INDIGENOUS TOOTHBRUSHES AND THEIR ANTIMICROBIAL POTENTIAL

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CERTIFICATION

The undersigned certifies that she has read and hereby recommends for acceptance by the University of Dar-es-Salaam a dissertation entitled :

Indigenous Toothbrushes And Their Antimicrobial Potential, in partial fulfilment of the requirements for the degree of Master of Pharmacy (Pharmacognosy).

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Date : .....October 15, 1998

DECLARATION

I hereby solemnly declare that this dissertation is my own original work and has never been submitted for a diploma or degree in any other University.

Name: Maryam Naim Khan.

Signature : ....

Date : 1st October 1998.

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DEDICATION

To my most wonderful Natural Products, Tony and Qainat, whose love and patience were the greatest inspiration. And to Tufi, who made it all possible.

ABSTRACT

Twigs of nine plants, twigs and roots of *Euclea natalensis* were collected from villages in Morogoro region, Tanzania, where they are used for brushing teeth. Barks were separated from the wood and each plant part was, separately, extracted with methanol by maceration at room temperature. The dried crude methanol extracts were screened for antimicrobial activity against oral microbes (*Streptococcus mutans, Actinomyces viscosus* and *Candida albicans*) and their minimum inhibitory concentrations were determined. Seven out of ten plants exhibited antimicrobial activity while three were inactive at 5 mg/well. The active plants were *Acacia senegal, Combretum molle, Diospyros usambarensis, Eriosema psoraleoides, Eucalyptus camaldulensis, Euclea natalensis* and *Hibiscus micranthus*. Barks were found to be more active than the woods. A phytochemical screening of *E. psoraleoides* and *H. micranthus* revealed the presence of terpenes, sterols, saponins and flavonoids in both plants. Tannins were also detected in *E. psoraleoides*. The stem bark of *E. psoraleoides* showed broad antimicrobial activity, hence it was subjected to further bioassay guided fractionation to give six active fractions.

In general, this study has demonstrated that most of the investigated chewing sticks have antimicrobial activity against the oral microorganisms used in this study. The barks were found to be more active than the woods, hence, it is advisable not to peel off the bark when using the chewing stick.

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1.0 INTRODUCTION

1.1 Background Information

In most developing countries the flora remains virtually unexplored from the point of view of practical utilization, yet past experience shows that many valuable drugs have been derived from plants. Information that a plant is used in traditional medicine is often an indication that it is worth a scientific study [1].

It has been estimated that 80% of the world's inhabitants rely chiefly on traditional medicines for their primary health care needs and it can safely be assumed that a major part of traditional therapy involves the use of plant extracts [2]. In many areas especially in the tropics, an abundance of medicinal plants offers people access to plant derived products for use in the prevention and treatment of illnesses, through self-medication. Such plants are also useful in modern medicine [2]. The use of plants has been intimately associated with dental hygiene and therapeutic practices since time immemorial. In the context of our prepackaged synthetic world it is easy to lose sight of the fact that *gutta percha* obtained from numerous sapotaceous *Palaguim* species and eugenol derived from clove oil [*Eugenia caryophyllata*] are noteworthy examples of plant products utilized in today's dentistry [3] and so is sanguinarine, a benzophenanthridine alkaloid isolated from *Sanguinaria canadensis* [4] and found in commercial mouthrinses.

A fascinating area of research which has proved worth studying is the use of plants by tribal societies for medicinal and other purposes. With reference to Tanzania, it is a multi-tribal country, which is divided into 25 regions [of which 5 are in the islands of Zanzibar and Pemba], each region has its own indigenous tribes, and each indigenous tribe has its own way of life, culture, beliefs, folklore, traditional healers, remedies and plants that are used to treat different ailments. Traditional ways of life should not be neglected, but rather wherever possible, they should be integrated into modern traditions. With the westernization of so many such peoples, there is a great need to register local knowledge before it is completely lost [5].

The advent of Primary Health Care as an approach for "Health for All by the year 2000" has made the health care providers strongly aware of the need to utilize proper technology and indigenous materials and resources. Dental health is one field in dire need for appropriate technology and indigenous materials [6]. Oral health as an important component of general health has not received its fair share of appreciation by many governments in the developing world. The reasons are obvious, there are too many killer diseases, such as malaria, measles and now AIDS/HIV, which attract more attention [7]. However, the economic effect on dental diseases due to loss of man-hours is well known. Indigenous toothbrush materials or traditional toothbrushes have long been used during the early years, long before the advent of commercial synthetic toothbrushes. Many African tribes used traditional chewing sticks called *"Mswaki"* [Kiswahili] from the toothbrush tree which was found to be as effective as the commercial toothbrush [6].

Curative dental treatment is very expensive and not so easily accessible. Therefore the only logical alternative is to resort to preventive dental measures. There is a clear need to identify indigenous plant sources containing biologically active substances against oral pathogens and to promote their use as chewing sticks. The main advantage of chewing sticks in developing countries is their ready availability, acceptability and low price. Besides, in poor communities, synthetic toothbrushes are often improperly used, usually with contaminated water [3].

Traditional methods of oral hygiene are highly practised in many developing countries, where the natural methods of teeth cleaning employ a wide variety of plant species that are chewed in the form of sticks, sponges, fruit or gums. Chewing sticks are still commonly employed for teeth cleaning in many parts of Africa and the majority are obtained from flowering plants. They are made from twigs, stems or roots, which are cut into pieces of 15-18 cm, and are usually frayed into a brush at one end with the teeth. In some communities, the bark is peeled off before use. It is assumed that the beneficial effects of the chewing stick are purely a result of its mechanical action, but several plants used as sources of chewing sticks do contain reasonable amounts of fluoride and many are claimed to have anti-cariogenic, anti-inflammatory or astringent properties [3].

1.2 Literature Review

1.2.1 Dental diseases

Dental caries and periodontal diseases are the two commonest dental pathologies known which are associated with dental plaque. Both conditions are caused by bacteria residing in the oral cavity that form dental plaque, a term used universally to describe the association of bacteria to the tooth surface. As far as dental caries is concerned, it can be described as a slow process of tooth decomposition resulting from the loss of hydroxyapatite crystals and leads to the reduced structural integrity of the tooth. Diet is the major factor in the development of cariogenic plaque. The gram-positive facultative cocci, *Mutans streptococci*, have been associated with initial caries formation, whereas *Lactobacilli* have been implicated in subsequent cavitation [8]. Periodontal disease can be described as a condition that affects the gingiva, gingival attachment, periodontal ligament, cementum and the supporting alveolar bone. The disease involves chronic bacterial infections caused by bacteria from the dental plaque. The pathological organisms implicated in these conditions include, *Actinomyces*, *Bacteroides*, *Actinobacillus*, and *Fusobacterium* species and the spirochetes [8]. Oral hygiene is mainly concerned with the prevention of dental plaque formation, which is the main etiological factor in dental diseases. This is achieved by the simple act of brushing teeth regularly using a commercial synthetic toothbrush and a toothpaste or the traditional method of tooth cleaning using the indigenous toothbrush [chewing sticks], or other mechanical forms of plaque

removers such as barks, chewing gums and sponges, or abrasive powders prepared from dried parts of plants [9].

Application of anti-microbial agents to the oral cavity is possible in three ways, namely, systemic, topical and controlled release agents. Since this study is involved with chewing sticks, emphasis is based on the topical application. Chewing and brushing is the mechanical property that is utilized in plaque removal. The action of chewing the stick involves the mixing of the substances present, with saliva, which has a local action on the tooth surface and the supragingival area, and these substances act against the oral pathogens responsible for plaque formation, which causes caries and periodontal diseases like gingivitis. The chewing sticks are believed to have anti-microbial substances, and the target area for these anti-microbial substances would be infections adjacent to the supra-gingival plaque. The use of chewing sticks is related to the reduction of dental caries and periodontal disease [10], and these are ideally suited for this target area and therefore there is no justification of using systemic anti-microbial agents in the treatment of caries or gingivitis [11].

The control or reduction of dental plaque is directly associated with the reduction and control of caries formation and periodontal diseases. This can be achieved by mechanical means or by use of topical antimicrobial agents. This study is concerned with the topical application since it is assumed that the chewing sticks may contain compounds which possess antimicrobial activity. Topical anti-microbial application can alter the caries rate by changing the resistance of the tooth, reducing the quantity

of the bacterial plaque and inhibiting specific caries associated micro-organisms (Table I) [4].

