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Medicinal value of *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) to a small community living at Gochas, southern Namibia

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Traditional medicine is widely used, but its effectiveness is often questioned. Biofilm-producing bacteria and fungi are important in difficult-to-treat persistent and recurrent infections. The present study investigated the anti-biofilm properties of crude methanolic extracts from three medicinal plants used in Namibia, namely *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.). Biofilm formation, inhibition and eradication were determined using microtiter plate assay. Extracts were tested against *Escherichia coli* ATCC 700928, *Staphylococcus aureus* ATCC 12600, *S. aureus* U3300, *Bacillus subtilis* ATCC 13933, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Pseudomonas aeruginosa* and *Candida albicans*. All isolates were strong biofilm producers. *A. albomarginatum* root extract moderately inhibited biofilm formation in *S. mutans* ATCC 25175 (60.0%), *E. coli* ATCC 700928 (51.6%) and *P. aeruginosa* (49.1%). *A. anthelmintica* twigs caused 58.4% biofilm inhibition in *C. albicans* and eradicated *S. aureus* U3300 biofilm by 74.8%. *D. schinzii* leaf extract inhibited *P. aeruginosa* biofilm by 67.3%, and in addition broke down *S. mutans* ATCC 25175 biofilm by 44.2%. These results validate the usefulness of the three plants as traditional medicine in some instances.

Key words: *Aptosimum albomarginatum*, *Albizia anthelmintica*, *Dicoma schinzii*, traditional medicine, anti-biofilm activity, flavonoids, saponins.

INTRODUCTION

All cultures across the globe have developed knowledge of local plants, enabling them to use these plants for medicinal purposes (Silvério and Lopes, 2012). Globally, many people still rely on such traditional medicine to remain healthy (van Wyk and Wink, 2015), because they often do not have access to modern medicine and

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antibiotic-based therapies (Silvério and Lopes, 2012).

African traditional medicine, which probably dates back to the origins of humankind, represents the most diverse of all medicinal systems, but is also the least systematized and most poorly documented of these systems. Traditional or folk medicine may be just as effective as conventional drugs, but its effectiveness is often questioned (van Wyk and Wink, 2015). Some strains of bacteria and fungi are able to form biofilms. Such biofilm producers are often responsible for difficult-to-treat persistent and recurrent infections. Hutcherson et al. (2015) define biofilms as dense, surface-attached communities of bacteria or fungi encased within a microbial-derived matrix that helps with colonization and survival. According to Mack et al. (2013) and Nazzari et al. (2014), this formation helps bacteria to withstand the host's natural immune defense mechanisms and to resist antibiotic treatment. In other words, as stated by Speziale and Geoghegan (2015), this is a survival strategy adapted by bacteria.

With drug-resistance being a never-ending problem, one should look into alternative treatment options, such as use of natural products, for example plant-derived products with antimicrobial and/or anti-biofilm activity. Such natural products may in some instances also be used as adjuvants together with antibiotics. Namibian people are using plants as natural medicine in the traditional setting to treat various illnesses, including biofilm-related infections. To rule out the possibility of placebo effects, such plants should be tested in the laboratory to validate their medicinal value.

The present study aimed to test crude methanolic extracts from three plants, namely, *Albizia albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) for their anti-biofilm properties in seven bacterial strains and a fungus. Extracts were screened for phytochemicals that may contribute to these properties.

MATERIALS AND METHODS

Selection of traditional medicinal plants and collection of plant material

Plant material was collected from the veld at Gochas (Altitude: 1139m; GPS coordinates: 24°47'S, 18°49'E), located in the Karas Region, southern Namibia in the month of February 2015. Plants were selected based on indigenous knowledge of local people about their medicinal value in the traditional setting. Voucher specimens were prepared and submitted to the herbarium at the National Botanical Research Institute (NBRI) in Windhoek for scientific identification of the plants. The selected plants (Figure 1) as identified by the NBRI are the shrub *Aptosimum albomarginatum* (Marloth and Engl.), the tree *A. anthelmintica* (A. Rich Brongn.) and the shrub *D. schinzii* (O. Hoffm.).

