Namibia Journal for Research, Science and Technology
VOLUME 3 Issue 1
May 2021
p-ISSN: 2026-8548
e-ISSN: 2026-8912

Original Research Article

Antibacterial and anti-biofilm properties of *Aptosimum albomarginatum* (Marloth & Engl.) and *Dicoma schinzii* (O. Hoffm.) crude methanolic extracts against *S. aureus* and MRSA.

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ARTICLE INFO

Received: May 2020
Accepted: May 2021

Keywords:
Aptosimum albomarginatum, *Dicoma schinzii*, antibacterial, anti-biofilm, *S. aureus*, MRSA

ABSTRACT

Background: The effectiveness of phytomedicine is often questioned by scientists. This study therefore aimed to test crude methanolic extracts from two traditional medicinal plants currently being used in Namibia for their antibacterial and anti-biofilm activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). ***Materials and methods***: *Aptosimum albomarginatum* (Marloth & Engl.) roots and *Dicoma schinzii* (O. Hoffm.) roots and leaves were used to prepare crude methanolic extracts by maceration, filtration, rotary evaporation and freeze-drying. Thin layer chromatography (TLC) was used to detect flavonoids, saponins and anthraquinones in the plant material. For antibacterial activity, two *S. aureus* reference strains (one susceptible and one multi-drug resistant MRSA) and 10 *S. aureus* nasal isolates from school children were used in disk diffusion assays with crude methanolic plant extracts. The microtiter plate assay with crystal violet stain was used to determine if these extracts could inhibit and/or eradicate bacterial biofilms. ***Results***: *Aptosimum albomarginatum* root extract displayed moderately antibacterial activity against five nasal isolates (one MRSA isolate) and two reference strains, of which one was multi-drug resistant MRSA. This extract was also the best biofilm inhibition agent, with highly active inhibition (86.0%) observed in *S. aureus* ATCC 33591 (MRSA). *Dicoma schinzii* root extract had moderate antibacterial activity against six nasal isolates and the two reference strains; its leaf extract was moderately active against two nasal isolates. The *D. schinzii* leaf extract moderately inhibited biofilms in two nasal isolates and *S. aureus* ATCC 25923. Flavonoids and saponins detected in both the roots and leaves of the two plants may have contributed to the extracts' antibacterial and antibiofilm activity. ***Conclusion***: *Aptosimum albomarginatum* roots and *D. schinzii* roots and leaves displayed anti-staphylococcal activity, indicating potential use against staphylococcal infections involving the bacteria under study. Noteworthy is both antibacterial and anti-biofilm properties of *A. albomarginatum* root extract against MRSA.

1. Introduction

Globally many communities make use of traditional medicine to remain healthy and treat a variety of ailments. Traditional medicine may be just as effective as conventional drugs, but its effectiveness is often questioned by scientists (van Wyk & Wink, 2015). *Aptosimum albomarginatum* (Marloth & Engl.) is commonly known as "!Guxa" by the Nama tribe in Namibia. 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 (Coetzee, 2015, personal communication; Frederick, 2015, personal communication). *Staphylococcus* may be associated with infections of the uterus, for example in the medical 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 (Dorland's illustrated medical dictionary, 2003). The tea also helps to alleviate cold symptoms (Coetzee, 2015, personal communication; Frederick, 2015, personal communication). Colds are viral infections, however, according to Bischoff et al. (2006) sneezing as a result of cold symptoms allows for rapid spreading of bacteria in the nose, including Staphylococcus aureus, to a person's surroundings and other people. *Dicoma schinzii*

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(O. Hoffm.) is also known as "Gu-!aru" in the Nama language (Coetzee, 2015, personal communication) or the "Kalahari fever bush" (Dugmore & van Wyk, 2008), since unspecified parts of the plant are used to treat febrile convulsions in babies (van Wyk & Gericke, 2000; Dugmore & van Wyk, 2008). The roots and leaves are pulverized, boiled as tea and drunk or used to steam oneself in the treatment of measles, chickenpox, the flu, colds and a blocked nose (Coetzee, 2015, personal communication). Measles, chickenpox, the flu and colds are caused by viruses, but staphylococci may be involved in congested nose or sinus infections.