Control of gingivitis, a periodontal disease, can be achieved by reducing the plaque quantity (mechanical effect of the chewing sticks), selectively altering gingivitis associated micro-organisms and also selectively altering host response to bacteria, when an anti-inflammatory agent is combined with the anti-microbial agent [11]. Some chewing sticks have been shown to have this anti-inflammatory activity [3]. Recently the use of an anti-inflammatory agent for controlling periodontal disease has been of interest. In the future, such agents may be used in the prevention and treatment of gingivitis if combined with mechanical or chemical properties, in reducing the microbial load in plaque formation [11]. This ideal combination of properties needed for plaque reduction in a topical agent, is present in nature in the form of the indigenous toothbrushes. The identification of these chewing sticks utilized by different tribes in Tanzania can lead to identifying which one(s) of these is (are) the ideal one(s). Topical applications of these anti-microbial agents can be in the form of dentifrices, oral rinses or irrigants. These may be used non-specifically on the supra gingival plaque or may be used against a specific bacterial component in the plaque [4].

Table I : <u>**Treatment goals for caries reduction**</u>

Goal	Treatment approach
Alter resistance of tooth	• Systemic fluorides (water fluoridation or fluoride supplements) during tooth development • Topical fluorides
Reduce plaque quantity	• Mechanical plaque control • Topical antiplaque agents
Inhibit caries-associated microorganisms	• Elimination of reservoirs of bacteria • Topical antibacterial agents used intensively for short periods to alter the ecological positions of these bacteria and to allow fewer pathogenic organisms to capture that ecological niche.

Before the 1950's, agents that showed some effectiveness as topical anti-microbial agents in reducing plaque, were the phenol derivatives, such as hexylresorcinol, surfactants such as sodium ricinoleate, heavy metals such as mercurials and ammoniated compounds. The *first generation agents* have antibacterial activity *in vitro*, but have a minimal substantivity (prolonged and effective availability of the agent). These include topical antibiotics, quaternary ammonium compounds, phenolic compounds, sanguinarine, fluorides and peroxides. Mouth rinses that are available over the counter belong to this group [4]. The *second generation agents* have antibacterial activity and in addition also have an appreciable substantivity. Chlorhexidine belongs to this group. The *third generation agents* are those which demonstrate selective effects on specific bacteria or bacterial products that are implicated in disease development. These agents have a theoretical advantage over the *second generation agents* in that they do not need to inhibit all plaque bacteria and are therefore considered to be more effective and safe for long term use [4]. Inhibition of plaque and plaque induced disease has been achieved by use of systemic and topical applications of penicillin, tetracycline, polymyxin B, vancomycin, kanamycin, erythromycin, clindamycin, metronidazole and spiromycin [4]. Past evaluations of the use of topical antibiotics in dentifrices or rinses have been unimpressive because of the presence of very low doses and lack of prolonged effect from the anti-microbial agent. The use of systemic antibiotics for plaque control should be limited because of the risk of hypersensitivity reactions to penicillins and a few other antibiotics and also the development of bacterial resistance to important antibiotics. The use of these antibiotics for routine control of supragingival plaque is inappropriate.

Topical anti-microbial agents for use in oral preparations in dental practice, should have the following properties, to be rendered as ideal [4] :-

(a) Low acute and chronic toxicity - These agents should not damage the oral mucósa with daily use. To achieve a fatal dose, extremely large volumes of these agents will have to be consumed.

(b) Potency - This refers to the concentration required to inhibit growth of specific types of bacteria. The lower the minimum inhibitory concentration (MIC) the higher the potency. This concentration should be the one that is available to the supra-gingival plaque.

(c) Substantivity - The important factor in a compound's anti-plaque activity is the retention of the compound in the oral cavity and release kinetics that favor prolonged antibacterial action. The following characteristics are required for an agent with good substantivity :-

      (i) Retention of the compound in the oral cavity should be high.

      (ii) The agent should be released to maintain therapeutic levels.

      (iii) The released agent should be in an active form.

1.2.2 Plant derived anti-plaque agents

Over the past years biological therapies in dentistry have been gaining interest. The use of essential oil ingredients like thymol for plaque control is one such example. Thymol is the active ingredient of Listerine®, and it has been in use for more than 100 years [12]. Another example is the extract of *Salvadora persica* which has been

incorporated into a commercially available tooth paste known as "Sarakan" [13].

Sanguinarine, a benzophenanthridine alkaloid from *Sanguinaria canadensis*, is a

widely used herbal remedy in the United States, has also been incorporated in a

commercially available toothpaste called Viadent ®.   The chemical structure of

sanguinarine is similar to that of the benzophenanthridine alkaloidal components of

Zanthoxylum zanthoxyloides, a chewing stick used by Nigerians [14], as shown in

Figure 1 [15, 16].

Figure 1 : <u>Structures of Berberine and Chelerythrine from *Zanthoxylum*</u>

<u>*zanthoxyloides* and Sanguinarine from *Sanguinaria canadensis*</u>
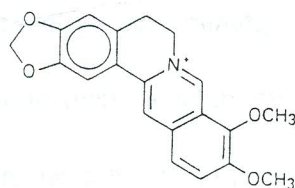
Berberine
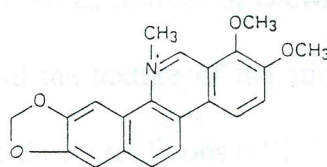
Chelerythrine
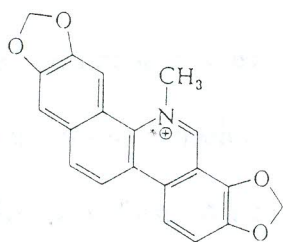
Sanguinarine

As many as 173 species of flowering plants are used as chewing sticks in various parts of the world. The majority (76.3%) originate from Africa where their use for cleaning teeth is widespread. In Southern Asia, however, where such use is also common, the number of different kinds of known species totals only 12.7%. In Europe and the Americas, where use is sporadic, there is the least number of species used as chewing sticks [17]. Ethnobotanical surveys on the use and effectiveness of these chewing sticks have shown that these species and others have a medicinal basis, in that many possess anti-cariogenic, antibiotic, healing, anti-inflammatory and astringent properties [3]. Users who often prefer chewing sticks to modern tooth brushing technique attribute their dental health to the traditional practice. It is noteworthy that dental caries rates are often very low among such users in spite of the high carbohydrate diets they normally consume [9].

The factors determining which plants are used as sources of chewing sticks in Africa include their availability, colour, taste and the texture of the sticks, the age of the user, the culture and religious customs and family traditions [18]. The chewing stick is still commonly used amongst many tribes of sub-Sahara Africa and is well known amongst individuals living in the rural communities. Many ethnobotanical surveys have been done in West-Africa in relation to the use of chewing sticks. While almost 100 species have been recorded as being used in West-Africa [13], the number known to South or East Africa is very much smaller. While some species are common to tropical Africa, little information exists regarding their use in areas where they grow. It is however known that species of *Acacia* and *Salvadora* are popular in East North

Africa, *Rhus cotinus* and *Vellozia equisetoids* in Tanzania, *Diospyros lyciodes* and *Syzygium guineense* in Zambia, *Euclea fructicosa* and *Clausena anisata* for Kenya and the *Zanthoxylum* species *Zanthoxylum dermensis* and *Zanthoxylum chalybea* for South Africa. Ethnobotanical surveys in these regions are lacking, thus, it is not known which one of these is important [13]. Previous reports also indicate that plants belonging to the families of Ebenaceae, Leguminosae, Malvaceae, Combretaceae, Myrtaceae, and Labiatae are also utilized as chewing sticks in other parts of the African continent [17, 21].

The commonest chewing stick in sub-Sahara Africa is that from *Salvadora persica*. This is a small tree, the stem of which has been used for many centuries by different communities as an oral hygiene aid. The stem of this plant is presented in the form of a short stick which when softened with water will become like a toothbrush. Chewing sticks are believed to contain chemical substances which inhibit plaque formation and gingivitis [19]. *Salvadora persica* has recently been used in the commercial manufacture of toothpaste in Egypt, India, Pakistan, Switzerland and the United Kingdom [20]. Some of the chemical components previously isolated from various chewing sticks include : trimethylamine, salvadorine, chlorides, fluoride in large amounts, silica, sulfur, vitamin C, and small quantities of tannins, saponins, flavonoids, and sterols [20]. The fluoride content is well known to be beneficial, and the silica present in many chewing sticks helps to clean the teeth, acting as an abrasive agent. Chewing sticks derived from the *Rutaceae* contain alkaloids that have a bactericidal effect. Chewing sticks obtained from *Aegles marmelos, Salvadora*

persica, *Azadirachta indica*, *Zanthoxylum zanthoxyloides* and some other plants contain essential oils and exert carminative, antiseptic and analgesic action. Tannins and resins in many chewing sticks have an astringent effect on the mucous membrane and form a layer over the enamel, thus, giving protection against dental caries [20].

