

Full Length Research Paper

Antimicrobial activity of *Euclea undulata*, *Euclea divinorum* and *Diospyros lycioides* extracts on multi-drug resistant *Streptococcus mutans*

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Accepted 18 September, 2013

The antimicrobial activities of three Zimbabwean chewing sticks (*Euclea undulata*, *Euclea divinorum* and *Diospyros lycioides*) on multi-drug resistant (MDR) *Streptococcus mutans* were determined. Thirty one isolates of *S. mutans* were obtained from 47 carious teeth and antibiotic sensitivity testing was done using the disc diffusion assay for all isolates. The resistance pattern of each isolate against eight antibiotics was noted. Eight resistance patterns were identified, and 19 isolates showed resistance to at least 4 antibiotics; these were considered MDR. Eight MDR and 2 multi-drug sensitive isolates were then assayed against methanol, acetone, diethyl ether and aqueous extracts of *E. undulata*, *E. divinorum* and *D. lycioides*. The broth macro-dilution assay was used to determine the minimum inhibitory concentration (MIC) of the extracts. All *E. divinorum* extracts showed no antimicrobial activity. Stem methanol extracts of *E. undulata* and root and stem methanol extracts of *D. lycioides* showed anti *S. mutans* activity. The largest inhibition zones were exhibited by methanol extracts of *E. undulata* (60 mm) and *D. lycioides* (54 mm). MICs ranged from 0.385 to 11.22 mg/ml and minimum bactericidal concentration (MBCs) from 0.485 to 20.20 mg/ml. *D. lycioides* and *E. undulata* extracts have potential use as anticariogenic agents.

Key words: *Streptococcus mutans*, dental caries, antimicrobial activity, multi-drug resistant.

INTRODUCTION

Dental caries is the most prevalent and costly oral infectious disease worldwide (Jeon et al., 2011). *Streptococcus mutans* is a very important oral pathogen, long thought to be the leading cause of dental caries (Jarvinen et al., 1993). Dissemination of this bacterium into the blood stream is caused by professional dental treatment and daily oral care practices such as toothbrushing, flossing, and even chewing (Nakano et al., 2007). To prevent possible occurrence of bacteremia and

endocarditis by *S. mutans*, antibiotic administration prior to invasive dental procedures is usually recommended. An important factor influencing the emergence of resistance in a bacterial population is the selective pressure applied by antibiotics. Indiscriminate use of antibiotics has led to the development of multi-drug resistant pathogens (Chitemere and Mukanganyama, 2011). Oral bacteria resistant to tetracyclines, aminopenicillins and cephalosporins have been reported

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(Sweeney et al., 2004).

To cope with the widespread problem of antibiotic resistance, strategies may include a significant reduction in antibiotic use or alternative medicines. There have been many studies on antibacterial activities of plant and natural product derivatives (Al-hebshi et al., 2006) including chewing sticks. Few research studies are available on the antibacterial effects of traditional plants on *S. mutans* (Dalirsani et al., 2011; Nalina and Rahim, 2007) and even fewer have investigated these effects on multi-drug resistant isolates (Fani et al., 2007). Although chewing sticks play a role in the promotion of oral hygiene, evaluation of their effectiveness still requires further research (Al-Otaibi et al., 2004). Despite incomplete information concerning their mechanism of action, many natural biocidal agents have been incorporated in oral healthcare products (Joel et al., 2011). Extracts of miswak, tea tree oils, essential oils, peppermint and green tea have been added to mouth rinses and toothpastes to enhance their overall antimicrobial activities (Allaker and Douglas, 2009). A number of traditional Chinese medicines are being used in oral healthcare products such as toothpaste (Wong et al., 2010).

In the Middle East, the most common source of chewing sticks is Arak (*Salvadora persica*). In West Africa, the lime tree (*Citrus aurantifolia*) and the orange tree (*Citrus sinensis*) are used. In the Central and North of Togo, it is especially the roots of *Terminalia glaucescens* that are mostly used (Food and Agriculture Organization (FAO), 2000). Other species such as *Napoleona vogelii*, *Pseudocedrella kotschyii* and *Anogeissus leiocarpus* are noted for their importance in the treatment of oral diseases. Neem (*Azadirachta indica*) is widely used in the Indian subcontinent (Sadhan and Almas, 1999). The most commonly used plants in Zimbabwe include *Euclea* spp and *Diospyros lycioides*. *Euclea undulata*, *Euclea divinorum* and *Diospyros lycioides* belong to the Ebenaceae family. Medicinal uses of plants belonging to the Ebenaceae for the treatment of oral diseases have been reported (Hadissa and Jean-Pierre, 2005).