Some strains of staphylococci are biofilm producers. 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 Speziale and Geoghegan (2015) the nasopharynx, heart valves, lungs and oral cavity are all sites for biofilm growth involving staphylococci and streptococci. Mack et al. (2013) and Nazzari et al. (2014), say that this formation helps the bacteria to withstand the host's natural immune defense mechanisms and to resist antibiotic treatment. Cowan (2012) explains that biofilm bacteria are often resistant to the same antimicrobials that work against them when they are free-floating. When attached to surfaces their gene expression is altered, leading to different antibiotic susceptibility profiles. According to Stefanović et al. (2012) bacterial resistance to antibiotics is a significant health problem. Solving this problem and the search for novel sources of antimicrobial agents is a global challenge and the aim of many researchers. Scientists have been screening plant extracts with the goal to discover new compounds effective for treatment of bacterial infections.

The present work assessed the antibacterial activity as well as biofilm inhibition and eradication properties of crude methanolic extracts from *A. albomarginatum* roots and *D. schinzii* roots and leaves against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) nasal isolates and reference strains. Extracts were also screened for secondary metabolites that may contribute to their activity. We concluded that *Aptosimum albomarginatum* roots and *D. schinzii* roots and leaves displayed anti-staphylococcal activity, indicating potential use against staphylococcal infections involving the bacteria under study.

2. Materials and methods

2.1 Selection of 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 February 2015. The two 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. Plants were identified as the shrubs *Aptosimum albomarginatum* (Marloth & Engl.) and *Dicoma schinzii* (O. Hoffm.).

2.2 Plant parts and extracts used

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

2.3 Preparation of crude extracts

To prepare crude methanolic extracts for bioassays, the maceration methods of Njateng et al. (2013) were followed, with some modifications. Plant material from the different plant parts was macerated in methanol (Skylabs, Johannesburg, SA). Flasks containing the extracts were parafilmed, placed in a cupboard and left to stand for three days with occasional swirling. After three days, the extracts were gravitationally filtered through Whatman 110mm 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, Schwabach, Germany) did not exceed 45°C. The flasks were labeled, sealed with parafilm and frozen at -86°C for a few hours. Thereafter, the frozen extracts were connected to an Alpha 1-2 LD Plus freeze-dryer (Christ®,Osterode, 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 (Greiner Bio-One, Kremsmünster, Austria) at -86°C for further use.

2.4 Phytochemical screening for flavonoids, saponins and anthraquinones

Antibacterial 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 thin layer chromatography (TLC) (Wagner & Bladt, 1996) on TLC gel 60 F_{254} aluminium sheets 20 x 20cm (Merck, Darmstadt, Germany). Five 2-ml Eppendorf tubes (Eppendorf, Germany) containing methanol were used, three for plant extracts and two for phytochemical standards (positive controls). Plant extracts and phytochemical standards were added to the respective tubes for spotting the TLC plates. Mobile phases (solvents) were prepared according to the ratios given in Table 1. Spraying reagents for confirmation of compounds belonging to the two

phytochemical classes were also prepared (Table 1). The developed TLC plates were viewed at visible and under UV light at 366nm (blue light, long wavelength). A pencil was used to trace around the most prominent spots/bands and observed colours were recorded. Chromatograms were placed on paper towels in a fume hood and sprayed with the appropriate spraying reagents. These were dried in the fume hood, and viewed again under UV light. Any colour changes and new spots/bands were circled and recorded. As described by Maobe et al. (2012) distances travelled by the solvents and the spots/bands were measured with a ruler and used to calculate retention factor (R_f) values: R_f = distance travelled by compound / distance travelled by solvent.

Table 1 Mobile phases (solvents), controls and spraying reagents used for TLC, adapted from Wagner and Bladt (1996).

Phytochemical class and standards (controls) used	Mobile phase	Spraying reagent and expected result
Flavonoids Control: Quercetin dihydrate (97%)	Ethyl acetate : Formic acid : Acetic acid : Water 100 : 11 : 11 : 27	1.0% Antimony (III) chloride in chloroform. Dark yellow, orange, green, or blue fluorescent spots at 366nm (intensified by spraying). Also detectable in visible light. Quercetin is orange-yellow or yellow-green. Flavonoid extracts often contain coumarins, which form blue, light blue or green fluorescent zones.
Saponins Control: Saponin	Butanol : Ethyl acetate : Acetic acid : Water 10.8 : 3.6 : 0.2 : 2.7	Vanillin-sulphuric acid reagent; sprayed plates heated at 110°C for 5-10 minutes. Blue, blue-violet, red, or yellow-brown zones in visible light. With some exceptions, saponins are not detectable under UV light and need spraying reagents.