Research has shown that the regular use of the chewing stick compares favourably with that of the tooth brush as a practical means of achieving oral hygiene. A clinical study done on Ethiopian school children comparing "mefaka" *[miswak]* with the conventional toothbrush found *miswak* to be as effective as the toothbrush on oral deposits [21]. A study on five types of chewing sticks used by the Yorubas in Nigeria was undertaken to determine the fluoride concentration in the bark and the stems, it was found that the bark has a higher fluoride concentration in the range of 2.3-17.2 microgram/gram compared to the stem which had 1.0 microgram/gram [18]. Another study was done on the anti-microbial activity of alkaloids from a Nigerian chewing stick *Zanthoxylum zanthoxyloides*. The finding provided further evidence on the justification of the use of this root as a chewing stick and for treating toothache caused by bacterial infection [22]. The antiplaque effect of a toothpaste containing an extract of *Salvadora persica* compared with chlorhexidine gluconate was studied. It was found that the toothpaste containing the chewing stick extract, used on daily basis with the aid of a toothbrush, had some merits over chlorhexidine, on the reduction of plaque and gingivitis. The study also demonstrated that the toothpaste with the chewing stick extract significantly inhibited streptococcal growth, but this was not the case with chlorhexidine [19].

There have been few reports on the effects of extracts from chewing sticks on the growth or physiological properties of the bacteria suspected of involvement in periodontal diseases. These pathogenic bacteria include the proteolytic Gram-negative organisms, *Bacteroides gingivalis* and *Bacteroides intermedius* and the anaerobic spirochete *Treponema denticola*. Inhibition of protease activities of periodontopathic bacteria by extracts of plants used in Kenya as chewing sticks *[Mswaki]* showed that extracts of *Rhus natalensis* and *Euclea divinorum* were the most inhibitory on protease activity. Therefore, the use of these two plants as chewing sticks might have a greater influence on the proteolytic activity of *B .gingivalis, B. intermedius*, and *T. denticola*, than would the use of the other three plants studied. [Note: This same study showed that 70% of the primary school children in Kenya use the *Mswaki*] [23]. A study of the *in vitro* antibacterial effects of eight Nigerian tooth cleaning sticks on five periodontopathic bacteria was done. It was found that seven of the eight tooth cleaning sticks tested showed anti-microbial activity. Extracts of *Terminalia glaucescens* showed the broadest spectrum, by inhibiting the growth of four out of the five test bacteria, while the extracts of *Pseudocedrela kotschyi* and *Sorindeia warneckei* showed the least spectrum by inhibiting the growth of only one out of the five periodontopathic bacteria utilized for the test [24].

To date no literature is available on the anti-microbial potential of the indigenous toothbrushes used in Tanzania. Indeed there is very little information on the utilization of indigenous methods of oral hygiene in this country.

1.2.3 Plants under study

Acacia senegal var. *senegal* (L) Willd.

Acacia senegal belongs to the family Mimosaceae, whose members are trees or shrubs and include about 35 genera and 2000 species, mainly in dry, tropical or sub tropical regions. The genus *Acacia* has around 750-800 species [5, 25]. In Brazil the dried leaf decoction of *Acacia senegal* is used for reducing fevers and in Tanzania the fresh root is used in treating gonorrhea. It has antinflammatory, antispasmodic, and molluscicidal activities. Triterpenes, steroids, flavonoids, carbohydrates, and indole alkaloids have been reported to be present in this plant [26, 27].

Combretum molle G. Don.

It belongs to the Combretaceae, one of the 17 families in the order Myrtiflorae. The Combretaceae consists of 20 genera and 600 species, tropical and sub-tropical trees and shrubs usually rich in tannins [5]. *Combretum molle* is used as an antihelminthic, for stomach ailments, as an abortifacient, as a laxative, for diarrhoea accompanied with mild anal bleeding and is combined with other plants for the treatment of infertility [27].Previous phytochemical studies revealed the presence of saponins, benzenoids and polycyclic compounds [28, 29].

Diospyros usambarensis F. White

Diospyros usambarensis is a member of the Ebenaceae, one of the 7 families within the order Ebenales. The Ebenaceae consists of 3 genera, which are tropical trees and shrubs. *Diospyros* is one of the chief genera, with about 500 species [5]. *Diospyros usambarensis* has molluscicidal antibacterial, mitogenic and antifungal activities [30] and the roots are used for the treatment for snake bites [27].General screening of *D. usambarensis* has revealed the presence of sesquiterpenes, monoterpenes, phenylpropanoids, alkaloids, and naphthoquinones [30].

Eriosema psoraleoides (Lam) G. Don.

Eriosema psoraleoides belongs to the family Papilionaceae which is composed of herbs, shrubs or trees with leaves which are simple or compound, zygomorphic and papilionaceous flowers, fruit a legume; stamens 10 monodelphous or diadelphous. The family is made of 11 larger genera with about 4430 species [31]. *Eriosema psoraleoides* (Lam) G. Don. is synonymous to *Crotalaria psoraleoides* Lam., *Rhynchosia cajanoides* Guill & Perr., *Eriosema cajanoides* (Guill & Perr.) Hook and *E. proschi* Brig.[32]. The plant (Figure 2) can be described as :-

Erect, branched subshrub or woody herb about 2 m tall. The branches are strongly ribbed, and covered with brownish hair, often velvety and covered with small orange-red glands. The leaves have three leaflets, which are paler beneath, darker on top and elliptic or oblong in shape with an entire margin. They are finely to densely velvety

pubescent above and velvety beneath. The venation is prominent and usually with buff hairs. Racemes are many-flowered, with terminal and axillary flowers. The corolla is deep golden-yellow and glabrous or rarely with a few hairs outside. Pods are oval or ovaloblong, covered with long ferruginous hairs. The seeds are shiny, dark reddish-brown with blue-black mottling and the aril is creamy brown. Its habitat is in grassland, or grassland with scattered *Commiphora*, *Acacia* or *Protea*, thicket, bushland or sometimes on margins of cultivation. Grows at height of 0-1950 m above sea-level. It is distributed in East Africa i.e. Kenya, Uganda, Tanzania (including Zanzibar), West Africa to Sudan, Central Africa to South Africa (Transvaal, Natal), Angola and also Madagascar [32].The plant is used in the treatment of syphilis in Northern Zimbabwe (Nyenja) and the root and leaf are also used for the same purpose in the Congo. In Northern Nigeria the decoction of the root is a remedy for venereal disease, and in West Africa the leaves are rubbed on the dog for control of lice. In Central Africa the leaf is used as an oxytocic, while in Tanzania the leaf is used for the treatment of threatening miscarriage, in combination with the root of *Piliostigma thonningii* [33]. No previous biological, pharmacological or phytochemical studies have been done on this plant.

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Figure 2   _**Eriosema psoraleoides**_ **: flowering shoot**

Eucalyptus camaldulensis Dehn.

Eucalyptus camaldulensis belongs to the family Myrtaceae, within the order Myrtiflorae. The Myrtaceae consists of about 100 genera and 3000 species of evergreen shrubs and trees, and is well represented in Australia, East Indies, and Tropical America. The family is divided into 2 sub-families the Leptospermoideae and Myrtoideae. The genus *Eucalyptus*, which has over 500 species belongs to the former [5]. The plant has antibacterial, antifungal, and antitussive activities. Alkanes (C_{21}-C_{31}), sesquiterpenes, monoterpenes benzenoid, coumarins, flavonoids, triterpenes, saponins, and tannins have been isolated from this species [34].

Euclea natalensis A. DC.

It also belongs to the order Ebenales, and the family Ebenaceae. The genus *Euclea* consists of about 20 species [5]. Various parts of *Euclea natalensis* are used in treating toothache, for bleeding gums, hookworm and other infestations, leprosy, headache, abdominal complaints, as laxative, dentifrice, gonorrhea, diarrhoea ,dysentery and as an emetic [27, 30]. Compounds that are present in *Euclea natalensis* include sesquiterpenes, monoterpenes, phenylpropanoids, alkaloids and napthoquinones [30, 35].

Hibiscus micranthus L.

