaerophilic conditions (Anaerocutt, Baringstoke, England); the diameter of the zone of inhibition was measured and recorded in millimeters. The experiment was repeated 2 times for each strain.

Determination of minimum inhibitory concentration (MIC_{50})

MIC_{50} determination was carried out by the microdilution test method in 96 well plates as earlier described (Banfi et al., 2003; Njume et al., 2010) with slight modifications. Briefly, Two-fold dilutions of the most potent extracts and antibiotic (ciprofloxacin) were prepared in the test wells in complete Brian Heart Infusion (BHI) broth (Oxoid, UK); the final extracts and antibiotic concentrations ranged from 0.0024 to 5 mg/mL. Twenty microlitres of each bacterial suspension was added to 180 µL of extract containing culture medium. Control wells were prepared with culture medium and bacterial suspension only. Also included was culture medium and extract only at different concentrations. An automatic ELISA micro plate reader (Model 680, Bio-Rad, Japan) adjusted to 620 nm was used to measure the absorbance of the plates before and after 24 h incubation. The absorbencies were compared to detect an increase or decrease in bacterial growth. The lowest concentration of the test extract resulting in inhibition of 50% of bacterial growth was recorded as the MIC.

Determination of minimum bactericidal concentration (MBC)

To determine the MBC, 0.2 mL of the contents of the MIC was serially diluted tenfold in 0.9% physiological saline (Ndip et al., 2007). A loop full was taken from each tube and inoculated onto BHI agar plates. The MBC was recorded as the lowest concen-

tration of the extract that gave complete inhibition of colony formation of the test bacteria at the latter cultivation.

Phytochemical screening of the extracts

A small portion of the dry extract was subjected to phytochemical test using previously established methods (Akinpelu et al., 2009) to test for alkaloids, tannins, flavonoids, steroids and saponins.

Test for alkaloids

Exactly 0.5 g of the plant extract was dissolved in 5 mL of 1% HCl on steam bath. A millilitre of the filtrate was treated with drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids.

Test for tannins

About 1 g of the extract was dissolved in 20 mL of distilled water and filtered. Two to three drops of 10% $FeCl_3$ were added to 2 mL of the filtrate. The production of a blackish-blue or blackish-green colouration was indicative of tannins. To another 2 mL of the filtrate was added 1 mL of bromine water. A precipitate was taken as positive for tannins.

Test for flavonoids

A 0.2 g of the extract was dissolved in 2 mL of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration was an indication of the flavonoids.

Test for saponins

Two grams of the extract was boiled in 20 mL of distilled water in a water bath and filtered (Acrodisc syringe filter pall, USA). Aprroximately 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frosting was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of an emulsion.

Test for steroids

About 0.5 g of the extract was dissolved in 3 mL of $CHCl_3$ and filtered. Concentrated H_2SO_4 was added to the filtrate to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

Statistical analysis

Analysis was performed using the SPSS Version 17.0 (Illinois USA, 2009). The one way ANOVA test was used to determine if there was any statistically significant difference in the zone diameters of inhibition of the different solvent extracts; the MIC_{50} of the extracts and the control antibiotic (ciproxacillin). P-values < 0.05 were considered significant.

RESULTS

Extract yield

Different solvents including ethyl acetate, acetone, ethanol, methanol and water were used for extraction because the type of solvent used may have an effect on the nature of the compounds extracted, the quantity extracted and the resulting bioactivity of the extract. Water extracted the highest quantity followed by methanol, and ethyl acetate the least (Figure 1).

Antimicrobial susceptibility testing

The extracts showed *in vitro* activity against the test bacterial pathogens with the exception of water. The mean zone diameter of inhibition ranged from 0 to 22 mm (Table 1). Acetone, methanol and ethyl acetate were the most active extracts for *S. aureus*, *A. hydrophila* with mean zone diameter of inhibition ranging from 13 to 22 mm, while for *H. pylori,* methanol and ethyl acetate extracts showed activity with mean zone diameter ranging from 14 to 21 mm. The most active crude extracts (methanol and ethyl acetate) against all test microorganisms were statistically significant ($P < 0.05$) compared to all other extracts, but not to ciprofloxacilin ($P > 0.05$) the positive control, with mean zone diameter ranging from 14 to 17 mm. DMSO used as negative control, showed no activity. An inhibition zone of ≥ 12 mm was chosen as representative of bacterial susceptibility to the extracts. The breakpoint of ciprofloxacin (0.05 mg/mL) was taken at 21 mm (CLSI, 2008).

