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Research Article

Antimicrobial and antibiofilm activity of certain South African plants against the oral pathogen - *Streptococcus mutans*

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ABSTRACT

Dental caries is a serious oral infection caused by microorganism in the oral cavity. *Streptococcus mutans* can colonize the teeth surfaces and forming biofilm leading to dental caries. The investigation of the antibacterial activity of the plants' secondary metabolites may lead to the development of alternative therapeutics to treat multi-resistant bacterial infections. South Africa has unique floral biodiversity especially in the Cape region and many of these plants have been used locally for medicinal use. In this study, the antibacterial activities of several South African plant extracts have been tested against *S. mutans in-vitro*. *Psoralea pinnata* and *Otholobium fruticans* showed the highest antimicrobial activity with MIC value of 31.25 µg/mL against *S. mutans*. The same extracts also effectively inhibited the biofilm formation by *S. mutans*.

Keywords: dental caries, *Streptococcus mutans*, Cape flora, plant extracts, antibacterial activity, antibiofilm

INTRODUCTION

Dental caries (cavities) is a chronic oral infectious disease that is initiated through a series of complex chemical and biological reactions caused by microorganisms found in the oral cavity. Biofilm and fermentable dietary carbohydrates in the oral cavity are key environmental factors involved in the initiation and development of dental caries (Lien *et al*., 2014).

The gram-negative bacteria *Streptococcus mutans,* is a member of the human oral flora, which is recognized widely as one of the main factors that can cause teeth decay (Hamada and Slade, 1980). *S. mutans* colonize the tooth surface and initiate the biofilm formation by its ability to biosynthesize different types of polysaccharides. These polysaccharides allow the adhesion of the bacterial cells to each other and on teeth surfaces. They also protect the bacterial cells from the fluid movements and prevent the penetration of antibiotics and help the bacteria to evade the host immune attack (Limoli *et al*., 2015). Indeed, the formation of the biofilm in the oral cavity is an important determinantal factor in most of the oral infectious diseases. *S. mutans* has been identified as the primary etiological bacteria in the dental biofilm (Kouidhi *et al*., 2015). Because of their high prevalence and severity, oral diseases impose a huge economic burden on the South African health

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system (Kouidhi *et al*., 2015). Over half of the children under the age of 12 in South Africa have dental caries and over two thirds of these cases do not receive proper treatment (van Wyk *et al*., 2004).

The emergence of antibiotic-resistant bacterial strains has motivated natural product researchers to screen the biomolecules found in plant extracts for their effective use as antibiotics (Agbor and Naidoo, 2016). The Cape Floristic Kingdom, located in South Africa, contains around 9000 indigenous plant species, of which two thirds of these species are endemic to the region (Manning and Goldblatt, 2000). The use of South African plants for treatment of infectious diseases has been reported (Van Vuuren and Holl, 2017). To exemplify, the *Acacia karroo* plants, which is used to alleviate the symptoms associated with listeriosis, was found to exhibit a minimum inhibition concentration (MIC) value of 3.1 mg/mL against *Listeria monocytogenes* (Nyila *et al*., 2012). *A. karroo* and *Salvia africana-lutea*, another famous South African medicinal plant, were able to inhibit the growth of several fungi, mycobacteria, gram negative and gram positive bacteria (Nyila *et al*., 2012). However, only few studies have been conducted to evaluate the antimicrobial activity of South African plants against oral pathogens (Akhalwaya *et al*., 2018). A study by Akhalwaya *et al.* (2018) screened 31 South African plants against nine oral pathogens. Two of these plant species, *Clematis brachiate* and *Englerophytum magalismonatanum*, which are used to treat oral infections in folk medicine, displayed noteworthy antimicrobial activity against the tested oral pathogens including *S. mutans* (Akhalwaya *et al*., 2018). Therefore, the systematic antibacterial screening of plants extracts, especially those with documented use in folk medicine, may offer solutions against multi-resistant bacteria.