This plant belongs to the order Malvales, an order of 7 families, which are herbs, shrubs, or trees growing in tropical and temperate countries. Many species contain mucilage but alkaloids are rare. The Malvaceae, to which *Hibiscus micranthus* belongs, consists of 75 genera and about 1000 species of tropical and temperate herbs, shrubs and trees. *Hibiscus micranthus* is one of the 300 species in the genus *Hibiscus* [5]. The plant is used in the treatment of fevers, threatened abortion, ear-ache, tonsillitis, leucorrhea, venereal diseases, and as a anti-venom. *Hibiscus micranthus* has exhibited anti-inflammatory , antipyretic, hypotensive, and antifungal activities [27, 33]. However, no previous phytochemical investigations have been done on this plant.

Ocimum suave Willd.

It belongs to the suborder Verbenineae of the order Turbiflorae, which is made up of 6 sub-orders and 26 families. *Ocimum suave* belongs to the family Labiatae which has about 200 genera and 3300 species, most of which are aromatic, annual or perennial herbs or under shrubs. The family is well represented in the Mediterranean area and Britain. The genus *Ocimum* has 150 species [5, 25]. *Ocimum suave* is used as a stomachic, mosquito repellent, decongestant, for stomatitis, constipation, haemorrhoids, abdominal pains, coughs, disinfectant, insecticide, to speed labor and

facilitate delivery of after-birth. The volatile oil from the plant contains 53% of phenols, sesquiterpenes and monoterpenes [27, 33, 36].

Opilia celtidifolia (Guill. & Perr.) Walp.

Opilia celtidifolia belongs to the order Santalales, which is an order of 7 families. The family Opiliaceae is made up of trees, shrubs or woody climbers limited to the tropics with a majority of representatives in Asia and Africa. The genus *Opilia* consists of about 25 species, 10 of which occur in Africa [37]. The root decoction of *Opilia celtidifolia* is drunk for the treatment of fever and influenza [27]. Triterpenoid saponins have been previously isolated from this plant [38].

Xerophyta suaveolens (Greves) N. Menezes

Xerophyta suaveolens belongs to the order Haemodorales. The order as defined by Hutchinson includes 6 families which are mainly tropical and sub-tropical in the Southern hemisphere. It includes plants that are somewhat intermediate in character between the Liliales and the Orchidales. The family Velloziaceae to which *X. suaveolens* belongs, are perennial herbs or sometimes woody plants or rhizomes or corms. Leaves are mostly entire, rarely lobed and in tufts at the ends of branches in woody ones [25]. No previous studies have been done on *X. suaveolens*.

1.3 Statement of the problem

There are many plants which are used as *Mswaki* in Tanzania but only *Salvadora persica* has been documented [39]. Some of these plants, though widely used and reputed as effective in maintaining oral hygiene, have not been scientifically tested for their biological effect. For instance, neither their anti-microbial potential against oral pathogens nor their chemical composition have been studied. The aim of this study was to identify the types of chewing sticks used in one of the regions of Tanzania, Morogoro, to screen them for activity against oral pathogens, and carry out phytochemical investigation on those with promising antimicrobial activity.

2.0 RESEARCH OBJECTIVES

2.1 Main Objective

To evaluate the anti-microbial potential of chewing sticks against oral pathogenic microbes.

2.2 Specific Objectives

[a] To collect and identify plants which are commonly used as chewing sticks.

[b] To screen extracts of the collected plants for anti-microbial activity against oral pathogenic microbes.

[c] To carry out phytochemical screening of the chewing sticks with significant anti-microbial activity.

[d] To carry out bioassay guided preliminary purification of the most promising plant extract(s).

3.0 EXPERIMENTAL

3.1 Preparation of plant extracts

3.1.1 Collection and identification of plant materials

For the purpose of this study 10 plant species were collected as chewing sticks from Morogoro, Tanzania, in October 1997. Morogoro is one of the regions where the villagers use chewing sticks for tooth cleaning and it is an area which is very familiar to me. Collection was done through visits and interviews with villagers in various localities within 40 km range from Morogoro town. The plant parts used mostly are twigs, but for *Euclea natalensis* the root is also used as a chewing stick. The plants (Table II) were identified by the staff of the Herbarium of the Department of Botany, University of Dar-es-salaam, Tanzania. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Muhimbili University College of Health Sciences, Dar-es-Salaam, Tanzania.

3.1.2 Extraction of plant material for antimicrobial screening

For each plant, the bark was separated from the wood, air dried and separately ground in a mill to give a coarse powder. The powdered materials (50 gm for the bark and 100 gm for wood) were macerated with methanol for 72 hours.

Table II : **Plants used as chewing sticks in Morogoro Region**

Plant species	Family	Part Used	Vernacular name
Acacia senegal var.*senegal*	Mimosaceae	Stem	Aiti
Combretum molle	Combretaceae	Stem-wood	Mrama
Diospyros usambarensis	Ebenaceae	Stem-wood	Mgiriti
Eriosema psoraleoides	Papilionaceae	Stem, stem-wood	Kipangi-pangi
Eucalyptus camaldulensis	Myrtaceae	Stem, stem-wood	Mdaini
Euclea natalensis	Ebenaceae	Root and stem-wood	Mdaa
Hibiscus micranthus	Malvaceae	Stem, stem-wood	Mchunga
Ocimum suave	Labiatae	Stem-wood	Mswameno
Opilia celtidifolia	Opiliaceae	Stem-wood	Msaka
Xerophyta suaveolens	Velloziaceae	Stem	Mswaki

All wood samples were each macerated with 500 ml of methanol with the exception

of *Eriosema psoraleoides*, for which 600 ml of solvent was used since it was bulky.

All bark samples were each macerated with 400 ml of methanol. Extracts were filtered

and the filtrates dried with a rotary-evaporator under reduced pressure at temperature

of 55°C. The thick syrupy liquids left behind were later poured into crucibles together

with the methanol that was used to rinse the flask to remove any extract sticking to

the walls and left to dry in an oven at 50°C for 2 days. The percent yields are shown

in Table III.

Table III : <u>Percentage yield of bark and wood extracts</u>

Plant name	bark extract (% w/w)	wood extract (% w/w)
Acacia senegal	7.36	1.59
Combretum molle	4.10	1.64
Diospyros usambarensis	3.10	1.02
Eriosema psoraleoides	2.72	1.09
Eucalyptus camaldulensis	3.82	0.46
Euclea natalensis (stem)	3.06	0.74
Euclea natalensis (root)	26.94	2.06
Hibiscus micranthus	2.24	1.13
Ocimum suave	1,88	2.03
Opilia celtidifolia	5.30	2.32
Xerophyta suaveolens	1.90	1.10

3.2 Microbiological Aspects

3.2.1 Reconstitution of microorganisms

Lyophilized standard strains *Steptococcus mutans* (HG 982), *Actinomyces viscosus* [(HG 485 (= NY 335)] and *Candida albicans* (HG 392) were kindly supplied by the Academic Center for Dentistry Amsterdam (ACTA), the Netherlands. They were reconstituted in 1ml each of pre-reduced sterile peptone water and normal saline (1 ml each) except for *C. albicans* where the sterile peptone water and normal saline were not pre-reduced. For pre-reducing, each medium in a Bijou bottle, with a slightly loosened cap, was placed in a candle jar, with CO_2 generating gas packs, the jar was quickly closed and incubated at 37°C, for 3 hours. Each medium was then inoculated with a loopful of the lyophilized micro-organism strain, and placed in a candle jar, under anaerobic conditions for *S. mutans* and *A. viscosus* and in aerobic conditions for *C. albicans*. They were then incubated at 37°C, for the bacteria and at 25°C for the yeast, for 2 hrs, after which the bottles were examined for any growth, which was visible as turbidity in the medium. Turbidity was observed in all the inoculated media while no turbidity was observed in the control bottles. This indicates that the growth of the lyophilized standard strains of the test microorganisms was achieved and that no contamination was introduced since the control bottles did not have any growth.

3.2.2 Inoculation and culturing of microorganisms

A loopful of the inoculum from each of the bottles was streaked in duplicate on blood agar plates, for all the organisms. The plates were once again placed in anaerobic jars

with the CO_2 generating gas packs and incubated at 37°C for 3 days for bacteria. In the case of *C. albicans* incubation was at 25°C for 3 days. A control was run for blood agar with no micro-organisms. Growth was observed for all the 3 micro-organisms and no growth was seen on the uninoculated blood agar plate.

3.2.3 Storage of Standard Strains

Skim milk (1.0 ml) in cryo-tubes was sterilized by autoclaving at 121°C for 15 min, and was allowed to cool down to room temperature. Each tube was then inoculated, with one well formed colony of the micro-organism. Four cryo-tubes were stocked for each micro-organism and these were placed in a plastic bag, labelled, and stored at -79°C.

