

Antimicrobial activity of medicinal plants against oral Microorganisms

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Abstract

Ethanol extracts of eight plant species used traditionally in South Africa for the treatment of oral diseases were investigated for *in vitro* antimicrobial activity against oral pathogens namely *Actinobacillus actinomycetemcomitans*, *Actinomyces naeslundii*, *Actinomyces israelii*, *Candida albicans*, *Porphyromonus gingivalis*, *Prevotella intermedia* and *Streptococcus mutans* using the disk diffusion method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanol extracts were determined against these microorganisms using micro dilution. The cytotoxicity and therapeutic index (TI) of selected active extracts were also determined. Out of eight plants, six (*Annona senegalensis*, *Englerophytum magalismontanum*, *Dicerocarym senecioides*, *Euclea divinorum*, *Euclea natalensis*, *Solanum panduriforme* and *Parinari curatellifolia*) exhibited MIC values ranging from 25.0 mg/ml to 0.8 mg/ml. Gram negative bacteria were found to be more resistant to the plant extracts than Gram positive bacteria, except for *Euclea natalensis* which inhibited all three Gram negative bacteria tested in this study. All plant extracts showed moderate cytotoxicity on the Vero cell line. The fifty percent inhibitory concentration (IC₅₀) of all plants tested range from 92.3 to 285.1 µg/ml.

1. Introduction

Periodontal diseases and dental caries are two main common dental pathologies affecting humankind (Marsh and Martin, 1992). These conditions are caused by plaque forming bacteria and yeast, which reside in the oral cavity. Periodontal diseases have mainly been associated with *Actinomyces*, *Actinobacillus*, *Streptococcus* and *Candida* species (Van Oosten et al., 1987). *Candida albicans* is not cariogenic, but was included in this study because it is a pathogenic microorganism causing oral thrush particularly in immunocompromised individuals (Samaranayake, 2000).

Dental treatment usually is expensive and not so easily accessible, especially in developing countries, therefore humans have turned to the use of tooth sticks/chewing sticks to prevent dental caries (Akpatha and Akinrimisi, 1977; Homer et al., 1990).

Some of these remedies include bisguanide-antiseptics, quaternary ammonium-antiseptics, phenolic-antiseptics and other remedies such as oxygenating agents, and metal ions (Addy, 1986). Common side effects of some these may be the staining of the teeth and restorations, taste of food and a burning sensation at the tip of the tongue (Gründermann et al., 2000). The use of plants has been closely associated with dental hygiene and therapeutic practices since time immemorial. Chewing sticks are used by the many people in various African tribes such as Namibians, Zimbabweans, Vhavenda and Ethiopians (Olsson, 1978; Lewis, 1980; Mabogo, 1990). Ndukwe et al. (2004) conducted a study investigating the antibacterial activity of chewing stick, his study confirms that chewing sticks have a potential preventing oral ailments. A majority of the plants tested in his study reveals that chewing sticks are capable to inhibit Gram-positive and negative bacteria such as *Bacillus subtilis*, *Porphyromonus gingivalis* and *Fusobacterium nucleatum*. Several African tribes use the common traditional

chewing stick called 'Muthala', scientifically known as *Diospyros lysioides* DESF (Khan, 1978) and/or *Euclea natalensis* A.D.C. (Lall and Meyer, 2000). The use of medicinal plants belonging to the family of Fabaceae, Ebenaceae, Bombaceae and Annonaceae has been reported for treatment of oral diseases (Hadissa and Jean-Pierre, 2005).

The rationale of this study was to determine the antimicrobial activity of the traditional South African medicinal plants used as chewing sticks against oral microorganisms which are responsible for dental caries. Chewing sticks with antimicrobial activity could become a potential source of new drugs for oral diseases.

Oral microorganisms are known for their pathogenesis in tooth decay, gingivitis, periodontitis and their ability to cause teeth loss (Samaranayake, 2000).

2. Materials and methods

2.1. Plant material

Different plant parts (twigs, leaves, bark and roots) from eight plant species commonly used in the Venda region (Limpopo Province of South Africa) for various tooth problems such as gingivitis, periodontitis and teeth decay were collected during March 2006. Voucher specimens were prepared and identified at the H.G.W.J. Schwelcherdt Herbarium (PRU), University of Pretoria (Table 1).