The present study aims at the investigation of the antibacterial activity of certain South African plants against *S. mutans*. The study includes the determination of their MIC, minimum microbicidal concentration (MMC) and their antibiofilm properties.

MATERIALS AND METHODS

Bacterial culture

S. mutans ATCC® 25175 was procured from American type culture collection (ATCC). Brain heart infusion media, nutrient broth media and tryptic soy agar from Quantum biotech (Mumbai, India). *p*-iodonitrotetrazolium chloride (INT), neomycin, dimethyl sulfoxide (DMSO), Fluoroshield™ with DAPI, acetic acid, dichloromethane (DCM), methanol (MeOH) and phosphate buffer saline (PBS) were used from Sigma-Aldrich (Cape Town, South Africa).

Plants collection

27 South African plant species were collected from both Cape Flats natural reserve and University of the Western Cape campus, Cape Town, South Africa, in November 2012. Voucher specimens were identified by Mr. Weitz Franz (Biodiversity Department, University of the Western Cape) and deposited at the Chemistry Department, University of the Western Cape.

Preparation of extracts

Dry aerial plant materials (~100 g) were blended with MeOH for 24 h. After extraction, the filtrates of each extract were combined and evaporated under reduced pressure at 45°C and the extracts were kept at -5°C till further use.

Bioautography qualitative screening

Thin Layer Chromatography (TLC) bioautography was done according to the procedure reported before (Horváth *et al*., 2010). In short, 1.0 mg of each plant extract was dissolved in 1.0 mL of MeOH and then one drop (~ 10 µL) of the mixture was spotted on the TLC plates (TLC silica gel 60 F_{254}). The Plate was developed in DCM : MeOH (97 : 3) and was left to dry. After, the plate was sprayed with *S. mutans* and incubated in humid atmosphere to allow for bacterial growth for 24 h at 37°C. After incubation, the plate was sprayed with INT and incubated for 2 h at 37°C before analysing the data.

Determination of MIC and MMC

The microdilution protocol was adapted according to the procedure reported (Eloff, 1998). First, the seven active extracts were serially diluted in 96 well plates. The final concentration of the extracts from 500 to 31.025 µg/mL. The final concentration of Neomycin was used as a positive control. Nutrient broth was used instead of the extracts or the antibiotic as a negative control. The density of bacteria was adjusted at 0.5 OD McFarland at λ_{450} nm using a

POLARstar omega micro plate reader (BMG Labtech, Ortenberg, Germany). After 24 h of incubating the tested samples with the bacteria at 5% CO_2 saturation in a humified chamber, 40 µL of INT was added to each well and incubated at 37°C for 2 h. The MIC was defined as the lowest concentrations that inhibit the colour change of INT. The MMC was determined by adding 50 µL of the suspensions from the wells that did not show any growth in the MIC assay, to 150 µL of fresh nutrient broth. These suspensions were re-incubated in humified chamber at 5% CO_2 and 37°C for 24 h. The MMC was determined by adding 40 µL of INT and incubated at 37°C for 1 h. The MMC value was identified as the lowest concentration of extract that completely inhibit the growth of bacteria (Nyila *et al*., 2012).

Biofilm inhibition assay

The bacteria were activated in Brain heart infusion media and incubated for 24 h at 37°C. After the incubation period the culture was left to develop a biofilm on glass slides in 6 well plates. Approximately 50 µL media containing the bacteria and 50 µl fresh media was cultured on the glass slide cover and incubated for 24 h at 37°C at 5% CO_2 in a humified incubator. After incubation, the slides were either left untreated (negative control), treated with neomycin (positive control) or the MIC values of the plant extracts. After 24 h of incubation in the same conditions, the slides were rinsed with PBS. The fixation was done using MeOH for 10 min, then acetic acid: MeOH (70:30) for 30 min followed with MeOH again for 2 min. Then the slides were rinsed again with PBS and dried. The slides were stained with DAPI and examined using a florescence microscope according to the methodology reported earlier (Nyila *et al*., 2012).