Plant material/extracts used

Crude methanolic extracts were prepared at the Biomedical Research Laboratory, Biological Sciences Department at the University of Namibia. Plant parts used were *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* roots and leaves.

Preparation of plant material

A. albomarginatum roots, *A. anthelmintica* twigs, and *D. schinzii* roots and leaves were prepared for extractions in the laboratory. Plant material was washed with tap water, placed on towel paper on the benches and left to air-dry at room temperature for two weeks. Thereafter, the material was cut and crushed to finer pieces and blended to powder form using a Philips Problend 5 household blender. Blended material was sieved, weighed, put into labeled 50 ml Falcon centrifuge tubes and stored in the freezer at -20°C.

Preparation of crude extracts

To prepare crude methanolic extracts, 10 g of plant material from the different plant parts was added to 100 ml methanol. Flasks containing the extracts were parafilmed, placed in a cupboard and left to stand for three days (maceration) with occasional swirling. After three days, the extracts were gravitationally filtered through Whatman 110 mm filter papers. The extracts were rotary evaporated in round bottom flasks at reduced pressure (91mbar) and temperature (45°C) to evaporate the methanol, and to dry and concentrate them. To avoid thermal decomposition of compounds in the plant material, the temperature set for the rotary evaporator (Heidolph, Germany) did not exceed 45°C. The flasks were labeled, sealed with parafilm and kept at -86°C for a few hours. Thereafter, the frozen extracts were connected to a Christ Alpha 1-2 LD Plus freeze-dryer (Germany) for two to four days to further dry and concentrate them. Dried extracts were scraped off with a spatula, weighed and stored in labeled 50 ml centrifuge tubes, and kept at -86°C for further use. Yields of extracts were calculated using the formula used by Osungunna and Adedeji (2011):

Percentage yield = Quantity of dried extract (g) / Quantity of powdered sample (g) × 100.

Phytochemical screening for flavonoids, saponins and anthraquinones

Antimicrobial and anti-biofilm activity of plant extracts may be attributed to the presence of secondary metabolites such as flavonoids, saponins and anthraquinones. These compounds were screened for using the methods for qualitative chemical assays described by Farnsworth (1966), with minor modifications.

For screening of flavonoids, 0.5 g of powdered plant material was added to a conical flask and extracted with 15 ml water and methanol (Merck, Germany) mixture in the ratio 2:1. The mixture was left to stand at room temperature for 30 min after which it was filtered using Whatman 110 mm filter paper. Thereafter, some magnesium turnings were added to the filtrate, and concentrated hydrochloric acid (HCl) (Merck, Germany) was added dropwise. Appearance of a yellow color indicated the presence of flavonoids.

To screen for saponins, 0.5 g powdered plant material was mixed with 15 ml distilled water. The mixture was then heated in a water bath at 100°C for 30 min, and the filtrate was left to cool down to room temperature. It was then vigorously shaken in a test tube for 10 seconds and observed for formation of froth. Froth measured 2cm or higher that persisted for 10 minutes or more confirmed the presence of saponins.

To screen for anthraquinones, 0.5 g plant material was extracted with 10 ml ether-chloroform (Merck, Germany) in the ratio 1:1 for 15 min at room temperature. The mixture was filtered and 1 ml of the

Table 1. Screening for flavonoids, saponins and anthraquinones.

Variable	*A. albomarginatum* roots	*A. anthelmintica* twigs	*D. schinzii* roots	*D. schinzii* leaves
Flavonoids	+	-	-	-
Saponins	+++	++	-	+
Anthraquinones	-	-	-	-

Key: +++ High presence; ++ Moderate presence; + Low presence; - Absent.

filtrate was treated with 1 ml of 10% (w/v) sodium hydroxide (NaOH) (Sigma, USA) solution. Development of a red color indicated the presence of anthraquinones.