2.5 Antibacterial and anti-biofilm activity

2.5.1 Microorganisms used

Ten *S. aureus* isolates (including one MRSA isolate) originating from nasal specimens of healthy school children aged 6-14 years in the Mariental District, Namibia, as well as commercially obtained *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591 (MRSA) (Microbiologics®, St. Cloud, USA) were used in disk diffusion and microtiter plate assays with crude methanolic plant extracts.

2.5.2 Antibacterial activity

2.5.2.1 Disk diffusion assays

Disk diffusion assays based on the Kirby-Bauer technique (Harley & Prescott, 2002) were performed to determine the antibacterial effects of the different plant extracts on staphylococci. Extract concentrations of 60mg/ml, 30mg/ml, 10mg/ml, 5mg/ml, 0.5mg/ml, and 0.1mg/ml were prepared in 2-ml Eppendorf tubes (Eppendorf, Germany) each containing undiluted dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and vortexed well to dissolve. A tube with only DMSO was kept to use as control. The assay was done in triplicate for each isolate. Whatman filter paper disks (110mm) were used to punch out smaller 6mm disks. The small disks were autoclaved in a screwed cap test tube before use. Bacterial-saline suspensions adjusted to 0.5 McFarland standard were swabbed onto Mueller-Hinton agar (Mast Diagnostics, Merseyside, UK) and the plates were left to dry for 5 minutes before applying the filter paper disks with a flamed tweezer onto the agar. Extracts (10µl) were pipetted onto the disks. Gentamicin (10µg) or chloramphenicol (30µg) antibiotic disks (Mast Diagnostics, Merseyside, UK) were applied as positive controls and disks containing DMSO as negative controls. Plates were incubated at 37˚C for 18-20 hours and inhibition zones measured with a ruler to the nearest millimeter. The classification by Nematollahi et al. (2011) was used to interpret results: ≤ 7mm inhibition (negative), 8-10mm (weak activity), 11-14mm (moderate activity), 15-24mm (strong activity), and ≥ 25mm (very strong activity). Results were compared with the antibiotic susceptibility profiles of isolates to see if the extracts have potential to be used as antimicrobial agents. Minimum inhibitory concentrations (MICs) were taken as the lowest concentration of extract that were able to inhibit bacterial growth on each plate.

2.5.3 Anti-biofilm activity

2.5.3.1 Microtiter plate assay for biofilm inhibition

For biofilm assays, the methods of Christensen et al. (1985), Merrit et al. (2011), and Monte et al. (2014) were used, with some modifications. Cultures were inoculated into 50-ml centrifuge tubes (Greiner Bio-One, Kremsmünster, Austria) containing brain heart infusion broth (Merck, Darmstadt, Germany) and grown to stationary phase at 37°C for 24 hours. The stationary phase cultures were then diluted 1:100 (a 0.5 McFarland standard) and some of the diluted culture was added to each of six wells in a sterile flat-bottomed 96-well microtiter plate (Thermo Fisher Scientific, Newport, UK). Figure 1 shows the plate layout. Three of these six wells were each inoculated with previously determined sub-minimum inhibitory concentrations (sub-MICs) of plant extract. Eight wells each contained sterile brain heart infusion broth only as control. 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 three times by pipetting sterile distilled water into it and inverting the plates 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 0.1% crystal violet, incubated for 15 minutes at room temperature and the crystal violet discarded. Excess stain was removed by washing (pipetting) three times with sterile distilled water. Plates were air-dried for a few hours. Wells were de-stained with 33.0% glacial acetic acid (Merck, Darmstadt, Germany) for 10-15 minutes. The contents of each well was briefly mixed by pipetting and transferred to corresponding wells of a new clean microtiter plate. The optical densities (ODs) of stained biofilms were determined with a SpectraMax M2 Multi-mode Microplate Reader (Molecular Devices, China) at 595nm. Readings from the sterile brain heart infusion broth control wells were averaged and subtracted from the test readings. Test readings were averaged and standard deviations calculated. Results for biofilm formation/inhibition were interpreted using the classification by Christensen et al. (1985). The equation I% = [1 – (A595 of test / A595 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 (≥ 85.0% inhibition); moderately active (≥ 40.0% inhibition); inactive (< 40.0% inhibition).