RESULTS

Plant collection and identification

27 plants from 15 different families were collected from Cape Flats natural reserve. The plants were identified, and voucher specimens were kept in the University of the Western Cape for reference. Plant materials were extracted with methanol and evaluated against *S. mutans* growth. Table 1 lists the names of the collected plants and their herbarium numbers.

Antibacterial screening using TLC bioautography

TLC bioautography was used to the plant extracts to identify which plant species posses potent antibacterial activity. Of the 27 plant species collected in this study, only seven species namely; *A. karroo, H. cymosum, H. zeyheri, O. fruticans, P. pinnata, S. africana-caerulea and S. africana-lutea*, showed inhibition zones on the TLC plate after spraying with *S. mutans* and INT (Figure 1). The formation of colouress (yelleowish) spots indicate the inibition of the bacterial gowth.

Determination of MIC and MMC

After screening the plant extracts using the TLC bioautography, the antibacterial active plant extracts were further tested to determine their MIC and MMC vlues. This was done by using microdilution assay and, as in the TLC autobiography, the plates in this essay were also visualized using INT. The results indicated that *O. fruticans* and *P. pinnata* extracts were the most active against *S. mutans*, exhibiting MIC and MMC values of 31.25 and 62.5 µg/mL respectively (Table 2). Both *H. cymosum*, *H. zeyheri* showed the least antibacterial activity with MIC and MMC

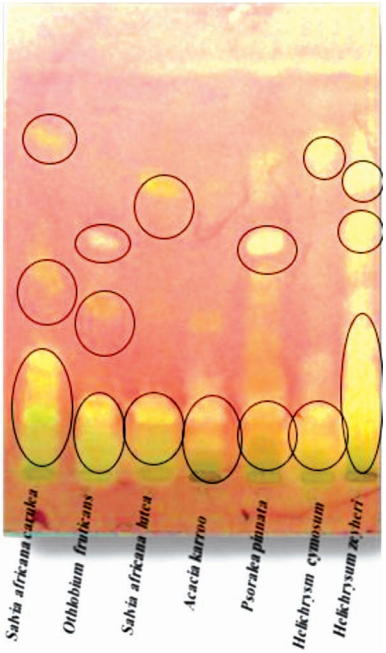

Figure 1: TLC-bioautograph (silica gel plate; developed in CH_2Cl_2:MeOH (97:3)) sprayed with *S. mutans* and showing the inhibtion zones of *A. karroo, H. cymosum, H. zeyheri, O. fruticans, P. pinnata, S. africana-caerulea and S. africana-lutea* plant extracts.

Table 1. List of the collected plants and their herbarium numbers

Family	Plant Name	Herbarium number
Amaryllidaceae	*Brunsvigia orientalis* (L.) Aiton ex Eckl.	Hu- 44
Anacardiaceae	*Searsia lucida* (L.) F.A. Barkley	Hu-22
	Searsia laevigata (L.) F.A. Barkley	Hu-46
Apiaceae	*Lichtensteinia lacera* Cham. & Schltdl	Hu-17/1
Asteraceae	*Arctotheca populifolia* (P.J. Bergius) Norl.	Hu-22/16
	Athanasia crithmifolia (L.)	Hu-22/1
	Athanasia trifurcata (L.)	Hu-33
	Helichrysum cymosum (L.) D. Don	Hu-47
	Helichrysum indicum (L.) Grierson	Hu-43
	Helichrysum zeyheri (L.)	Hu-18
	Metalasia muricata (L.) D. Don	Hu-1b
	Tarchonanthus camphorates L.	Hu-22/9
Compositae	*Senecio halimifolius* L.	Hu-8
Cyperaceae	*Isolepis antarctica* (L.) Roem. & Schult.	Hu-59
Fabaceae	*Acacia karroo* Hayne	Hu-52
	Aspalathus linearis (Burm.f.) R. Dahlgren*	N/A
	Otholobium fruticans	Hu-5
	Psoralea pinnata L.	Hu-55
Faboideae	*Aspalathus hispida* Thunb.	Hu-54
Lamiaceae	*Salvia africana-caerulea* L.	Hu-47a
	Salvia africana-lutea L.	Hu-50
Montiniaceae	*Montinia caryophyllacea* Thunb.	Hu-34
Myricaceae	*Myrica quercifolia* L.	Hu-24
Myrtaceae	*Myrtus communis* L.	Hu-42
Rhamnaceae	*Phylica ericoides* L.	Hu-4
Stilbaceae	*Halleria lucida* L.	Hu-27
Thymelaeaceae	*Passerina rigida* Wikstr.	Hu-3