2.2. Preparation of extracts

The plant material was air dried at 25 °C and ground in a Junke and Kunkel grinder to a fine powder. The powdered material (20 g) was extracted with 200ml of ethanol at room temperature for 48 h. Extracts were filtered and the solvent was evaporated on the rotary evaporator under reduced pressure at 40 °C. The extracts were further dried at room temperature after which they were subjected to antimicrobial tests.

2.3. Antimicrobial activity

2.3.1. Microbial strains

The microorganisms used in this study included *Actinomyces naeslundii* (ATCC 19039), *Actinomyces israelii* (ATCC 10049), *Actinobacillus actinomycetemcomitans* (ATCC 33384), *Candida albicans* (Med I), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC 25611) and *Streptococcus mutans* (ATCC 25175). Bacteria were grown in the Casein-peptone Soy Agar medium (CASO) (Merck SA (Pty) Ltd.) under anaerobic conditions in an anaerobic jar with an aerocult A (Merck SA (Pty) Ltd.), at 37 °C for 72 h. Sabouraud Dextrose Agar medium (SDA) (Merck SA (Pty) Ltd.) was used for the culturing of *Candida albicans* and incubated at 25 °C for 24 h under aerobic conditions. Sub-culturing was done once weekly.

2.3.2. Disc diffusion method

The microbial growth inhibitory potential of the extracts was determined by using the agar disc diffusion method as described by Washington (1981). Inocula were prepared by mixing a few microbial colonies with sterile ringer's solution and comparing the turbidity with that of the standard 0.5 McFarland solution (McFarland, 1907) which is equivalent to 10^6 – 10^8 CFU/ml. One hundred microlitres of inocula of all tested microorganisms were inoculated on Casein-peptone Soy Agar medium for bacteria and Sabouraud Dextrose Agar medium for *Candida albicans* was used.

Extracts were dissolved in 10% DMSO (Merck SA (Pty) Ltd.) to a final concentration of 100mg/ml. Twenty microlitres (2 mg/disc) and thirty microlitres (3 mg/disc) of the plant extracts was transferred onto sterile filter papers (5mmdiameter). One hundred microlitres of positive drug control (chlorhexidine 5%) and 10% DMSO (solvent control) soaked on filter papers served as positive and negative control, respectively. The plates were then incubated at 37 °C for 48 h anaerobically except for *Candida albicans* which was incubated at 25 °C for 48 h. All tests were performed in triplicate and zones of inhibition were measured from the edge of each disc after the incubation period.

2.4. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Although the results of the disc diffusion assay cannot always compare to the MIC data (Njenga et al., 2005), six plant extracts which showed positive antimicrobial activity against

most of the microorganisms tested in the disc diffusion bioassay were further tested for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The microdilution technique using 96-well micro-plates, as described by Eloff (1998) was used to obtain the MIC and MBC values of the crude extracts against the microorganisms under study. Selected extracts were serially diluted in the 96-well plate with 48 h old microorganisms (0.3 CFU/ml) grown at 37 °C and the final concentration of extracts and positive control (CHX) ranged from 25.0 mg/ml to 0.8 mg/ml.

Microbial growth was indicated by adding 40µl of (0.2 mg/ml) *p*-iodonitrotetrazolium violet (INT) (Sigma–Aldrich, South Africa) to micro-plate wells and incubated at 37 °C for 48 h. The MIC was defined as the lowest concentration that inhibited the colour change of INT. The MBC was determined by adding 50µl of the suspensions from the wells, which did not show any growth after incubation during MIC assays, to 150µl of fresh broth. These suspensions were reincubated at 37 °C for 48 h. The MBC was determined as the lowest concentration of extract which inhibited 100% growth of microorganisms (Cohen et al., 1998).

2.5. Determination of cytotoxicity

2.5.1. Cytotoxicity assay

A microtiter plate with Vero cells were used for testing all the ethanol extracts for cytotoxicity following the method of Zheng et al. (2001). Cytotoxicity was measured by the XTT (sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) method using the cell proliferation kit II (Roche Diagnostics GmbH). Hundred microlitres of Vero cells (1×10^5 ml) was seeded onto a micro-plate and incubated for 24 h to allow the cells to attach to the bottom of the plate. Dilution series were made of the extracts and the various concentrations (400–3.1µg/ml) were added to the micro-plate and incubated for 48 h. The XTT reagents were added to a final concentration of 0.3 mg/ml and the cells were incubated for 1–2 h.