Biofilm assays

Microorganisms used

The following biofilm-producing strains were obtained from the University of Pretoria's Biochemistry Department:

Escherichia coli ATCC 700928, *Staphylococcus aureus* ATCC 12600, *S. aureus* U3300, *Bacillus subtilis* ATCC 13933, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Pseudomonas aeruginosa* and *Candida albicans*. Strains were inoculated into Falcon centrifuge tubes containing 5 ml brain heart infusion broth (Merck, Germany) and grown to stationary phase at 37°C for 24 h.

Biofilm formation and inhibition

The methods of Christensen et al. (1985), Merritt et al. (2011), and Monte et al. (2014) were used for biofilm assays, with some modifications. To obtain a 0.5 McFarland turbidity standard of 1.5 x 10^8 CFU/ml, stationary-phase cultures were diluted 1:100 and 100µl of diluted culture was added to each of six 400 µl wells of a sterile flat-bottomed 96-well microtiter plate (Lasec, SA). Three of these six wells were each inoculated with 10 µl of crude methanolic extract dissolved in 100% Dimethyl sulfoxide (DMSO) just below minimal inhibitory concentration (MIC). MICs were determined by standard microbiological procedure, prior to this assay. Eight wells each contained 100 µl sterile brain heart infusion broth only as control. The plates were parafilmed at the lids to prevent them from drying out and incubated at 37°C for 24 h.

After incubation, planktonic cells were removed by placing the microtiter plate upside down on towel paper, and allowing for the paper to soak up any liquid. To remove remaining planktonic cells, each well was washed 3x by pipetting 400 µl triple distilled water into it, and inverting the plate onto towel paper. The biofilms in the wells were fixed by oven-drying the microtiter plates for 45 minutes at 60°C.

Wells were stained with 125 µl of 0.1% crystal violet, incubated for 15 min at room temperature and the crystal violet discarded. Excess stain was removed by washing (pipetting) 3x with 400 µl triple distilled water. Plates were air-dried for a few hours or overnight. Wells were de-stained with 200 µl of 33% glacial acetic acid (Merck, Germany) for 10 to 15 min. The contents of each well were briefly mixed by pipetting, and 125 µl was transferred to corresponding wells of a new clean microtiter plate. The optical densities (ODs) of stained biofilms were determined with a Multiskan Ascent plate reader (Thermo Labsystems, USA) at 595 nm. Readings from the broth control wells were averaged and subtracted from the test readings. Test readings were averaged and standard deviations calculated. Results for biofilm formation were interpreted using the classification by Christensen et al. (1985).

The equation I% = (1- (A_{595} of test / A_{595} of non-treated control) x 100) was used to calculate percentage inhibition (Kawsud et al. 2014). According to Manner et al. (2013), selection criteria (activity-based) for antimicrobials are as follows:

Highly active: ≥85% inhibition; moderately active: ≥40% inhibition; inactive: <40% inhibition.

Biofilm formation and eradication

Stationary-phase cultures were diluted 1:100, and 100µl of diluted culture was added to each of six 400 µl wells of a sterile flat-bottomed 96-well microtiter plate (Lasec, SA). Eight wells each contained 100 µl sterile brain heart infusion broth only as control. The plates were parafilmed at the lids to prevent them from drying out, and incubated at 37°C for 24 h. After incubation, planktonic cells were removed by decanting onto towel paper. To remove the remaining planktonic cells, each well was washed 3x by pipetting 400 µl triple distilled water into it and inverting the plate onto towel paper. Three wells with the grown biofilms were inoculated with 10 µl of extract (just below MIC) and 190 µl sterile triple distilled water. The remaining wells were filled with 200 µl water and plates were incubated at room temperature for another 24 h. After the second incubation and removal of liquid in the wells, the same steps for fixing, staining and de-staining were followed according to inhibition assay. The equation E% = (1- (A_{595} of test / A_{595} of non-treated control) x 100) (Kawsud et al., 2014; Teanpaisan et al., 2014) was used to calculate percentage eradication. The classification of activity for inhibition by Manner et al. (2013) was also used to interpret eradication results.