*Purchased from Afriplex, Cape Town, South Africa.

values of 126 and 250 µg/mL, respectively. The MIC value of the positive-control, Neomycin, was 0.01 µg/mL, and showed MMC value of 0.31 µg/mL.

Inhibition of *S. mutans* biofilm

S. mutans can adhere to the dental surfaces and form biofilm known as dental plaque, this biofilm is essential for development of dental caries. After incubation with the MIC values, the florescence microscope images of the slides showed different degrees of biofilm inhibition (Figure 2). Interestingly, *O. fruticans* and *P. pinnata* showed visible reduction of the biofilm. Apart from *S. africana-caerulea,* the other plant extracts (*A. karroo, H. cymosum, H. zeyheri*

Table 2: MIC and MMC values (µg/mL) for the antibacterial active plant extracts

Plant extracts	MIC	MMC
A. karroo	62.5	125
H. cymosum	125	250
H. zeyheri	125	250
O. fruticans	31.25	62.5
P. pinnata	31.25	62.5
S. africana-caerulea	62.5	125
S. africana-lutea	62.5	125
Neomycin	0.01	0.31

*The values were obsereved from three experemints which gave identical results.

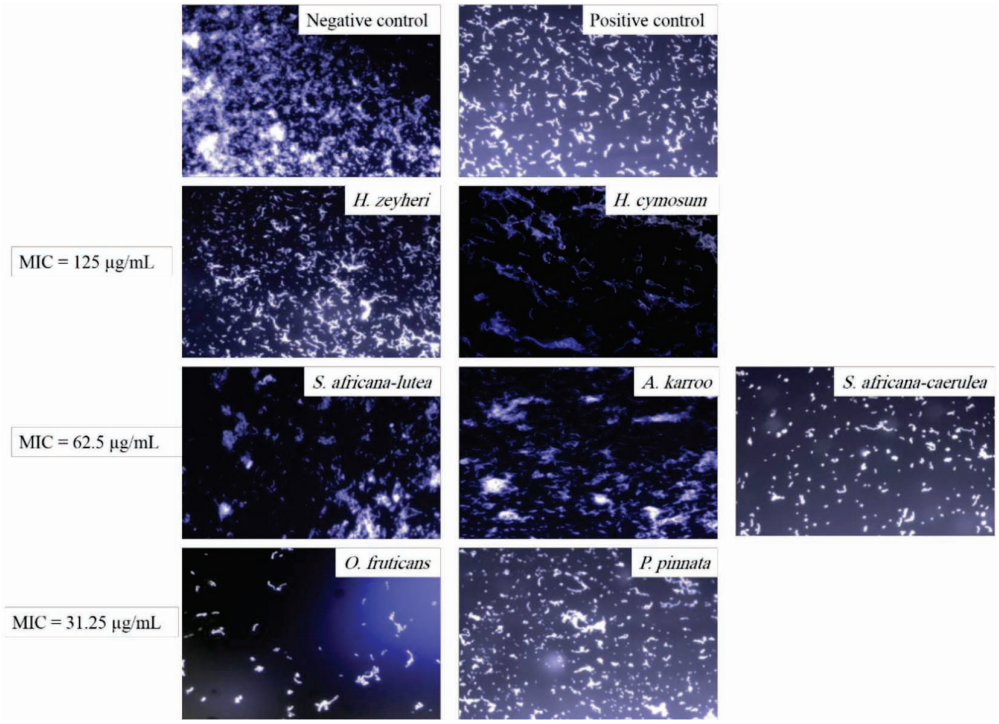

Figure 2: Florescence microscope images of *S. mutans* biofilm formation after treatment with selected active plant extracts, Neomycin (positive control) and nutrient broth (negative control)

and *S. africana-lutea*) did not exhibit a strong biofilm reduction like *O. fruticans* and *P. pinnata*, however they showed bacterial reduction when compared to the negative control.

DISCUSSION

In this study, methanolic extraction was selected for the preparation of the total extracts from the collected plants. The use of methanol allows the extraction of higher amounts of endo-cellular biomolecules (Silva *et al*., 1998), which could lead to higher antibacterial activity in the total extract. Indeed, alcoholic plant extracts were found to have more antibacterial activity than aqueous extracts (Panda *et al*., 2016).

The initial screening of the methanolic plant extracts, using TLC bioautography, led to the identification of seven antibacterial active plant extracts (Figure 1). The use of TLC bioautography is considered more sensitive than agar diffusion method to screen potential antibacterial agents. This is because there are several factors that can interfere with the results in agar diffusion technique such as; incubation time, temperature, agar salt concentration and, depending on their molecular sizes, the diffusion rate of the antimicrobial agents (Eloff, 1998). TLC bioautography can also indicate the polarity of the biomolecules