MIC and MBC determination

The active extracts were further assayed to determine their MIC_{50} and MBC against the bacterial pathogens. Although only methanol and ethyl acetate extracts showed activity against *H. pylori*; methanol, acetone, ethanol and ethyl acetate extracts were active against *S. aureus* and *A. hydrophila*. Subsequently methanol and ethyl acetate extracts were used for the determination of MIC_{50} and MBC for *H. pylori* (ATCC 43526 and PE 252C) and methanol, acetone, ethanol and ethyl acetate extracts were used to determine MIC_{50} and MBC for *S. aureus* and *A. hydrophila*. The MIC_{50} and MBC ranged from 0.078 to 2.5 mg/mL and 0.78 to 25 mg/mL, respectively for all studied microorganisms (Tables 2 and 3), while for ciprofloxacin it ranged from 0.0098 to 0.078 mg/mL and 0.78 to 0.098 mg/mL, respectively.

The Gram positive bacterium (*S. aureus*) was most susceptible to *H. africana* compared to the Gram negative bacteria (*A. hydrophila and H. pylori*). However, there was no statistically significant difference ($P > 0.05$) between the MIC_{50} and MBC of the different solvents against Gram negative and Gram positive organisms.

Phytochemical analysis

Phytochemical analysis of the four extracts is

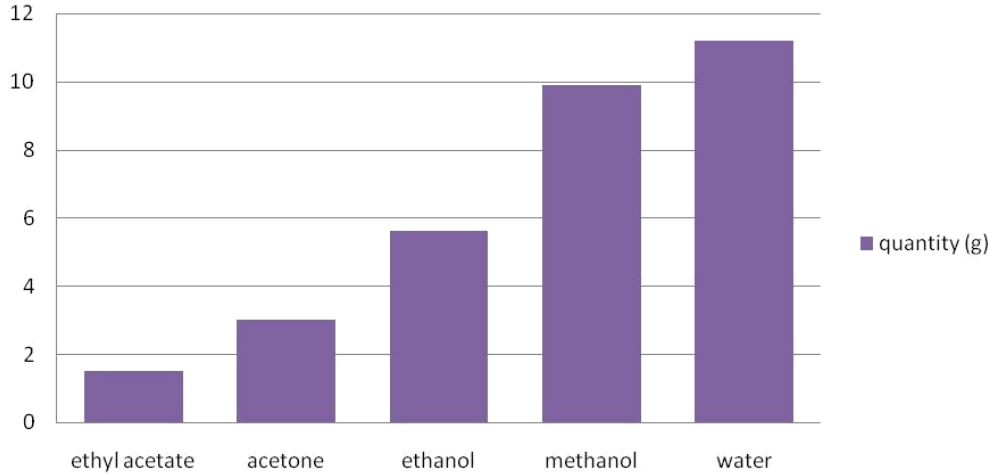

Figure 1. Quantity (grams) of *H. africana* flower extracted with different solvents. X-axis shows the different solvents used for extraction and Y-axis shows quantity extracted in grams.

Table 1. Antibacterial activity of extracts of *H.africana* against selected bacterial pathogens.

	Zone diameter at different concentration (mm)*																
	Methanol			**water**			**Acetone mg/mL**			**Ethyl acetate**			**Ethanol**			**Cipro**	
SBP	200	100	50	200	100	50	200	100	50	200	100	50	200	100	50	0.025	0.0125
S.a	17±2.1	21±2.1	22±2.1	0	0	0	20±0.7	19±1.4	22±3.5	16±0.7	18±0.7	19±0.7	14±1.4	16±1.4	17±1.4	17±0.7	17±0.7
A.h	17±0.7	16±0.7	15±1.4	0	0	0	17±2.1	17±1.4	18±1.4	14±1.4	13±1.4	15±0	13±0.7	16±1.4	14±0.7	17±1.4	17±1.4
H.p1	20±0.7	16±1.4	15±1.4	0	0	0	0	0	0	15±2.8	17±1.4	14±1.4	0	0	0	14±1.4	14±1.4
H.p2	17±2.1	21±2.1	18±0.7	0	0	0	0	0	0	16±0.7	19±1.4	17±0.7	0	0	0	15±0.7	15±0.7