RESULTS AND DISCUSSION

Average percentage yields for *A. albomarginatum* root extract, *A. anthelmintica* twig extract, *D. schinzii* root extract and *D. schinzii* leaf extract were 12.5, 3.5, 6.1 and 7.3%, respectively. The plant material (roots, leaves and twigs) were used for qualitative detection of flavonoids, saponins and anthraquinones. According to Kamonwannasit et al. (2013) and Lee et al. (2016), these secondary metabolites can aid in a medicinal plant's antimicrobial properties, including anti-biofilm activity. The presence or absence of the compounds is indicated in Table 1. Flavonoids were detected only in *A. albomarginatum* roots. Saponins were present in *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* leaves. Anthraquinones were not detected.

Biofilm formation over 24 h is shown in Figure 2. All isolates were classified as strong biofilm formers, with *B. subtilis* ATCC 13933 being the strongest one. Single-

factor analysis of variance (ANOVA) in Microsoft Excel revealed that there was a significant difference in biofilm formation between the eight strains (P = 0.0002; F = 4.86; F crit = 2.18). A Tukey-Kramer Multiple Comparisons Procedure indicated that this difference was between *B. subtilis* ATCC 13933 and *S. sanguinis* ATCC 10556, *B. subtilis* ATCC 13933 and *E. coli* ATCC 700928, and *S. mutans* ATCC 25175 and *E. coli* ATCC 700928. The medicinal uses of the plants in this study, as well as anti-biofilm properties of their methanolic crude extract against seven bacterial strains and a fungus is discussed hereafter.

A. albomarginatum (Marloth and Engl.) as seen in Figure 1A is commonly known as "!Guxa" in Namibia (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015), also spelled "!Kuxa" (Sullivan, 1998) or "!Khuxa" (Huggins et al., 2013). The roots are pulverized, boiled as a tea and drunk to purify the blood and cleanse the uterus. Some believe that it can cure women who experience difficulty in conceiving. It also helps to relieve the symptoms of colds (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015).

Bacteria such as staphylococci may be associated with infection of the uterus, for example in the condition known as endometritis (inflammation of the endometrium). One form of this condition is known as bacteriotoxic endometritis, where it is caused by the toxins of bacteria rather than the presence of the pathogens themselves (Allen, 2004). Phytochemical screening revealed the presence of saponins in the roots (Table 1), and according to Wink and van Wyk (2008) saponins have anti-inflammatory effects. It is thus possible that the plant may be effective to treat conditions of the uterus, and saponins can play a role in its effectiveness.

According to Sullivan (1998), du Pisani, (1983) and Steyn (1981), Nama people shred the plant's roots and use it as a spice or coffee substitute. A decoction from the crushed root is drunk by the Nama to treat chest complaints, stomach disorders and coughs. Huggins et al. (2013) say that tea made from the plant is used to treat headaches, to induce vomiting and as a general body cleanser. Flavonoids were detected in the roots (Table 1). According to Wink and van Wyk (2008), flavonoids act as antioxidants and free-radical scavengers. Flavonoids may therefore be partly responsible for the plant's cleansing properties.

At a sub-MIC concentration of 0.625mg/ml, *A. albomarginatum* root extract caused 60.0% inhibition of biofilm formation in *S. mutans* ATCC 25175, and at 1.25 mg/ml it inhibited *E. coli* ATCC 700928 and *P. aeruginosa* biofilms by 51.6 and 49.1%, respectively. This is moderate inhibition, according to Manner et al. (2013). With inhibition below 40.0%, it was inactive against the fungus *C. albicans*, as well as the bacteria *S. aureus* U3300, *S. aureus* ATCC 12600, *S. sanguinis* ATCC 10556, and *B. subtilis* ATCC 13933, and could not eradicate pre-formed biofilms.

From these inhibition results, it is evident that *A. albomarginatum* roots may be used as traditional medicine to treat biofilm-related infections involving *S. mutans* ATCC 25175, *E. coli* ATCC 700928, and *P. aeruginosa*. The roots are not expected to be effective against biofilm infections caused by *C. albicans*, *S. aureus U3300*, *S. aureus* ATCC 12600, *S. sanguinis* ATCC 10556, and *B. subtilis* ATCC 13933.