3.2.4 Screening for antimicrobial activity

The agar hole-plate diffusion method was employed for screening of antimicrobial activity [40, 41]. Each plant extract was dissolved in 10% DMSO to give a solution with a concentration of 100 mg/ml. Media were reconstituted according to the manufacturers' instructions on the containers and autoclaved at 121°C for 15 minutes. They were maintained at a temperature of 40° - 45°C in a water bath. A turbidity standard was prepared which was equivalent to 0.5 McFarland scale, corresponding to 10^6 - 10^8 CFU/ml, by mixing 0.5ml of 1.175% w/v barium chloride solution with 99.5 ml. of 1% v/v sulfuric acid [42]. The inocula of *Streptococcus mutans*,

Actinomyces viscocus , and *Candida albicans* were prepared by mixing a few colonies with sterile 0.9% sodium chloride solution and comparing the turbidity with that of the standard 0.5 McFarland solution. If the turbidity was low then few more colonies of the test organism were added, and if the turbidity of the inoculum was more than that of the standard then more sterile 0.9% sodium chloride was added. The inoculum (12.5 ml) was added to 237.5 ml of the Sabouraud dextrose agar medium (Difco) in the case of *C. albicans* and Iso-Sensitest agar medium (Oxoid) for *S. mutans* and *A. viscosus*. The inoculated media were thoroughly shaken to disperse the micro-organisms, and 20 ml was dispensed into sterile petri dishes. The media were allowed to set at room temperature for 15 minutes, after which they were placed in a warm oven at 37°C so as to dry any moisture present, for 15 minutes. Wells (10 mm diameter each) were made in the solidified media by punching with a sterile hole borer, then 50 µl of the plant extract was introduced into each well, so that the amount of extract used was 5 mg/well. For positive controls, 50 µl of the solutions of standard antimicrobial agents were introduced into the wells. These were thymol (15,000 µg/ml) and chlorhexidine (32 µg/ml). A blank was also run, consisting of wells with 50 µl of the solvent only (10% DMSO). This was done in order to check sterility and the growth inhibitory potential of the solvent. After addition of the plant extracts into the wells the plates were left undisturbed, at room temperature for 2 hours before incubation, to allow the diffusion process to take place. In the case of *C. albicans* the plates were incubated at 25°C for 3 days, while those of *S. mutans*, and *A. viscosus* were incubated at 37°C for 24 hrs and for 24 - 48 hrs, respectively. After the incubation period the zones of inhibition were measured from

the edge of the well. Controls were run for checking the sterility and the growth supporting ability of the media for the test micro-organisms. All the tests were performed in duplicates.

3.2.5 Determination of Minimum Inhibitory Concentrations

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method [41, 42, 43], using six concentrations of: 10, 5, 2.5, 1.25, 0.625, and 0.313 mg/ml. All the MIC determinations were done in duplicate. In this method a stock solution of the plant extract with a known concentration was thoroughly mixed with the medium, and maintained at a temperature of 40°C- 45°C to prevent it from setting, before being poured into sterile petri dishes. The medium, containing the extract, was then poured into petri dishes as 20 ml aliquots and was allowed to set at room temperature. The plates were placed in the oven at 37°C to get rid of any moisture if formed during setting. A standardized inoculum of the micro-organism was prepared by dispersing a few well formed colonies in sterile normal saline and comparing it with 0.5 McFarland standard, and a loopful of the inoculum was streaked onto the plates. The plates were then incubated, at different temperatures and varying periods, depending on the micro-organism used. In the case of *S. mutans* and *A. viscosus* , Iso-Sensitest agar was used and incubation was at 37°C for 24 hrs and for 24 - 48 hrs, respectively in a candle jar with CO_2, gas packs. Sabouraud dextrose agar plates inoculated with *C. albicans* were incubated at 25°C for 3 days under aerobic conditions. The minimum inhibitory concentration was taken as the

lowest concentration which inhibited growth. The results are presented in Table IX and X.

3.3 Phytochemical Aspects

3.3.1 Phytochemical screening of *Eriosema psoraleoides* and *Hibiscus micranthus*

The methanol extracts of the stem wood and stem bark of *Eriosema psoraleoides* were subjected, separately, to tests for saponins, tannins, flavonoids, alkaloids and other plant metabolites as described by Fong *et al* [44]. In case of *Hibiscus micranthus,* the stem wood and stem bark were not extracted separately because the amount of this plant was small. Thus, extraction with methanol was carried out for the whole twig. The extracts were then subjected to the various chemical tests.

3.3.2 Fractionation of *Eriosema psoraleoides* stem bark

Eriosema psoraleoides twigs were re-collected in bulk, and the bark was separated from the wood. Air dried and milled stem bark (2 kg) was macerated with methanol (16 L) for 72 hours at room temperature. The extract was decanted, filtered, and the filtrate was dried *in vacuo* using the rotary evaporator under reduced pressure to yield a thick syrupy liquid. The latter was dissolved in methanol (150 ml) and was extracted with n- hexane (3 x 150 ml) to yield the n-hexane and methanol extracts and an insoluble residue. The methanol and n-hexane fractions were dried under

reduced pressure to yield ME (72.86 g) and HE (10.4 g), respectively. The insoluble material was allowed to dry at room temperature and gave IE (3.4 g).The partitioned extracts were subjected to antimicrobial tests on *S. mutans* and *A. viscosus* by the agar hole diffusion method as already described, at 5 mg of extract per well.

3.3.3 Chromatographic fractionation of fraction ME of *E. psoraleoides* stem bark

3.3.3.1 Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out to guide the fractionation of *Eriosema psoraleoides* stem bark. It was done on aluminium-backed plates pre-coated with silica gel 60, GF_{254} (E. Merck, Darmstadt, Germany). Plates were developed using various solvent systems comprising mixtures of chloroform and methanol. After drying, the plates were visualized in daylight, UV (at 254 and 365 nm) and by spraying with 1% vanillin in concentrated sulphuric acid, followed by heating at 110°C for 10 minutes. From the TLC profiles, mixtures of methanol and chloroform were used in varying polarities as eluents for column vacuum liquid chromatography. Mixtures of the same solvents were also used to monitor the collected fractions.

3.3.3.2 Vacuum liquid chromatography

The methanol-soluble fraction (ME) which showed antimicrobial activity and was available in a larger amount, was given priority for further purification by using vacuum liquid chromatography [45, 46]. An amount of 60gm of the extract was dissolved in a small volume of methanol and impregnated on silica gel for column chromatography (80 g).It was then dried, powdered and then applied dry on top of a column (7.5 x 11 cm) dry-packed with silica gel for thin layer chromatography (Silica gel 60 GF $_{254}$; 200 g; E. Merck, Darmstadt, Germany). The column was eluted initially, with chloroform, then with mixtures of chloroform and methanol, of increasing polarity and finally, with pure methanol. Fractions with similar TLC profiles were combined and dried *in vacuo* to give a total of 10 major (A-J). The fractions were subjected to antimicrobial tests by the hole diffusion method, at 5 mg and 2.5 mg per hole. The weights of the fractions are given in Table IV and the antimicrobial tests results are presented in Table XIII and XIV.

3.3.3.3 Gel Filtration

Fractions H and I showed antimicrobial activity and were obtained in appreciable amounts. Each was subjected to further purification by gel filtration on Sephadex LH-20 gel (Sigma Chemicals, London), pre-soaked in methanol, and packed as a slurry in a column (2 x 41 cm) in the same solvent and was allowed to settle. The sample (Fraction H, 4.5 g) was dissolved in a minimum volume of methanol and was carefully applied on top of the column. The column was eluted under gravity with

methanol (500 ml).Based on TLC profiles 6 major fractions (H-1 to H-6) were obtained. These were dried *in vacuo* and their weights are shown in Table V. Similarly, fraction I (12 g) from VLC was purified further on Sephadex LH-20 to give 6 major fractions (I-1 to I-6). Their weights are indicated in Table VI. The fractions H-1 to H-6 and I-1 to I-6 were subjected to anti-microbial test using *S. mutans* and *A. viscosus* as test microorganisms. Their results are indicated in Tables XV - XVIII.