In this study, the antimicrobial activity of three Zimbabwean chewing sticks against multi-drug resistant *Streptococcus mutans* was investigated. Chewing sticks with antimicrobial activity could become a potential source of new drugs against multi-drug resistant *S. mutans*.

MATERIALS AND METHODS

Plants

The *E. undulata* and *E. divinorum* plant specimens (stems) were collected at the National University of Science and Technology (NUST) campus in Bulawayo, Zimbabwe. The *D. lycioides* plant

specimens (stems and roots) were collected from Shagari, Zimbabwe. The plant specimens were positively identified by the Forestry Commission of Zimbabwe.

Preparation of plant extracts

Fresh *E. undulata*, *E. divinorum* and *D. lycioides* plant specimens were cut into pieces and ground using a Waring blender at a speed of 3000 rpm for 3 min. The solvents used for extraction were methanol, acetone, diethyl ether, cold distilled water and warm distilled water (42°C). The powdered material (15 g) was extracted with 60 ml of solvent for 24 h at 25°C, on an orbital shaker. Extracts were filtered using Whatman number 1 filter paper and then sterilized using the Wagtech International Filtration apparatus which uses a 47 mm Whatman sterile membrane filter (0.45 µm). The extracts were then used in antimicrobial tests.

Isolation of *S. mutans* from carious teeth

Extracted carious teeth were used to isolate *S. mutans*. The teeth were incubated in 10 ml Brain heart infusion broth at 37°C for 24 h and then subcultured onto sterile blood agar (Oxoid, South Africa). The plates were then put in a candle jar (providing about 5% carbon dioxide) and incubated at 37°C for 48 h. The protocol for isolating and identifying *S. mutans* was modified from the methods described by Beighton et al. (1991) and Fani et al. (2007). *S. mutans* were also identified on the basis of colony morphology (for example, greenish hemolysis) as well as biochemical tests which included the gram stain and catalase test. *S. mutans* was further identified by positive fermentation of glucose, mannitol, and sorbitol. In this study, 47 carious teeth were examined and a total of 31 isolates of *S. mutans* were obtained.

Antibiotic sensitivity testing

The antibiotic sensitivity profiles of the 31 *S. mutans* isolates were determined using a modification of the Bauer-Kirby (Bauer et al., 1966) disc diffusion method. A pure culture of each isolate was standardized using 0.5 McFarland standard and cultured on blood agar prior to antibiotic discs being placed on the medium. A pair of forceps was used to apply the commercial antibiotic discs (Abtek biologicals). The plates were then incubated at 37°C for 48 h under anaerobic conditions. The antibiotics used were ampicillin (25 µg), tetracycline (100 µg), fusidic acid (30 µg), penicillin (25 µg), cotrimoxazole (25 µg), gentamicin (10 µg), erythromycin (25 µg) and clindamycin (30 µg). Interpretation of resistance was based on the Committee for Clinical Laboratory Standards (CCLS) (2007). Nineteen isolates which were resistant to four or more antibiotics were considered as multi-drug resistant (MDR) strains and 12 which were susceptible to 5 or more antibiotics were considered as multidrug sensitive (MDS) strains. Ten isolates with different resistance patterns (8 MDR and 2 MDS) were then assayed against plant extracts.