responsible for the antibacterial activity. *H. zeyheri* for instance showed strong inhibition zones in the medium polar zone (Figure 1). This can facilitate the bio-guided isolation for the tested plant extracts. Moreover, the utilization of the colourless INT as a visualizing agent in the TLC bioautography is preferable than other tetrazolium salts to avoid absorbance interferences, especially when using plant extracts of intense colours (Hamburger and Cordell, 1987).

The results also showed that the growth of *S. mutans* was inhibited at concentrations as low as 31.25 µg/mL for *O. fruticans* and *P. pinnata,* and as high as 125 µg/mL for *H. cymosum* and *H. zeyheri*. Interestingly, these values are less than the MIC values reported against *S. mutans* of commercial mouthwash products (Yousefimanesh *et al*., 2015). The development of oral bacterial resistance against common chemical ingredients of mouthwash products, such as fluorides, chlorohexidine gluconate or cetylpyridinum chloride is a known issue (Liao *et al*., 2017; Masadeh *et al*., 2013). Additionally, these chemical-based mouthwash products often develop side effects such as tooth discoloration, taste change, the formation of enhanced

supragingival calculus and, less frequently, oral mucosal erosion (Addy, 1986; Flötra *et al*., 1971). Moreover, Ethanol content in certain mouthwash products have been found to cause genotoxicity *in vitro* (Rodrigues *et al*., 2007). Therefore, the use of herbal-based oral hygiene products is encouraged to avoid such problems (Malhotra *et al*., 2011). Herbal-based mouthwash products have shown high activity to treat oral cavity diseases. For example, the mixture of three Indian medicinal plants known as "Triphala" scored higher plaque index and gingival index more than 0.2% chlorhexidine (Naiktari *et al*., 2014).

Though *S. mutans* is considered the main cause of dental caries, any anticariogenic agent that inhibit *S. muta*ns alone with no broad spectrum antibacterial activity can facilitate the colonization of other cariogenic microorganisms (Smullen *et al*., 2007). No antibacterial activity from *O. fruticans* or *P. pinnata* was studied before to the best of our knowledge, however the growth inhibition of gram-positive bacteria have been reported for *A. karroo* (Madureira *et al*., 2012), *H. cymosum* (van Vuuren *et al*., 2006), *H. zeyheri* (Lourens *et al*., 2011), *S. africana-caerulea and S. africana-lutea* (Kamatou *et al*., 2007). Therefore, these plants can prove useful as efficient anticariogenic agents.