A. anthelmintica (A. Rich Brongn.) seen in Figure 1B have many common names including "kersieblomboom", "worm-cure albizia" (Orwa et al., 2009; Hoffmann, 2014), "aruboom" and "oumahout" (Hoffmann, 2014). In traditional practice, the outer parts of the twigs are scraped off and the inner part is used as a chewing stick or toothbrush to clean the teeth and tongue (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015). Both bacteria and fungi may be associated with mouth infections, where they can be part of biofilm communities as dental plaque.

The bark, wood or root from *A. anthelmintica* is boiled and milk is added to treat an upset stomach or intestinal worms. Tea made from the roots and bark is drunk to treat malaria. The Samburu pastoralists in Kenya treat gonorrhea by boiling the roots, bark and leaves, mixing it with sheep fat and giving it as an enema. Otherwise the boiled bark and roots are consumed with milk (Sullivan, 1998; du Pisani, 1983; Fratkin, 1996). The stem bark is widely used as a purgative (Orwa et al., 2009). Consumption of this plant can also be hazardous. The seeds and bark contains alkaloids which have a toxic effect. Animal poisoning can occur in cattle and sheep; livestock can die from heart failure. Overdoses of the plant can also cause death in humans (Wink and van Wyk, 2008).

Twig extract (1.25 mg/ml) from *A. anthelmintica* showed inhibition of biofilm formation only in the fungus *C. albicans*, with moderate activity (58.4%). The twigs can thus be useful as a toothbrush to remove this fungus from the teeth and tongue. The extract eradicated biofilm also only in one strain – *S. aureus* U3300. The extract's activity may partly be due to saponins.

D. schinzii (O. Hoffm.) as seen n Figure 1C is also known as "Gu-!aru" (Coetzee, personal communication, February, 2015) or the "Kalahari fever bush" (Dugmore and van Wyk, 2008). The roots and leaves are pulverized, boiled as tea and drunk or used to steam yourself in the treatment of measles, chickenpox, the flu, colds and a blocked nose (Coetzee, personal communication, February, 2015). Unspecified parts are used to treat febrile convulsions in babies in the Kalahari, hence the name "Kalahari fever bush" (Sobiecki, 2002; Dugmore and van Wyk, 2008).

Measles, chickenpox, flu and colds are caused by viruses, but bacteria can be involved in congested nose

(A)

(B) (C)

Figure 1. *A. albomarginatum (A), Image credit:* southafricanplants.net; *A. anthelmintica (B), and D. schinzii (C)* growing in the veld at Gochas, Image credit: Sunette Walter

or sinus infections. There is an interesting folk tale behind the plant's traditional use in the Kalahari to treat febrile convulsions in babies. This tale known as "Dicoma's shadow" can be read in the book "Muthi and myths from the African bush" written by Dugmore and van Wyk (2008). van Wyk (2015) explains the story in short. It is said that if the shadow of the black shouldered kite (*Elanus caeruleus*) falls on a baby, the child will get sick, and this illness will be recognized by the spastic movements of the baby's arms, similar to the movements made by the bird's feathers when it is hanging over its prey. It is furthermore said that if the condition is not treated, the infant can develop feathers on its arms. An extract of the plant can be given both topically and internally, which will counteract the symptoms and cure the child. In the traditional African context, the symbol of the bird represents fever, since birds have a higher natural body temperature (40°C) compared to that of humans (37°C). "The condition of the bird" refers to fever. Referral to feathers on the arms is actually "the gooseflesh of fever" – one of the symptoms of febrile convulsions in infants.