The positive drug control (Zelaralene) at concentrations range of (10–0.6µg/ml) was included in the assay. After incubation the absorbance of the colour was spectrophotometrically quantified using an ELISA plate reader, which measured the optical density at 490 nm with a reference wavelength of 690 nm. The assay was carried out in triplicate.

Table 1

Plants collected used as chewing sticks

Plants	Plant family	Plant part number	Voucher specimen	Medicinal use and references
<i>Annona senegalensis</i> Pers.	Annonaceae	Bark	PRU 074974	Toothache, venereal, Diarrhoea (Mabogo, 1990)
<i>Ennglerophytum magalimontanum</i> (Sond.) T.D. Penn.	Sapotaceae	Bark	PRU 095683	Pains, contraceptive rheumatism, Edible (Hutchings et al., 1996)
<i>Dicerocarym senecioides</i> (Klotzsch) Abels.	Pedaliaceae	Roots	PRU 095681	Soap substitute, expulsion of placenta (Van Wyk and Gericke, 2000)
<i>Euclea divinorum</i> Hiern	Ebenaceae	Bark, Leaves	PRU 095682 PRU 095685	Toothache, headache (Mabogo, 1990; Hutchings et al., 1996)
<i>Euclea natalensis</i> A.DC.	Ebenaceae	Leaves	PRU 095686	Laxative, toothache, epilepsy, gonorrhoea, Kidney calculi (Mabogo, 1990)
<i>Erythrina lysistemon</i> Hutch.	Fabaceae	Bark	PRU 066798	Toothache, ornamental, wounds (Mabogo, 1990)
<i>Parinari curatellifolia</i> Planch. Ex Benth	Chrysobalanaceae	Bark	PRU 096215	Toothache, ornamental (Mabogo, 1990)
<i>Solanum panduriforme</i> E.Mey.	Solanaceae	Roots	PRU 090203	Pelvic pains, wounds, toothache (Hutchings et al., 1996)

Table 2

Antimicrobial activity of plants tested against oral microorganisms with zones of inhibition in millimetre of the extracts and Chlorhexidine

Plant extracts	Zones of inhibition (mm ^a) of bacteria and yeast in the presence of plant extract dissolved in 10% DMSO														
	2mg						3mg								
	A.n	A.i	S.m	A.a	P.i	P.g	C.a	A.n	A.i	S.m	A.a	P.i	P.g	C.a	
<i>Annona senegalensis</i>	4.5	5.0	3.0	0.0	2.5	6.5	0.0	7.0	6.5	4.5	0.0	2.5	7.2	0.0	
<i>Englerophytum magalismsontanum</i>	6.5	4.0	3.5	0.0	2.5	4.0	0.0	7.5	3.5	3.5	0.0	2.2	5.5	0.0	
<i>Dicerocarym senecioides</i>	6.2	4.0	3.5	0.0	3.6	4.5	0.0	7.6	4.0	3.2	0.0	3.0	4.2	0.0	
<i>Euclea divinorum</i>	5.5	4.6	6.0	0.0	0.0	6.5	0.0	6.5	3.0	2.4	0.0	6.3	8	0.0	
<i>Euclea natalensis</i>	12.0	17.0	6.8	0.0	4.5	3.5	4.8	14.0	16.5	7.6	0.0	5.3	4.8	0.0	
<i>Erythrina lysistemon</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
<i>Parinari curatellifolia</i>	4.6	2.5	3.6	0.0	2.4	3.0	0.0	5.2	6.3	4.2	0.0	3.0	5.7	0.0	
<i>Solanum panduriforme</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	

A.n, *Actinomyces naeslundii*; A.i, *Actinomyces israelii*; S.m, *Streptococcus mutans*; A.a, *Actinobacillus actinomycetemcomitans*; P.i, *Privotella intermedia*; P.g, *Porphyromonus gingivalis*; C.a, *Candida albicans*.

^a Diameter of inhibition zone including disc diameter of 5.0mm.

3. Results and discussion

The extracts of *Annona senegalensis*, *Englerophytum magalismsontanum*, *Dicerocarym senecioides*, *Euclea divinorum*, *Euclea natalensis* and *Parinari curatellifolia* showed positive inhibitory activity against five microorganisms (*Actinomyces naeslundii*, *Actinomyces israelii*, *Porphyromonus gingivalis*, *Privotella intermedia* and *Streptococcus mutans*) at both 2 and 3mg per disc (Table 2). *Euclea natalensis* showed inhibition of *Actinomyces israelii* and *Actinomyces naeslundii* with zone of 16–17mm. When *Porphyromonus gingivalis* was exposed to the extract of *Euclea natalensis*, 3.5 and 4.8mm of zones of inhibition were observed. Of all the microorganisms, *Actinobacillus actinomycetemcomitans* and *Candida albicans* were found to be resistant to all the extracts at the highest concentration tested.