Table IV : <u>Fractions from vacuum liquid chromatography of fraction ME</u>

<u>obtained from *Eriosema psoraleoides*.</u>

Fraction code	Fraction No.	Eluent (Chloroform : Methanol)	Weight (g)
A	1-6	(100 : 0 - 95 : 5)	0.31
B	7-10	(95 : 5 - 90 :10)	0.28
C	11-16	(90 : 10 -80 : 20)	0.82
D	17-19	(80 : 20)	0.58
E	20-22	(70 : 30)	0.70
F	23-29	(70 : 30 - 50 : 50)	2.77
G	30-39	(50 :50 -30 : 70)	7.70
H	40-44	(30 : 70 - 0 : 100)	4.53
I	45-53	(0 : 100)	34.57
J	54-56	(0 : 100)	1.22

Table V : **Fractions obtained after gel filtration of fraction H of**

Eriosema psoraleoides

Fraction code	Fraction No.	Weight (g)
H-1	1-10	0.29
H-2	11-16	1.16
H-3	17-26	1.68
H-4	27-34	0.39
H-5	35-45	0.09
H-6	46-56	0.45

Table VI : **Fractions obtained after gel filtration of fraction I of**

Eriosema psoraleoides

Fraction code	Fraction No.	Weight (g)
I-1	1-6	0.24
I-2	7-13	2.00
I-3	14-20	4.12
I-4	21-36	3.35
I-5	37-47	0.34
I-6	48-54	0.35

The bioassay guided purification of the stem bark of *Eriosema psoraleoides* is summarized in Figure 3.

Figure 3 : <u>Fractionation scheme for *Eriosema psoraleoides* stem bark</u>

<u>Plant Material</u> (2kg)

<u>Crude Methanol Extract</u>   <u>Marc</u>

1.Concentrate

2. n-Hexane (3 x)

<u>n-Hexane soluble extract</u>   <u>Methanol soluble Extract</u>   <u>Insoluble residue</u>
(HE)   (ME)   (IE)

V LC

<u>Fraction A-G</u>   <u>Fraction H</u>   <u>Fraction I</u>   <u>Fraction J</u>

Sephadex LH- 20   Sephadex LH- 20

H-1 - H-6   I-1 - I-6
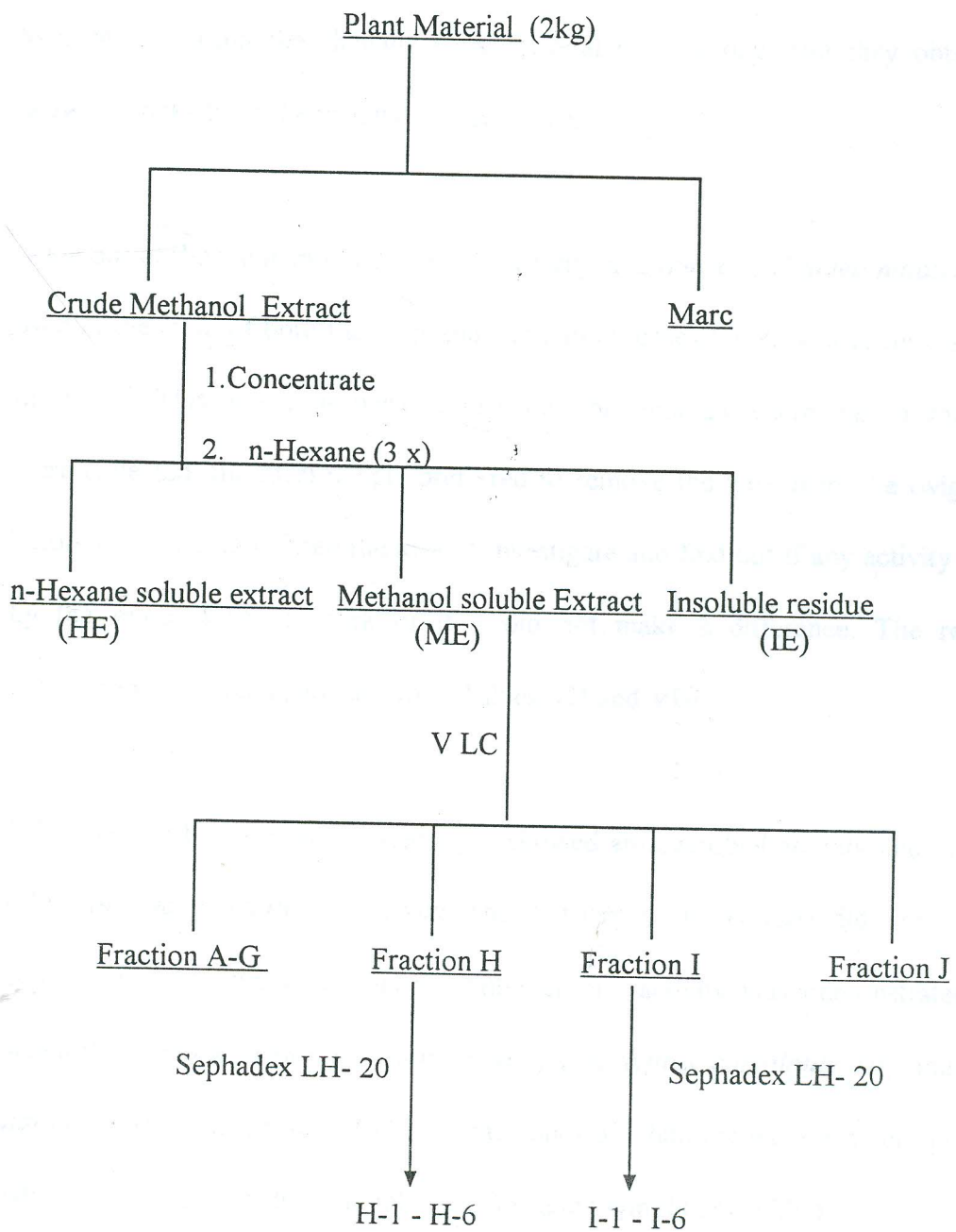
4.0 RESULTS AND DISCUSSION

The ten plant species which were used in this study are mainly utilized as chewing sticks in the villages where they were collected. The villagers claimed that they had excellent oral health. Many of them were adults who had never felt the need to see a dentist or get their tooth extracted because of a toothache. Their only means of oral hygiene was using the chewing stick several times a day, and they obtained the chewing sticks from the vicinity of their homes.

Nine out of the ten plants collected, were twigs and only one (*Euclea natalensis*) was used in the form of both the twig and root. In all cases the bark was separated from the wood. This was done because in some communities where the chewing sticks were collected, the local people preferred to remove the bark from the twig or root before use. This stimulated the idea to investigate and find out if any activity was lost by the removal of the bark or if it did not make a difference. The results of antimicrobial screening are shown in Tables VII and VIII.

Of the eleven bark extracts tested eight showed antimicrobial activity and only three (*Ocimum suave, Opilia celtidifolia* and *Xerophyta suaveolens*) did not have any activity. For *Streptococcus mutans*, antimicrobial activity was demonstrated by *A. senegal, C. molle, Eriosema psoraleoides, Eucalyptus camaldulensis,* and *Euclea natalensis* (stem and root). However, the zones of inhibition were not very prominent when compared with those of chlorhexidine and thymol (Table VII).

Table VII : <u>Antimicrobial activity of bark extracts at 5mg\well</u> [a]

Material tested	S. mutans	A. viscosus	C.albicans
Acacia senegal	4.75	9.25	3.0
Combretum molle	3.5	5.5	3.25
Diospyros usambarensis	-	10.75	-
Eriosema psoraleoides	4.0	6.5	2.5
Eucalyptus camaldulensis	2.0	5.5	-
Euclea natalensis (stem)	3.5	4.25	3.75
Euclea natalensis (root)	4.0	5.25	6.0
Hibiscus micranthus	-	6.5	-
Ocimum suave	-	-	-
Opilia celtidifolia	-	-	-
Xerophyta suaveolens	-	-	-
Chlorhexidine	11.0	6.5	6.5
Thymol	11.0	5.5	5.0

a = Results are presented as zones of inhibition (mm) measured from the edge of the

  well.

- = no zone of inhibition was observed.

The results suggest that *S. mutans* was not very sensitive to these extracts. On the other hand extracts of *D. usambarensis H. micranthus, Opilia celtidifolia, Ocimum suave* and *X. suaveolens,* were inactive on *S. mutans* at the concentrations tested. As for *A. viscosus,* it was sensitive to eight out of eleven bark extracts. Two extracts, *A. senegal* and *D. usambarensis* were even more active than chlorhexidine and thymol, while five (*C. molle, Eriosema psoraleoides, Eucalyptus camaldulensis, Euclea natalensis* root and *H. micranthus*) had similar activity to the standard antimicrobial agents. For *C. albicans* only five extracts were active, with *Euclea natalensis* root bark extract being the only one exhibiting a zone which was comparable with that of chlorhexidine and thymol. The other four extracts showed small inhibition zones.