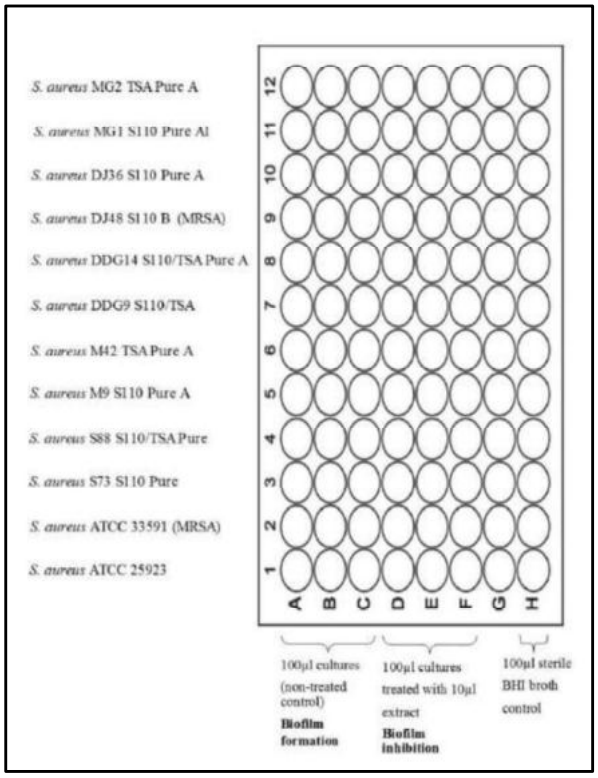

Figure 1 Schematic representation of the plate layout.

2.5.3.2 Microtiter plate assay for biofilm eradication

Stationary-phase cultures were diluted 1:100 and some diluted culture was added to each of six wells of a sterile flat-bottomed 96-well microtiter plate. Eight wells each contained 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 hours. After incubation, planktonic cells were removed by decanting onto towel paper. To remove remaining planktonic cells, each well was washed three times by pipetting sterile distilled water into it and inverting the plate onto towel paper. Three wells with the grown biofilms were inoculated with extract (just below MIC) and sterile distilled water. The remaining wells were filled with sterile distilled water and plates were incubated at room temperature for another 24 hours. After the second incubation and removal of liquid in the wells, the same steps for fixing, staining and de-staining were followed as for the inhibition assay. The equation E% = [1- (A_{595} of test / A_{595} of non-treated control) x 100] (Kawsud 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.

3. Results and Discussion

3.1 Phytochemical screening

Thin layer chromatography indicated the presence of flavonoids and saponins in all of the plant extracts, whereas anthraquinones were not detected (See Tables 2 and 3). For *A. albomarginatum* roots, one flavonoid spot of R_f 0.92 was observed. Close to our results, Hussain et al. (2011) detected a blue flavonoid spot with a R_f value of 0.94 at UV 365nm for methanolic extract of *Figonia critica*. In the present study, three saponin spots (R_f values 0.15, 0.49 and 0.94) were obtained. After derivatization with vanillin-sulphuric acid reagent and heating at 105°C, Priya et al. (2014) obtained a purple saponin spot with R_f 0.94 from chloroform extract of *Milagathi chooranam*. In this work, for *D. schinzii* roots, four flavonoid spots of R_f values 0.08, 0.28, 0.57 and 0.97 were present. Two saponin spots with R_f values 0.71 and 0.96 were observed. With *D. schinzii* leaves, there were four flavonoid spots (R_f 0.43, 0.59, 0.69 and 0.96) and three saponin spots (R_f 0.50, 0.71 and 0.96). Using TLC, Olivier (2012) detected steroids, terpenoids, bitter principles, saponins and flavonoids in *Dicoma* species, including *D. schinzii*.