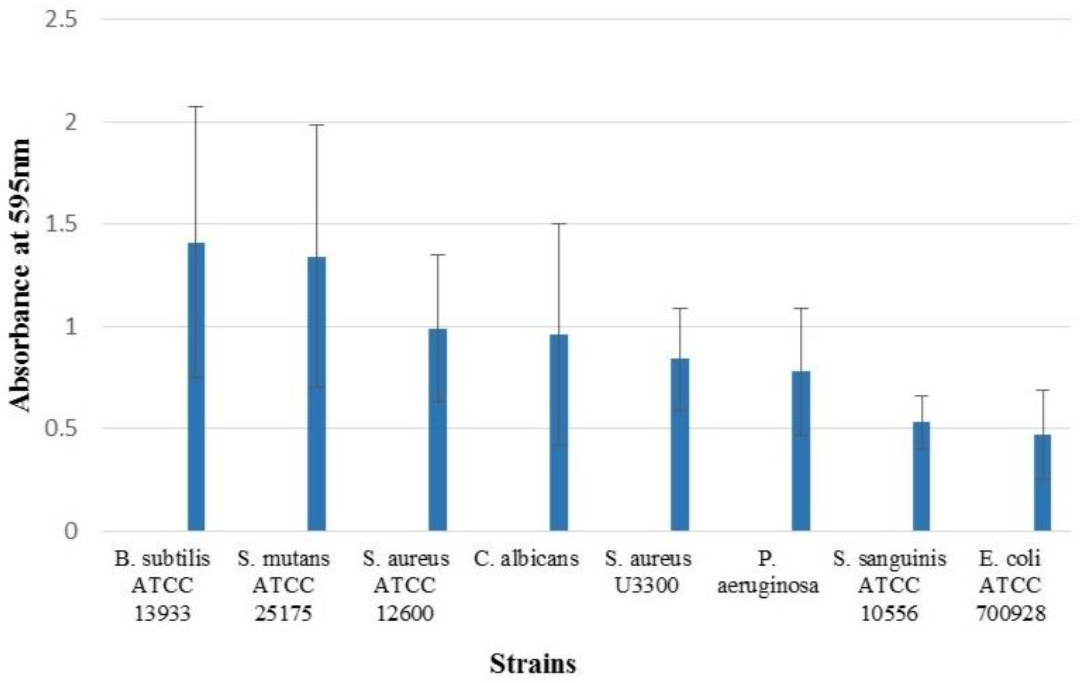

Figure 2. 24 h biofilm formation according to mean absorbance values at 595nm over eight microtiter plate assays. Formation was classified as strong/high with mean OD values > 0.240 (Christensen *et al*., 1985). Error bars indicate standard deviations. There was a statistically significant difference in biofilm formation between *B. subtilis* ATCC 13933 and *S. sanguinis* ATCC 10556, *B. subtilis* ATCC 13933 and *E. coli* ATCC 700928, and *S. mutans* ATCC 25175 and *E. coli* ATCC 7009298.

D. schinzii root extract was tested at a concentration of 1.25 mg/ml, and was unable to inhibit or eradicate biofilms. The plant's leaf extract (1.25mg/ml) however moderately inhibited biofilm formation in *P. aeruginosa* (67.3%) and is expected to help fight biofilm infections involving this bacterium. The extract eradicated the biofilm of *S. mutans* ATCC 25175 by 44.2%. Saponins in the leaves may be involved in biofilm eradication. *S. mutans* is normally not involved in the diseases mentioned by the locals at Gochas or in literature. The leaf extract may possibly be effective to treat other illnesses that are related to *S. mutans*, such as dental caries and endocarditis.

Conclusions

All strains in this study formed strong biofilms, with that of *B. subtilis* ATCC 13933 being the strongest. *A. albomarginatum* root extract moderately prevented biofilm formation in *S. mutans* ATCC 25175, *E. coli* ATCC 700928 and *P. aeruginosa,* and may therefore be effective as traditional medicine to treat biofilm infections involving these bacteria. *A. anthelmintica* twig extract inhibited *C. albicans* biofilm, and can thus be useful as a toothbrush or chewing stick to remove this fungus from the mouth. The twig extract may also be effective against biofilm infections involving the strain *S. aureus* U3300, as it was able to remove some of the bacterium's pre-formed biofilm. *D. schinzii* leaf extract moderately inhibited *P. aeruginosa* biofilm and moderately eradicated *S. mutans* ATCC 25175 biofilm. The leaves from this plant may thus be used in the traditional setting to treat biofilm infections related to these two strains. Anti-biofilm properties of the extracts under study may partly be attributed to the presence of flavonoids and saponins. The present work supports the use of the three medicinal plants in some instances.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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