The study conducted by Khan et al. (1978) confirms the antimicrobial activity of the bark extracts of *Euclea natalensis* on *Streptococcus mutans* and *Candida albicans* at a concentration of 100mg/ml. However, the zones of inhibition in the present study were not very prominent when compared with those of chlorhexidine (positive control). *Annona senegalensis* also exhibited activity against *Staphylococcus aureus* with zone of inhibition of 18mm and 16mm for both water and methanol extracts, respectively, but was not active against *Escherichia coli* a Gram-negative bacterium (Lino and Deogracious, 2006) (Table 3).

The plant extracts which demonstrated excellent results, were selected for MIC, MBC and cytotoxicity determination. *Actinomyces naeslundii*, *Porphyromonus gingivalis*, *Privotella intermedia*, *Streptococcus mutans* were found to be susceptible to most of the extracts tested in the present study, only two plant extracts (*Solanum panduriforme* and *Erythrina lysistemon*) which were not active, were not considered for MIC, MBC and cytotoxicity. *Euclea natalensis* and *Parinari curatellifolia* showed best activity against *Actinomyces israelii* exhibiting an MIC of 1.6 mg/ml. Little is known about *Englerophytum magalismsontanum* included in this study, but other plants such as *Euclea* spp., *Parinari* spp. and *Annona* spp. Have been studied extensively on many pathogenic microorganisms.

According to Lall and Meyer (2000) water and acetone extracts of *Euclea natalensis* at concentration range of 0.1–6.0 mg/ml inhibited growth of the *Bacillus* spp. *Micrococcus kristinae* and Gram-negative bacteria *Escherichia coli*. *Solanum panduriforme* did not show any inhibitory activity against the microorganisms included in this study. However, the study conducted by Prozesky (2001) confirmed the antiplasmodial activity of some species of the genus *Solanum*. Previous studies have shown that the acetone and water extracts of *Euclea natalensis* possess antibacterial activity against *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis* and *Staphylococcus aureus* at concentrations ranging from 0.1 and 6.0 mg/ml (Lall and Meyer, 2000). Fresh roots samples of *Euclea natalensis* were tested against *Streptococcus mutans*, human saliva and periodontal pockets isolates and it was found that aerobic as well as anaerobic bacterial growth was suppressed in all instances (Stander and Van Wyk, 1991).

Table 3
Mean MIC and MBC (mg/ml) results of six selected plants on oral microorganisms

Plant extracts	Microorganisms tested							MBC (mg/ml)						
	MIC (mg/ml)			Gram -ve			Yeast (C.a)	Gram +ve			Gram -ve			Yeast (C.a)
	A.n	A.i	S.m	A.a	P.i	P.g		A.n	A.i	S.m	A.a	P.i	P.g	
<i>Annona senegalensis</i>	1.6	3.1	12.5	Na ^a	Na	1.6	Na	1.6	3.1	12.5	Na	Na	1.6	Na
<i>Englerophytum magalimontanum</i>	12.5	6.3	12.5	Na	Na	12.5	Na	12.5	1.6	6.3	Na	Na	25.0	Na
<i>Dicerocarym senecioides</i>	25.0	6.3	12.5	25.0	Na	0.8	Na	25.0	6.3	25.0	Na	Na	25.0	Na
<i>Euclea divinorum</i>	6.2	12.5	25.0	6.3	Na	3.1	Na	25.0	12.5	25.0	12.5	Na	25.0	Na
<i>Euclea natalensis</i>	25.0	1.6	6.3	12.5	6.3	6.3	Na	25.0	1.6	6.3	12.5	6.3	6.3	Na
<i>P. curatellifolia</i>	1.6	1.6	6.3	12.5	Na	3.1	Na	1.6	1.6	6.3	Na	Na	25.0	Na
Chlorexhidine	1.6	3.2	1.6	1.6	6.3	1.6	6.3	1.6	3.2	1.6	1.6	6.3	1.6	12.5

^a Na: not active; A.n, *Actinomyces naeslundii*; A.i, *Actinomyces israelii*; S.m, *Streptococcus mutans*; A.a, *Actinobacillus actinomycetemcomitans*; P.i, *Prevotella intermedia*; P.g, *Porphyromonus gingivalis*; C.a, *Candida albicans*.