Evaluation of antimicrobial activity

Disc diffusion assay

The microbial growth inhibitory potential of the plant extracts was determined using the disc diffusion assay. Inocula were prepared by mixing a few microbial colonies in 0.9% saline solution and comparing the turbidity with that of the standard 0.5 McFarland

solution which is equivalent to 10^6 to 10^8 CFU/ml. A total of 100 μ l of the microbial suspension was spread onto Mueller Hinton agar (Oxoid, South Africa) for each isolate. Sterile paper discs (6 mm diameter) were impregnated with stem or root extracts (acetone, ethanol, diethyl ether, cold distilled water and warm distilled water) for each of the three assayed plants. A volume of 10 μ l of each extract was transferred onto each disc using a micropipette. The discs were allowed to dry then seeded on the inoculated Mueller Hinton plates prior to incubation at 37°C for 48 h. Discs with only the solvent (negative controls) were also seeded separately onto inoculated plates. Discs containing ampicillin (25 μ g) were used as positive controls. The plates were then inverted and incubated at 37°C for 48 h. The diameters of growth inhibition in two different areas were measured and the mean diameter of growth inhibition was calculated for each isolate.

Broth macrodilution assay

The plant extracts that showed potent antimicrobial activity against the tested isolates in the disc diffusion assay were selected for minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) determination using the standard broth macrodilution assay. The inocula for these tests were adjusted to the 0.5 McFarland standard. Using sterile broth as diluent, a dilution series for each assayed extract was made in a total volume of 10 ml and the final concentrations ranged from 0.385 to 50 mg/ml. Each tube in the dilution series was inoculated with 100 μ l of the test microbial suspension. The tubes were incubated aerobically at 37°C for 24 h and the MIC was recorded as the lowest concentration inhibiting growth. A culture suspension of 100 μ l was taken from each tube that showed no growth, using a micropipette, and subcultured onto Mueller Hinton agar. The plates were incubated aerobically at 37°C for 48 h. The lowest concentration of the extract which completely inhibited growth was expressed as the MBC. Each experiment was duplicated.

Phytochemical analysis

Phytochemical examinations were carried out for methanol, acetone and cold water extracts as per the standard methods (Harbone, 1998). Alkaloids, glycosides, saponins, phytosterols, resins, phenols, tannins, flavanoids and diterpenes were assayed for.

Statistical analysis

A one way analysis of variance (ANOVA) was used to evaluate statistical differences in antimicrobial activity amongst the different solvents for each plant species used and to evaluate the statistical difference between the two plant extracts that gave the best results in the disc diffusion assay. Differences were considered statistically significant if the P-value was less than 0.05. Microsoft Office Excel 2007 was used for the analysis.

RESULTS

To determine whether the commonly used chewing sticks, *E. divinorum*, *E. undulata* and *D. lycioides* are effective against multi-drug resistant *S. mutans* isolates, the disc diffusion and broth macrodilution assays were

performed. Methanol, acetone, diethyl ether and aqueous extracts were prepared from each plant and assayed against 10 *S. mutans* isolates which comprised 8 MDR isolates corresponding to each resistance pattern (Tables 1 and 2 randomly chosen MDS isolates). The results of the disc diffusion assay showed evidence of bacterial inhibition as determined by measured zones of clearance of bacterial growth (Table 2). Notably zones of inhibition for methanol extracts of *E. undulata* and *D. lycioides* were ≥ 36 mm for all the bacterial isolates (Table 2). Activity of these methanol extracts was significantly higher ($p < 0.05$) than that of extracts obtained with other solvents. None of the *E. divinorum* extracts showed antimicrobial activity (Table 2). Diethyl ether extracts showed no activity for all the plants assayed (results not shown).

MICs and MBCs were determined for the plant extracts that showed anti bacterial activity in the disc diffusion assay. Methanol, aqueous and acetone extracts of *E. undulata* inhibited the growth of MDR *S. mutans* isolates at concentrations between 2.424 and 9.776 mg/ml (Table 3). Extracts of *D. lycioides* showed the most inhibitory activity against the MDR isolates, as the lowest MICs of 0.385 and 0.485 mg/ml (Table 3) were obtained for the root and stem methanol extracts, respectively. A similar pattern was observed for the MBC values as the lowest bacteriocidal concentrations of 0.485 and 0.706 mg/ml were obtained for the stem and root methanol extracts of *D. lycioides*, respectively. However, there was no significant difference in the antimicrobial activity of extracts from *D. lycioides* and *E. undulata* (single factor Anova, $p > 0.05$). This study was sanctioned by the NUST ethical committee and no names were recorded during the study.