SBP, selected bacterial pathogens; *S.a*, *S. aureus*; *A.h*, A. *hydrophila*; *H.P*1, *H. pylori* 43526; *H.p*2, *H. pylori* PE 252C; Cipro, ciprofloxacin; *, experiment was repeated twice and zone of inhibition recorded as mean zone diameter ±SD. Sensitivity zone ≥ 12 mm.

Table 2. MIC_{50} of different solvents extract of *H. africana* and the antibiotic against selected bacterial pathogens.

	Extracts and Antibiotic (mg/mL)				
SBP	**Methanol**	**Acetone**	**Ethanol**	**Ethyl acetate**	**Ciproxacillin**
S. aureus	0.625	0.156	0.156	0.078	0.0098
A. hydrophila	0.3125	0.156	–	0.078	0.0098
H. pylori43526	2.5	ND	ND	1.25	0.078
*H. pylori*252C	–	ND	ND	2.5	0.078

SBP, selected bacterial pathogens; –, MIC_{50} values not within susceptible range; ND, not determined.

Table 3. MBC (mg/mL) of different solvent extracts of *H. africana* and antibiotic against selected bacterial pathogens.

	Extracts/ Antibiotic (mg/mL)				
SBP	**Methanol**	**Acetone**	**Ethanol**	**Ethyl acetate**	**Ciproxacillin**
S. aureus	6.25	1.56	1.56	0.78	0.098
A. hydrophila	3.125	1.56	_	3.125	0.098
H.pylori43526	25	_	_	12.5	0.78
*H.pylori*252C	ND	_	_	ND	0.78

SBP, selected bacterial pathogens; –, MBC values not within susceptible range; ND, not determined.

Table 4. Phytochemical constituents of different solvent extracts of *H.africana.*

	Solvent extracts			
Phytochemicals	**Methanol**	**Acetone**	**Ethanol**	**Ethyl acetate**
Alkaloids	+++	++	+++	++
Saponins	+++	++	++	+++
Tannins	+++	+++	+++	+++
Flavonoids	+++	+++	+++	+++
Steroids	+++	+++	+++	+++

+++, Present in large quantity; ++, Present in moderate quantity.

in Table 4. The results revealed the presence of the following secondary metabolites: alkaloids, saponins, tannins, steroids and flavonoids, based on colour, heamolysis, turbidity, layers, emulsification and precipitation following the reactions.

DISCUSSION

The phytoconstituents of various plants have longed been known and their antimicrobial properties have been widely reported (Nostro et al., 2000; Roy et al., 2006). The antimicrobial activities of plant extracts have been linked to the presence of some bioactive compounds. These secondary metabolites also serve to protect the plants against bacterial, fungal and viral infections (De and Ifeoma, 2002; El-Mahmood and Amey, 2007). These bioactive compounds are known to work synergistically to produce various effects on human and animal subjects (Amagase, 2006). However, most reports on *H. africana* have focused mainly on the morphology of the plant, while information on its activity against hospital based pathogens is scanty (Bolin et al., 2009).

The extraction of active compounds from plant material and their activitiy depends on the type of solvent used in the extraction process (Parekh et al., 2005; Majhenic et al., 2007). In this study, it was observed that plant extractions with organic solvents provided stronger antibacterial activity than extraction with water. This study confirms the results of previous studies, which reported that water is not a suitable solvent for extraction of antibacterial compounds from medicinal plants compared to organic solvents, such as methanol (Karaman et al, 2003; Moniharapon and Hashinaga, 2004; Parekh et al, 2005; Majhenic et al, 2007).