For wood extracts (Table VIII), only a few were active against the test micro-organisms. Four out of eleven extracts, *Eriosema psoraleoides, Euclea natalensis* root and stem, *H. micranthus* were inhibitory on *S. mutans,* while only one, *Euclea natalensis* root and two, *C. molle* and *E. psoraleoides* were active on *A. viscosus* and *C. albicans* respectively. Of these, only *E. natalensis* root wood and *C. molle* stem wood extracts gave appreciable zones of inhibition on *S. mutans* and *A. viscosus,* respectively. These results suggest that for all plant samples tested the wood samples have little activity towards the test microorganisms and that much of the activity is present in the bark.

Table VIII : <u>**Antimicrobial activity of wood extracts at 5mg\well**</u> [a]

Material tested	S. mutans	A. viscosus	C. albicans
Acacia senegal	-	-	-
Combretum molle	-	4.5	-
Diospyros usambarensis	-	-	-
Eriosema psoraleoides	4.0	3.0	-
Eucalyptus camaldulensis	-	-	-
Euclea natalensis (stem)	1.25	-	-
Euclea natalensis (root)	8.5	-	3.25
Hibiscus micranthus	1.75	-	-
Ocimum suave	-	-	-
Opilia celtidifolia	-	-	-
Xerophyta suaveolens	-	-	-
Chlorhexidine	11.0	6.5	6.5
Thymol	11.0	5.0	5.0

a = Results are given as zones of inhibition (mm) measured from the edge of the

well.

- = denotes absence of inhibition zone.

For the tests done, controls for the media, solvent and viability were run. The media control demonstrated the acceptability of the aseptic technique used since there was no growth of micro-organisms in it, showing no contamination was introduced while performing the experiment. The viability control for the test micro-organism exhibited the suitability of the media for growth. The solvent control showed no inhibition of the test micro-organism proving that the solvent, had no inhibitory effect. Thymol and chlorhexidine were used to demonstrate that the test microorganisms were sensitive to these standard antimicrobial agents, and also for comparison of zones of inhibition with those of the plant extracts.

Minimum inhibitory concentrations (MIC's) were determined for all the plant extracts, that is the eight bark extracts and the three wood extracts which showed appreciable inhibition zones. The MIC values of bark extracts are shown in Table IX and those of wood extracts are presented in Table X.

Table IX shows that the bark extract of *A. senegal* was twice as active as the other four bark extracts tested (*C. molle, Eriosema psoraleoides* and *Euclea natalensis* root and stem) against *S. mutans*. From the results it can be seen that the bark extract of *A. senegal* was twice as potent as that of *E. psoraleoides* and eight times more potent than the remaining six bark extracts, towards *A. viscosus*. The activity against *C. albicans* was shown by *A. senegal, C. molle, Eriosema psoraleoides* and *Euclea natalensis* (stem and root) all with a MIC value of 5 mg/ml. Overall, the bark extracts of *A. senegal, C. molle, Eriosema psoraleoides* and *Euclea natalensis* (stem and

Table IX : <u>Minimum inhibitory concentrations (mg/ml) of bark extracts</u>

Plant extract	*S. mutans*	*A. viscosus*	*C. albicans.*
Acacia senegal	2.5	0.625	5.0
Combretum molle	5.0	5.0	5.0
Diospyros usambarensis	-	5.0	-
Eriosema psoraleoides	5.0	1.25	5.0
Eucalyptus camaldulensis	-	5.0	-
Euclea natalensis (root)	5.0	5.0	5.0
Euclea natalensis (stem)	5.0	5.0	5.0
Hibiscus micranthus	-	5.0	-

- = Inactive, hence, not tested.

Table X : <u>Minimum inhibitory concentrations (mg/ml) of wood extracts</u>

Plant Extracts	*S. mutans*	*A. viscosus*	*C. albicans.*
Eriosema psoraleoides	5.0	5.0	-
Euclea natalensis (root)	5.0	-	5.0
Combretum molle	-	5.0	-

- = Inactive, hence, not tested.

root) showed activity towards all the three test microorganisms. This indicates that these four chewing sticks have a broad spectrum of activity.

The inhibitory zone produced by the extract of *D. usambarensis* was very big but the MIC value was relatively high, this might have resulted due to deactivation of the active compounds present in the extract when directly mixed with the growth media. Such that the amount of the active substances available for inhibition of the test microorganisms was considerably reduced.

Of the three wood extracts tested for antimicrobial activity, none was active against all three test micro-organisms. *Eriosema psoraleoides* extract had antibacterial activity for both *S. mutans* and *A. viscosus* but no antifungal activity was demonstrated on *C. albicans*. *Euclea natalensis* (root) wood extract exhibited both antibacterial activity (on *S. mutans* only) and antifungal activity on *C. albicans*. *C. molle* on the other hand was active against *A. viscosus* only, with no antifungal activity against *C. albicans*. All these three wood extracts had a MIC value of 5 mg/ml.

The antimicrobial tests show that, in the case of *C. molle, Eriosema psoraleoides,* and *Euclea natalensis* (root) the bark and wood have equipotency. *E. psoraleoides* bark was four times as potent as the wood against *A. viscosus*, but was active as the wood against *S. mutans*. And for the remaining five plant extracts of *A. senegal, D. usambarensis, Eucalyptus camaldulensis, E. natalensis* (stem) and *H. micranthus,* only the bark had the antimicrobial property. Therefore, the use of chewing sticks

from these plants will be more beneficial if the bark is not peeled off, otherwise the antimicrobial property of the bark will not be fully exploited. Hence, only the mechanical action of the chewing stick will be utilized during teeth cleaning. The results from this study confirm this and other reports that most of the plants used as chewing sticks possess antimicrobial potential [15, 22, 23, 24, 47] and further shows that they have varying spectra of activity against oral micro-organisms. In the case of *Euclea natalensis*, its activity against *S. mutans* has been reported previously [48].

Of the plants which showed antimicrobial activity, only *Eriosema psoraleoides* and *Hibiscus micranthus* had not previously been studied phytochemically. In this study efforts have been made to reveal the phytochemical profile of these plants. The groups of compounds present in these plants are indicated in Table XI. Terpenes, sterols, saponins, and flavonoids were detected, while anthraquinones and alkaloids were not present in both the plants. Tannins were present in *E. psoraleoides* and absent in *H. micranthus*.

Table XI : <u>Phytochemical analysis of *Eriosema psoraleoides* and *Hibiscus*</u>

   <u>***micranthus***</u>

Compound	Plant species		
	E. psoraleoides (SW)	*E. psoraleoides* (SB)	*H. micranthus* (WS)
Terpenes	+	+	+
Sterols	+	+	+
Alkaloids	−	−	−
Saponins	+	+	+
Anthraquinones	−	−	−
Flavanoids	+	+	+
Tannins	+	+	−

Key : SW : stem wood ; SB: stem bark ; WS: whole stem.

   + : present ; - : absent .

Eriosema psoraleoides was given priority for further bioassay guided fractionation because of its broad antimicrobial spectrum and the lack of any previous phytochemical work on the plant. Literature search revealed that an appreciable amount of research has been conducted on *Acacia senegal* and *Euclea natalensis* in various areas of biological activities and a lot of phytochemical studies have been done, compounds have been isolated and elucidated. On the other hand *Eriosema psoraleoides* lacked any kind of study and this prompted further investigation to be undertaken on this plant.

The fraction ME, from the partitioning of the methanol extract of *E. psoraleoides* was given priority for further fractionation because it was more active than the other fractions,(Table XII) and was available in amounts sufficient for further purification. Thus fraction ME was subjected to vacuum liquid chromatography (VLC) and yielded ten major fractions A-J. These fractions were subjected to antimicrobial test using *S. mutans* and *A. viscosus* as the test microorganisms, at 5 and 2.5 mg /well concentration (Tables XIII and XIV).