Table 4
Cytotoxicity ($\mu\text{g/ml}$) results and therapeutic index of six selected plants on Vero cell line

Species	IC50 ($\mu\text{g/ml}$) \pm S.D.	Microorganisms tested (therapeutic index)						
		Gram +ve			Gram -ve			Yeast (C.a)
		A.n	A.i	S.m	A.a	P.i	P.g	
<i>Annona senegalensis</i>	71.0 \pm 4.3	45	23	6	Na ^a	Na	45	Na
<i>Bequaertiodendron magalimontanum</i>	98.8 \pm 2.7	8	16	8	Na	Na	8	Na
<i>Dicerocarym senecioides</i>	122.1 \pm 2.2	5	19	10	5	Na	153	Na
<i>Euclea divinorum</i>	142.3 \pm 4.6	23	11	5.7	23	Na	46	Na
<i>Euclea natalensis</i>	285.1 \pm 4.9	11	16	45	23	Na	45	Na
<i>Parinari curatellifolia</i>	92.3 \pm 3.0	62	54	15	Na	Na	30	Na
Zelaralene	2.3 \pm 0.3							

^a Na not active at high concentration (25.0 mg/ml) tested, therefore no therapeutic index. A.n, *Actinomyces naeslundii*; A.i, *Actinomyces israelii*; S.m, *Streptococcus mutans*; A.a, *Actinobacillus actinomycetemcomitans*; P.i, *Prevotella intermedia*; P.g, *Porphyromonus gingivalis*; C.a, *Candida albicans*.

It has also been reported earlier that the crude methanolic extract of dried twigs of *Diospyros lycioides* demonstrated preferential growth inhibitory activity against the oral pathogens *Streptococcus mutans* and *Porphyromonus gingivalis* at 1.25 mg/ml (Cai et al., 2000). Khan et al. (1978) reported the activity of ten methanolic plant extracts including *Euclea natalensis* which showed minimum inhibitory concentration ranging from 0.63 mg/ml to 5 mg/ml against *Actinomyces viscosus*, *Streptococcus mutans* and *Candida albicans*.

According to Samie et al. (2005) the MIC of *Annona senegalensis* was found to be greater than >12mg/ml where as in this study it was found to be 12.5 mg/ml when tested against *Streptococcus mutans*. A study by Khan et al. (1978), confirmed the MIC of the bark of *Euclea natalensis* on oral *Candida albicans* and *Streptococcus mutans*, the recorded MIC is 5.0 mg/ml while in this study *Streptococcus mutans* was found to be susceptible at the MIC of 12.5 mg/ml. Stander and Van Wyk (1991) have also reported the activity of *Euclea natalensis* against *Streptococcus mutans*.

All plant extracts showed moderate cytotoxicity on the Vero cell line. Fifty percent inhibitory concentration (IC₅₀) of all plants namely, *Annona senegalensis*, *Englerophytum magalimontanum*, *Dicerocarym senecioides*, *Euclea divinorum*, *Euclea natalensis* and *P. curatellifolia* were found to range from 92.3 to 285.1 $\mu\text{g/ml}$. The extracts on the cell lines showed a trend of an increase in cell viability at lower concentrations, with a decrease in cell viability as the concentration increases (Table 4). The IC₅₀ value of the positive control Zelaralene used in this study was found to be 2.3 \pm 0.3 $\mu\text{g/ml}$.

The therapeutic index was determined as the cytotoxicity of plant extracts divided by the minimum inhibitory concentration value of the extracts on Vero cells (Lall et al., 2005). Values greater than 10 confirm the dose that can be administered in most physiological systems. *Annona senegalensis* showed excellent therapeutic indexes greater than 20 on *Actinomyces naeslundii*, *Actinomyces israelii* and *Porphyromonus gingivalis*. The leaves of *Euclea natalensis* on *Actinobacillus actinomycetemcomitans* and *Porphyromonus gingivalis* showed an index of 23 and 45, respectively. The results documented in this study correspond with the reports of Hutchings et al. (1996), Van Wyk and Van Wyk (1997) and Van Wyk and Gericke (2000).

The results of this study provided an insight into the antibacterial properties of the extracts used traditionally for the prevention and treatment of oral problems and other ailments, as well as opportunity for selection of bioactive extracts for initial fractionation and further studies in antibacterial assays.

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