Inhibitors of biofilm formation in oral cavity are of a particular interest in the treatment of dental caries. It is documented that bacteria are 1000 times less sensitive to antibiotics when it is aggregated in a biofilm form (Reffuveille *et al*., 2014). In this study, the antibacterial active plant extracts (as determined by the TLC autobiography) showed different degree of biofilm inhibition. *S. africana-caerulea*, *O. fruticans* exhibited strong disruption of the biofilm compared to the antibiofilm action of the positive control (Figure 2). The antibiofilm of plants have been attributed to the presence of phytochemicals such as flavonoids, diterpenes, tannins and alkaloids in their extracts (Borges *et al*., 2016; Dusane *et al*., 2014; Rubiano-Buitrago *et al*., 2019). Except for *S. africana lutea* and *A. karroo*, the chemistry of the seven antibacterial active plant extracts is not yet studied. Abietane diterpenoids such as carnosol, rosmadial, and carnosic acid were isolated from ethanolic extract of *S. africana-lutea* (Hussein *et al*., 2007). Flavonoids are also a major constituent of family Fabaceae including *O. fruticans* and *P. pinnata* (Figure 3). Isolated secondary metabolites were also found to exhibit stronger antibiofilm and bacterial growth inhibition than their respective plant extracts (Nyila *et al*., 2012). Therefore, it is recommended to evaluate the antibacterial activity of the isolated secondary metabolites from these plants.

CONCLUSION

In this study, plant species from Cape region showed antibacterial and antibiofilm activity against *S. mutans*, which indicates the importance of the Cape flora as a source of antibacterial plants for drug discovery. Using the combination of TLC bioautography and microdilution method, the study provides a scheme that can be implemented to screen plants for antibacterial activity. Of all the collected plants, only *P. pinnata*, *O. fruticans* and *S. africana-caerulea* showed strong antibacterial activity against *S. mutans* and its biofilm formation. These plants

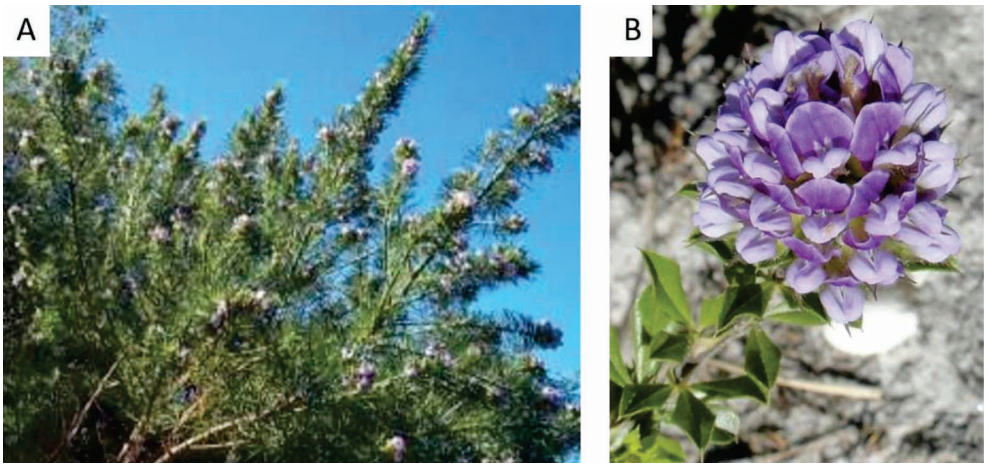

Figure 3: Photo images of (A) *P. pinnata* and (B) *O. fruticans* (sanbi.org).

can be then incorporated as mouthwash products for the treatment or the prevention of dental caries.

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