Table 2 Screening for flavonoid compounds in plant extracts based on R_f values and colour changes on chromatograms before and after spraying with 1.0% antimony (III) chloride reagent.

	Compound number and R_f in brackets	Colour before spraying	Colour after spraying	Identification
Extract 1: ***A. albomarginatum*** **roots**	1. (0.92)	Light fluorescent blue at UV 366nm	Yellow in visible light	Flavonoid
Extract 2: ***D. schinzii*** **roots**	1. (0.08)	Light fluorescent blue at UV 366nm	Became colourless	Flavonoid
	2. (0.28)	Light fluorescent blue at UV 366nm	Became colourless	Flavonoid
	3. (0.57)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid
	4. (0.97)	Light fluorescent blue at UV 366nm	Yellow in visible light	Flavonoid
Extract 3: ***D. schinzii*** **leaves**	1. (0.43)	Light fluorescent blue at UV 366nm	Yellow in visible light	Flavonoid
	2. (0.59)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid
	3. (0.69)	Fluorescent yellow at UV 366nm and yellow in visible light	Stayed the same	Flavonoid
	4. (0.96)	Light fluorescent blue, pink and orange mixture at UV 366nm	Yellow in visible light	Flavonoid
Control/standard: Quercetin dihydrate (97%)	1. (0.97)	Fluorescent yellow-green at UV 366nm and yellow-green in visible light	Brighter yellow-green	Quercetin dihydrate (flavonol)

Table 3 Screening for saponin compounds in plant extracts based on R_f values and colour changes on chromatograms before and after spraying with vanillin-sulphuric acid reagent.

	Compound number and R_f in brackets	Colour before spraying	Colour after spraying	Identification
Extract 1: ***A. albomarginatum*** **roots**	1. (0.15) 2. (0.49) 3. (0.94)	Colourless Colourless Light fluorescent blue at UV 366nm	Purple in visible light Purple-brown in visible light Purple-brown in visible light	Saponin Saponin Saponin

Extract 2: *D. schinzii* roots	1. (0.71) 2. (0.96)	Colourless Light fluorescent blue at UV 366nm	Purple-brown in visible light Purple-brown in visible light	Saponin Saponin
Extract 3: *D. schinzii* leaves	1. (0.50) 2. (0.71) 3. (0.96)	Yellow-brown in visible light Yellow-brown in visible light Fluorescent orange-pink mixture at UV 366nm	Stayed the same Stayed the same Green at UV 366nm and purple in visible light	Saponin Saponin Saponin
Control/standard: Saponin	1. (0.1)	Colourless	Red in visible light	Saponin

3.2 Antibacterial activity

3.2.1 *Aptosimum albomarginatum* root extract

Using disk diffusion assays, at the highest (60mg/ml) concentration, *A. albomarginatum* root extract was moderately active against five nasal *S. aureus* isolates (one MRSA isolate and the two *S. aureus* reference strains ATCC 25923 and ATCC 33591). The largest inhibition zone (13.67 ± 0.58mm) was observed against *S. aureus* DJ36 S110 A. This isolate was obtained from the nose of a 9-year-old boy. Based on inhibition zone size, the root extract was not more effective than the antibiotic control gentamicin (GM) 10µg, that had an average inhibition zone of 23.33mm. As observed in this study and other studies (Kamonwannasit et al., 2013; Carranza et al., 2015) flavonoids and saponins may play a role in the root extract's antibacterial activity.

3.2.2 *Dicoma schinzii* root extract

By disk diffusion assay, at the highest (60mg/ml) concentration, *D. schinzii* root extract was moderately active against six nasal isolates and the two reference strains, as depicted in Figure 2. This extract displayed weak activity against MRSA. The largest inhibition zone (14.0 ± 0mm) was observed against *S. aureus* M9 S110 Pure A, isolated from the nose of a 7-year-old girl. The root extract was not more effective than the control antibiotics chloramphenicol (C) 30µg or gentamicin (GM) 10µg, that had average inhibition zones of 23.5mm and 18.84mm, respectively. Flavonoids and saponins in the roots may have antibacterial properties.