Table XII : <u>Antimicrobial activity of fractions from solvent partitioning of</u>

<u>*Eriosema psoraleoides*</u> [a, b]

Test substance	*S. mutans*	*A. viscosus*
HE	0.0	0.0
ME	5.5	6.5
IE	4.25	5.25
Chlorhexidine	11.0	6.5
Thymol	11.0	5.25

a = Results are given as zones of inhibition (mm) measured from the edge of the well.

b = Amount of extract was 5mg/well

Table XIII : <u>**Antimicrobial activity of vacuum liquid chromatography**</u>

<u>**fractions of ME from *Eriosema psoraleoides* at 5 mg/ well**</u>[a]

Fraction	S. mutans	A. viscosus
A	2.0	3.0
B	2.25	4.0
C	3.0	6.0
D	1.75	3.0
E	3.0	6.0
F	-	4.0
G	1.5	5.0
H	2.0	6.0
I	2.0	6.0
J	-	3.0
Chlorhexidine	11.0	6.5
Thymol	11.0	5.5

a = Results are given as zones of inhibition (mm) measured from the edge of the

well.

- = Absence of inhibition zone.

Table XIV : <u>Antimicrobial activity of vacuum liquid chromatography</u>

<u>fractions of ME from *Eriosema psoraleoides* at 2.5 mg/ well[a]</u>

Fraction	*S. mutans*	*A. viscosus*
A	-	-
B	-	-
C	-	1.0
D	-	-
E	-	1.0
F	-	-
G	-	-
H	-	1.0
I	-	1.0
J	-	-
Chlorhexidine	11.0	6.5
Thymol	11.0	5.5

a = Results are given as zones of inhibition (mm) measured from the edge of the

well.

- = Absence of inhibition zone.

Table XIII shows that at 5 mg/well the fractions were much more active towards *A. viscosus* than *S. mutans* Fractions A, B, C, D, E, H and I demonstrated activity towards both the test micro-organisms, while F and J were active only towards *A. viscosus*. Fractions C and E had the same inhibitory capacity and so did fractions H and I. At 2.5 mg/well (Table XIV) the growth of *S. mutans* was not inhibited by any of the fractions, while *A. viscosus* was only slightly inhibited by fractions C, E, H and I. Further purification of fractions H and I was undertaken while C and E were not, because of the small amounts available. Hence H and I were subjected to gel filtration by using Sephadex LH 20 and for each one six further fractions were obtained which were coded as H-1 to H-6 and I-1 to I-6.

All these fractions were once again subjected to antimicrobial test. Fractions H-4, H-5 and H-6 were active at 5 mg/well but the activity was more pronounced on *A. viscosus* than *S. mutans* (Table XV). At 2.5 mg/well antimicrobial activity was reduced or lost (Table XVI). Similarly fractions I-1 to I-6 were subjected to antimicrobial test, of these I-5 and I-6 were active at 2.5 and 5 mg/hole (Tables XVII and XVIII). Once again it can be seen from the results that the growth inhibitory effect of fractions I-5 and I-6 was more prominent on *A. viscosus* than on *S. mutans*.

Fractions H-4 - H-5 and I-5 - I - 6 are of smaller size, that is later eluting from the gel column than H-1 - H-3 and I-1 -I-4 respectively, they are also among the polar substances, since they were eluted with methanol by VLC. And these are the fractions with activity towards *A. viscosus*. This varying susceptibility of the two micro-organisms to the plant fractions may be related to the different metabolic and

Table XV : **Antimicrobial activity of fractions H-1 to H-6 from**

Eriosema   psoraleoides **at 5 mg/ well**[a]

Fraction	*S. mutans*	*A. viscosus*
H-1	-	-
H-2	-	-
H-3	-	-
H-4	-	3.0
H-5	3.0	5.5
H-6	1.0	5.0
Chlorhexidine	10.0	6.5
Thymol	10.0	5.5

a = Results are given as zones of inhibition (mm) measured from the edge of the

well.

- = denotes absence of inhibition zone.

Table XVI : <u>Antimicrobial activity of fractions H-1 to H-6 from</u>

<u>*Eriosema   psoraleoides*</u> **at 2.5mg/well**[a]

Fraction	*S. mutans*	*A. viscosus*
H-1	-	-
H-2	-	-
H-3	-	-
H-4	-	1.0
H-5	1.5	3.25
H-6	-	2.5
Chlorhexidine	10.0	6.5
Thymol	10.0	5.5

a = Results are given as zones of inhibition (mm) measured from the edge of the

well.

- = denotes absence of inhibition zone.

Table XVII : <u>Antimicrobial activity of fractions I-1 to I-6 from</u>

<u>_Eriosema psoraleoides_ at 5 mg/well</u>[a]

Fraction	_S. mutans_	_A. viscosus_
I-1	-	-
I-2	-	-
I-3	-	-
I-4	-	-
I-5	3.0	6.0
I-6	1.5	5.0
Chlorhexidine	10.0	6.5
Thymol	10.0	5.5

a = Results are given as zones of inhibition (mm) measured from the edge of the

   well.

- = denotes absence of inhibition zone.

Table XVIII : <u>Antimicrobial activity of fractions I-1 to I-6 from</u>

<u>*Eriosema psoraleoides* at 2.5 mg/well[a]</u>

Fraction	S. mutans	A. viscosus
I-1	-	-
I-2	-	-
I-3	-	-
I-4	-	-
I-5	1.0	5.0
I-6	1.0	4.0
Chlorhexidine	10.0	6.5
Thymol	10.0	5.5

a = Results are given as zones of inhibition (mm) measured from the edge of the

well.

- = denotes absence of inhibition zone.

physiological processes of the individual bacterial species. The active substances present in the fractions tested, may vary in quantity and possibly in their constitution and hence this may affect these processes differently [24].

It was however noted that as the purification continued of the stem bark extract of *Eriosema psoraleoides*, the zones of inhibition against the test microorganisms were not increasing as much as expected. This might have been due to the effect of light, temperature changes, storage or repeated contact with the eluents on the stability of the purified extract, which to some degree could have been altered.

When the sizes of zones of inhibition of fractions H-4, H-5, H-6, I-5 and I-6 on *S. mutans* were compared with those of chlorhexidine and thymol it was noted that the activity of the fractions was very little. With respect to *A. viscosus* the zone sizes produced by H-5, H-6, I-5 and I-6 at 5 mg/well were almost of the same magnitude as those of the reference antimicrobials. From this observation it can be assumed that the active compound or compounds present in these fractions has or have an activity comparable to the reference antimicrobials, with regard to *A. viscosus*.

Thin-layer chromatography profiles of fractions H-4, H-5, H-6, I-5 and I-6 indicated that no pure compound was isolated, since all the fractions gave at least three spots, showing that a mixture of compounds was present. Further purification with different solvent systems and adsorbents may be needed before a pure compound could be isolated and identified.

5.0 CONCLUSION

The results of this study have shown that seven of the ten plants collected, (*Acacia senegal, Combretum molle, Diospyros usambarensis, Eriosema psoraleoides, Eucalyptus camaldulensis, Euclea natalensis,* and *Hibiscus micranthus*) had antimicrobial activity at varying levels, while three did not. When used as chewing sticks, the seven could have both mechanical and antimicrobial effect in the oral cavity. The rest (*Ocimum suave, Opilia celtidifolia* and *Xerophyta suaveolens*) would, most likely, have only a mechanical effect as they lack antimicrobial activity. Preliminary antimicrobial screening and minimum inhibitory concentrations (MICs) determination, with *S. mutans, A. viscosus* and *C. albicans* as the test micro-organisms, revealed that the stem and root barks were more active than the wood. Hence the habit of peeling off the bark from the chewing stick should be discouraged, if the full potential of its action is desired for maintaining oral health.

In this study efforts were made to establish the phytochemical profiles of *Eriosema psoraleoides* and *Hibiscus micranthus*, which were not previously known. In both plants, terpenes, sterols, saponins and flavonoids were shown to be present while alkaloids and anthraquinones were not detected. Tannins were present in *Eriosema psoraleoides*, but absent in *H. micranthus*. However, no conclusion can be made from this study, as to which compounds were responsible for the antimicrobial effect.

The stem bark from the plant *Eriosema psoraleoides* showed broad antimicrobial activity hence it was subjected to further purification. Only partial purification of the crude extract was achieved, therefore no pure compound was isolated in this study. The partially purified fractions showed activity to both *S. mutans* and *A. viscosus*, but the activity towards the latter was more prominent.

Since the investigation on *E. psoraleoides* was not exhaustive and MIC determinations of the partially purified fractions was not done due to small amounts obtained, further investigation may be done to isolate pure compounds and subjecting them to detailed antimicrobial tests against a higher number of oral pathogens. Structure elucidation of such compounds could be carried out by spectroscopic methods. More such studies of plants used in ethnomedicine should be encouraged, since it is through such investigations that some undocumented plants which possess therapeutic potential are known.

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