DISCUSSION

S. mutans is one of the most important oral bacteria which play a major role in dental caries, bacteremia and bacterial endocarditis among predisposed patients (Nakano et al., 2007). In this study, 61% of the *S. mutans* isolates were found to be multi-drug resistant. This is in contrast to a study by Fani et al. (2007) who found only 30% of 92 *S. mutans* isolates to be MDR. A limitation of our study was the small number of *S. mutans* (31) isolates used for the antibiotic susceptibility assay. This might not give a representative picture of the antibiotic resistance profiles. The occurrence of MDR *S. mutans* in our study is still a cause for concern and could point to the development of multi-drug resistance by this bacterium in Zimbabwe. However, a larger study needs to be done to determine the extent of MDR *S. mutans* throughout Zimbabwe. A total of 8 resistance patterns were determined for the 31 *S. mutans* isolates (Table 1) and an isolate from each resistant pattern was used in

Table 1. Resistance patterns of 19 MDR strains of *S. mutans* recovered from carious teeth.

Pattern no.	Resistance patterns	Number of isolates
1	AMP,E,CLN,GEN,FUS,TET,COT,P	4
2	E,CLN,GEN,FUS,TET,COT,P	5
3	E,CLN,GEN,TET,COT,FUS	2
4	AMP,E,CLN,FUS,COT,P	1
5	E,CLN,FUS,COT,P	2
6	E,GEN,TET,COT,P	2
7	E,CLN,FUS,COT	1
8	E,GEN,P,COT	2

AMP: Ampicillin, CLN: clindamycin, COT: cotrimoxazole, E: erythromycin, FUS: fusidic acid; GEN: gentamicin; P: penicillin and TET: tetracycline

Table 2. Antimicrobial activity of *E. undulata*, *E. divinorum* and *D. lycioides* against *S. mutans*.

Plant	Extract conc. (mg/ml)	Zones of inhibition (mm) of <i>S. mutans</i> isolates									
		S1*	S2*	S3*	S4*	S5*	S6*	S7*	S8*	S9	S10
<i>E. undulata</i> (stem)	Met (80)	60	48	44	54	45	43	49	40	44	44
	Ace (65)	5	11	10	4	6	4	3	2	4	1
	Aq(c) (88)	1	14	5	8	6	-	-	-	-	-
<i>D. lycioides</i> (stem)	Met (16)	54	36	54	52	43	50	39	38	40	42
	Ace (17)	14	8	8	19	6	7	-	-	-	-
<i>D. lycioides</i> (roots)	Met (20.7)	54	44	54	54	44	48	50	51	42	48
	Ace (13,8)	10	2	2	9	1	-	-	-	-	-
	Aq(w) (25)	26	18	20	16	18	12	14	-	-	-
<i>E. divinorum</i>	Met (71)	1	-	1	4	-	-	-	-	-	-
	Ace (90)	3	2	6	9	-	-	-	-	-	-
	Aq(w) (101)	2	-	6	10	-	-	-	-	-	-
Ampicillin	25 µg	15	10	11	7	25	6	8	18	18	14

S*, multi-drug resistant isolates: Met, methanol: Ace, acetone: Aq(c), aqueous cold: Aq(w), aqueous warm : (-), no inhibition zone. Zone of inhibition excludes diameter of disc.

plant extract antimicrobial assays.

We examined methanol, acetone, diethyl ether and aqueous extracts of 3 plants commonly used as chewing sticks in Zimbabwe. The results showed that methanol, acetone and aqueous extracts of *E. undulata* and *D. lycioides*, were active against MDS and MDR *S. mutans* isolates (Table 2). *E. divinorum* extracts showed limited antimicrobial activity against *S. mutans* suggesting that they may not be effective when used for dental hygiene purposes. This is however contrary to findings by More et al. (2008) who found the plant to have positive inhibitory activity against *S. mutans*. *E. divinorum* has also been

found to possess antimicrobial activity against *Staphylococcus aureus* (Mothana et al., 2009) and *Nesseiria gonorrhoea* (Geyid et al., 2005). The antimicrobial activities of *Euclea* spp, particularly *E. natalensis* (Khan et al., 1978; Lall and Mayer, 2000; More et al., 2008) and *E. divinorum* (Geyid et al., 2005; More et al., 2008; Mothana et al., 2009) have been extensively studied.