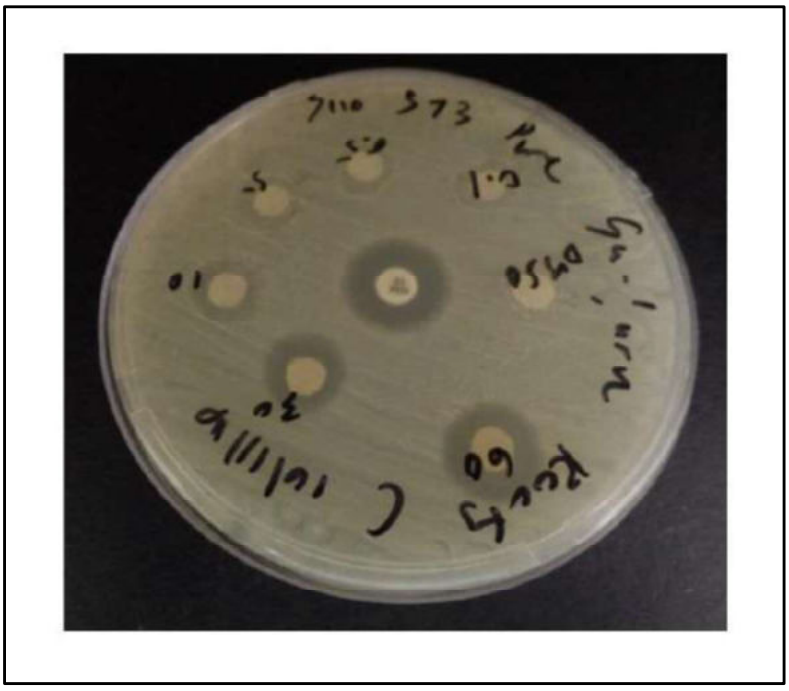

Figure 2 Moderate antibacterial activity of *D. schinzii* root extract at 60mg/ml and 30mg/ml concentrations, with inhibition zones of 13.1 ± 1.0mm and 11.67 ± 0.58mm, respectively.

3.2.3 *Dicoma schinzii* leaf extract

According to disk diffusion assays, at 60mg/ml, *D. schinzii* leaf extract was moderately active against only two *S. aureus* nasal isolates, with an average inhibition zone of 12.33 ± 0.58mm for each of them. This activity may partly be attributed to the presence of flavonoid and saponin compounds in the leaves. The extract showed very weak activity against the MRSA strain ATCC 33591. It was less active than the antibiotic control chloramphenicol (C) 30µg, that inhibited bacterial growth by an average of 17.89mm.

3.4 Anti-biofilm activity

3.4.1 Biofilm inhibition

As shown with the microtiter plate assay, overall, *A. albomarginatum* root extract was the best biofilm inhibition agent, with highly active inhibition (86.0%) observed in *S. aureus* ATCC 33591 (MRSA), and moderate activity in four other nasal isolates (Figure 3).

Flavonoids and saponins were detected in the plant's roots (Tables 2 and 3) and leaves. These secondary metabolites may play a role in its activity. Manner et al. (2013) observed 10 commercially bought flavonoids to be highly active, causing more than 85.0% biofilm inhibition and eradication against the clinical strains *S. aureus* ATCC 25923 and the Newman strain. Kamonwannasit et al. (2013) found that *Aquilaria crassna* leaf extract, containing flavonoids and saponins, could inhibit biofilm formation in *Staphylococcus epidermidis*. They explained that destruction of the bacterial cell wall by the plant extract prevents bacteria from growing and creating primary biofilm structures. In our study, *Dicoma schinzii* leaf extract moderately inhibited biofilm formation in two nasal isolates and the reference strain ATCC 25923. *Dicoma schinzii* roots were classified inactive against staphylococci in this study.