S. aureus and A. hydrophila were the most susceptible organisms to all solvent extracts except water, with mean zone diameter of inhibition ranging from 13 to 22 mm. Moreover *H. pylori* (43526 and PE 252C) were only susceptible to methanol and ethyl acetate with mean zone diameter that ranged from 14 to 21 mm. This variation in activity may be due to the solvent used for extraction, e.g., methanol and ethanol are used for alkaloid extraction; acetone for flavonoids and steroids and ethanol may also be used for sterols, polyphenols, and tannins (Büssing, 1996). However, lack of activity demonstrated by water extracts *in vitro* against the organisms, does not necessarily imply that they would demonstrate weak activities *in vivo*.

In line with the findings of this study, another study had demonstrated very potent antibacterial activity of *Hydnora abyssinica*. In their study, Saadabi and Ayoub (2009) screened crude extracts of the family *Hydnoraceae* and reported potent antibacterial activity against common pathogenic Gram-negative and positive bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*.

In the present study, MIC_{50} and MBC recorded for *H. africana* against all studied microorganisms ranged from 0.078 to 2.5 mg/mL and 0.78 to 25 mg/mL, respectively. However, the methanol extract showed no inhibition at MIC_{50} against *H. pylori* PE 252C. The MIC results confirm earlier findings by Nariman et al. (2004) who documented MIC ranges of 0.0037 to 2 mg/mL. Also, MIC values of

0.0625 to 0.5 mg/mL have been documented for East African medicinal plants against similar bacterial pathogens (Fabry et al., 1996). A Gram positive bacterium (*S. aureus*) was most susceptible to *H. africana* compared to the Gram negative bacteria (*A. hydrophila and H. pylori*). Most plants extracts are active against Gram positive bacteria; this has been attributed to the fact that the cell wall of Gram positive bacteria is easier to penetrate than the Gram negative bacteria which contains an outer membrane with a lipopoly-sacharide layer which is impermeable to certain antibiotics and antibacterial compounds (Fennell et al., 2004).

Phytochemical analysis of the extracts of *H. africana* revealed the presence of alkaloids, saponins, tannins, flavonoids and steroids (Table 4). These phytochemical compounds are known to be biologically active and thus aid the antimicrobial activities of plants. Alkaloids were one of the phytochemical compounds identified in this study. Most common biological properties of alkaloids are toxic against cells of foreign organisms, anti-inflammatory, anti-asthmatic, and anti- anaphylactic properties (Ganguly and Sainis, 2001; Staerk et al., 2002) and may be responsible for the observed activity.

The presence of flavonoids in crude extract of *H. africana* is important since they have been reported to exhibit antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic, antioxidant, antitrypanosomal and antileishmanial properties (Ferguson, 2001; Hodek et al., 2002). Flavonoids in human diet may reduce the risk of various cancers, coronary heart diseases as well as preventing menopausal symptoms (Xu et al., 2000; Hodek et al., 2002; Tasdemir et al., 2006). Saponins and tannins were also reported in this study. Saponins are responsible for numerous pharmacological properties and are known to produce inhibitory effects on inflammation (Estrada et al., 2000). Tannins exert antimicrobial activities by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells (Njume et al., 2009). Herbs that have tannins are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003). Motar et al. (1985) revealed the importance of tannins for the treatment of inflamed or ulcerated tissues. Tannins were observed to have remarkable activity in cancer prevention (Li et al., 2003). Steroidal compounds were also present in the crude extracts of *H. africana;* they have drawn much interest in pharmacy due to their relationship with such compounds as sex hormones (Okwu, 2001). The findings of our study demonstrated the *in vitro* activities of the crude extracts of *H. africana* and provide preliminary evidence for the use of this plant in traditional medicine. This plant may provide new lead molecules, which could become starting materials for the synthesis of new drugs. Further study using bioassay-guided fractionation is necessary to isolate and characterize active compounds of the plant.

ACKNOWLEDGEMENTS

The authors are grateful to the National Research Foundation, South Africa (grant reference CSUR2008052900010), and the Govan Mbeki Research and Development Centre, University of Fort Hare, South Africa for funding this study. They are also grateful to Dr Samie A and Mr. Tshikhawe P for technical assistance.

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