There is a paucity of data regarding the antimicrobial activities of *E. undulata*, with the only reported work being on its anti diabetic effects (Deutschländer et al., 2011). In this study, *E. undulata* methanol extracts demonstrated

Table 3. Minimum inhibitory concentration and minimum bactericidal concentration of *E. undulata* and *D. lycioides* extracts against MDR *S. mutans*.

Plant	Solvent extract	MIC (mg/ml)	MBC (mg/ml)
<i>E. undulata</i>	Methanol	2.424	2.424
	Acetone	3.611	7.220
	Aqueous(c)	9.776	17.60
<i>D. lycioides</i> (root)	Methanol	0.385	0.706
	Acetone	0.767	1.533
	Aqueous (w)	2.800	5.040
<i>D. lycioides</i> (stem)	Methanol	0.485	0.485
	Acetone	0.944	1.889

(c), cold; (w) warm.

excellent results against multi-drug resistant *S. mutans* (Table 2). The results suggest that the methanol extracts may contain active substances that could be useful in oral and dental hygiene. Plant extracts having MICs of less than 8 mg/ml are considered to have some antimicrobial activity, while those with MICs of less than 1 mg/ml are worth noting (van Vuuren, 2008). The MICs for the *E. undulata* extracts were mostly less than 8 mg/ml, with the methanol extract (0.475 mg/ml) being less than 1 mg/ml (Table 3). Our phytochemical analysis (results not shown) of the methanol extracts *E. undulata* showed the presence of alkaloids, saponins, glycosides, diterpenes, tannins and phytosterols. The major difference with the methanol extracts of *E. divinorum* was the presence of glycosides. This led us to postulate that the differences in antimicrobial activity between the two *Euclea* spp could be partly due to the presence of glycosides in *E. undulata*. However, further work needs to focus on identifying the active compounds in these methanol extracts and their mechanisms of action.

The antimicrobial activities of *D. lycioides* are well documented. Cai et al. (2000) demonstrated that crude methanolic extracts of *D. lycioides* inhibited the growth of oral pathogens *S. mutans* and *Porphyromonas gingivalis* at a minimum inhibitory concentration of 1.25 mg/ml. In our study, similar results were obtained as *S. mutans* isolates were inhibited by methanol extracts of *D. lycioides* at an MIC of 0.375 mg/ml (Table 3). Some researchers have isolated active compounds from *D. lycioides* such as binaphthalenone glycosides (Li et al., 1998), and naphthalene glycosides (Cai et al., 2000). Studies on other *Diospyros* species which are used as chewing sticks, and for other medicinal uses, have also been reported. These include work on *Diospyros piscatorial* (Adeniyi et al., 2000), *Diospyros bateri*, *Diospyros monbutensis* (Odelola and Okorosobo, 1988) and *Diospyros mespiliformis* (Lajubutu et al., 1995). It

was expected that *D. lycioides* would exhibit antimicrobial activity against *S. mutans*. This plant species is not well known in Zimbabwe as a chewing stick, with many communities in the rural areas of Zimbabwe preferring or being only aware of the *Euclea* species. Our results suggest that there may be a need to educate people who use chewing sticks for their oral hygiene on the efficacy of *D. lycioides*. Chewing sticks have been demonstrated to be as effective as toothpaste, mouthwashes and denitrifies (Muhammad and Lawal, 2010).

Two of the three chewing sticks we tested demonstrated *in vitro* activity against MDR *S. mutans*. MDR is increasing, and there is a need for new antibiotics which may treat pathogens like MDR *S. mutans*. Although conventional antibiotics are still efficacious, herbal medicines could offer an alternative treatment option (Miyasaki et al., 2010).

Conclusion

The results of this study showed that extracts of *E. undulata* and *D. lycioides* had the most antibacterial activity against MDR *S. mutans*. Possible pharmacological benefits of these plant extracts should be explored, as well as further studies to determine their modes of action. To our knowledge, this is the first report on the antimicrobial activities of Zimbabwean chewing sticks.

ACKNOWLEDGEMENTS

Special thanks go to the International Network for the Availability of Scientific Publications (INASP) for their assistance through AUTHORAID. This study was supported by the National University of Science and

Technology (NUST) Research Board. The authors also thank Thulani Sibanda and MaSiziba for the helpful discussions on chewing sticks.

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