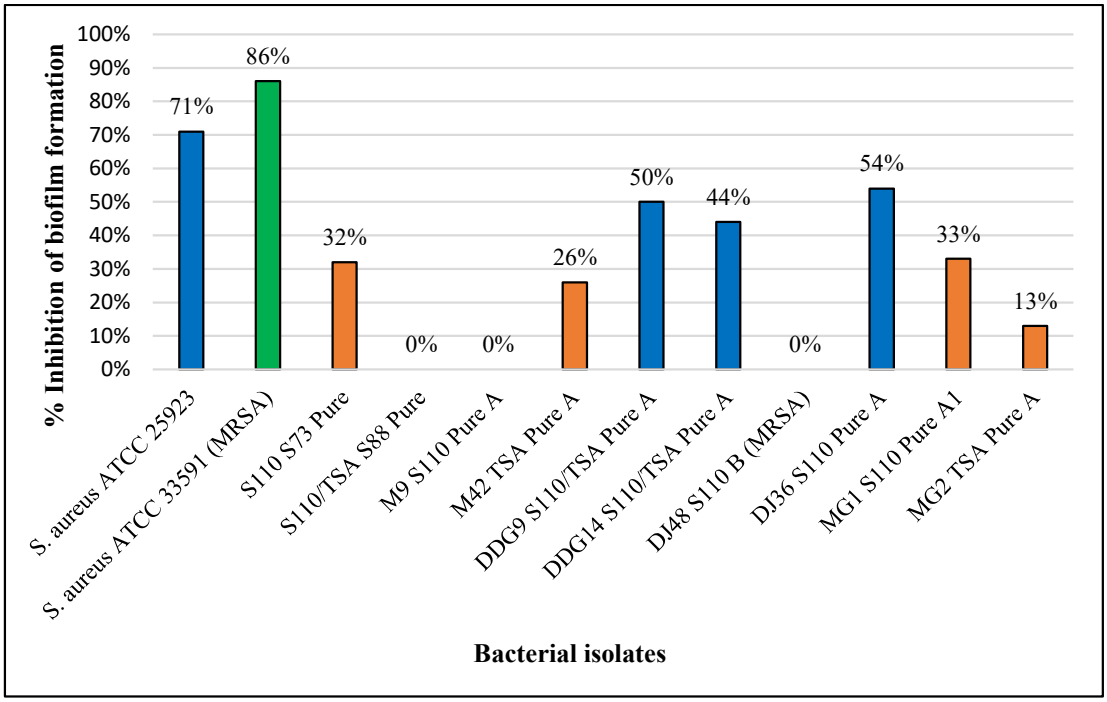

Figure 3 Percentage biofilm inhibition in microtiter plates by treatment with *A. albomarginatum* crude methanolic root extract. Classification of activity according to Christensen et al. (1985): ≥ 85.0% inhibition (highly active); ≥ 40.0% inhibition (moderately active); < 40.0% inhibition (inactive).

3.4.2 Biofilm eradication

Biofilm eradication was observed in microtiter plate assay only with *A. albomarginatum* root extract in only one *S. aureus* nasal isolate from an 11-year-old boy. It destructed the biofilm of this isolate by 40.0% (moderate activity). As stated previously, this activity may partly be attributed to the presence of flavonoid and saponin compounds in the plant's roots.

4. Conclusion

This study evaluated crude methanolic extracts from two traditional medicinal plants currently being used in Namibia for their antibacterial and anti-biofilm activity against *S. aureus* and MRSA. *Aptosimum albomarginatum* roots and *D. schinzii* roots and leaves displayed antibacterial activity, indicating potential use against staphylococcal infections. *Aptosimum albomarginatum* root extract was the best anti-biofilm agent against *S. aureus*. It was highly active in inhibiting

biofilm formation in one MRSA reference strain, and moderately active in inhibiting formation in four nasal isolates. This extract moderately eradicated the biofilm in one nasal isolate. Noteworthy is both antibacterial and anti-biofilm properties of *A. albomarginatum* root extract against MRSA. *Dicoma schinzii* leaf extract moderately inhibited biofilms in two nasal isolates and *S. aureus* ATCC 25923. Flavonoids and saponins may contribute to extracts' activity. The present work supports the traditional medicinal use of *A. albomarginatum* roots and *D. schinzii* roots and leaves as natural anti-staphylococcal agents in infections involving the bacteria under study.

Conflicts of interests

The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge and thank the University of Namibia's Biomedical Research Laboratory, Biological Sciences Department where the work was carried out. We also acknowledge and thank the Southern African Biochemistry and Informatics for Natural Products (SABINA) and the Regional Initiative in Science and Education (RISE) for financial support. Lastly, we thank the people at Gochas for sharing their knowledge on traditional medicinal plants with us. This work forms part of a larger Ph.D. study undertaken at the University of Namibia.

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