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Antimicrobial activity of *Streblus asper* (Moraceae) against bacteria causing dental caries

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Abstract: *Streblus asper Lour (Family: Moraceae) is a small tree which is indigenous to tropical countries.* The plant *is a rich source of cardiac glycosides which are pharmacologically very important because of its antimicrobial, antifilarial, anticancer, anti-allergic and insecticidal activities. S. aspera is most useful in oral hygiene because its leaf extract is active against Streptococcus mutans and some other bacteria causing dental caries.*
In the present investigation the antibacterial activity of four solvent extracts of leaves of S. asper was assayed against bacteria causing dental caries. The results revealed that the four solvent extract fractions were not equally antimicrobial against Gram positive and Garam negative bacteria. The solvent fractions are found to be more inhibitory to Gram negative bacteria in comparison to Gram positive bacteria. Among four solvent fractions Dichloromethane (DCM) and ethyl acetate (EAE) did not cause any growth inhibition to Streptococcus mutans and Streptococcus salivarus. The n-hexane (NHE) and Methanol (MEL) caused 17.50 mm and 12.25 mm growth inhibition in S. mutans respectively; and 18.50 mm and 13.35 mm growth inhibition in S. salivarus respectively. The n-hexane fraction was not inhibitory to Actinomyces odontolyticus, but dichloromethane (DCM), ethyl acetate (EAE) and methanol (MEL) fractions were less inhibitory to A. odontolyticus. DCM, EAE and MEL fractions of S. asper caused 11.35 mm, 9.7 mm and 10.25 mm inhibition in this Gram positive bacterium.The growth of Bifidobacterium dentium was inhibited by EAE and NHE fractions, but ineffective to DCM and MEL fractions. Similarly, the growth of Staphylococcus aureus was inhibited by EAE and MEL fractions in the order of 14.35 mm and 19.5 mm respectively.
It can be concluded that the mouthrinse containing S. asper *leaf extract can reduce the microbial count to safe level without changing an oral ecology.*
Key Words: *Streblus asper, dental caries, Streptococcus mutans, Streptococcus salivarus, Actinomyces odontolyticus, Bifidobacterium dentium, Escherichia coli, Enterobacter aerogens.*

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I. Introduction

Streblus asper Lour (Family: Moraceae) is a small tree which is indigenous to tropical countries such as India, Sri Lanka, Malaysia, the Philippines and Thailand. It is known by various names, e.g. Bar-inka, Berrikka, Rudi, Sheora, Koi, Siamese rough bush and Tooth brush tree [1] (Glasby, 1991). In India it is known by its several vernacular names, the most commonly used ones being Shakhotaka (Sanskrit), Siora (Hindi), Sheora (Bengali) and Piray (Tamil) [2] (Chopra *et al*., 1956). It is used traditionally in leprosy, piles, diarrhea, dysentery, elephantiasis [3] (Kirtikar and Basu, 1933) and cancer [4] (Bhakuni *et al*., 1969). It is a rigid shrub or gnarled tree; branchlets tomentose or pubescent. Leaves are 2–4 inch, rigid, elliptic, rhomboid, ovate or obovate, irregularly toothed; petiole 1/12 inch. Male heads globose, solitary or 2-nate, sometimes androgynous; peduncle short scabrid, flowers minute. Female flowers longer peduncled. Fruit pisiform; perianth yellow. It is found in the drier parts of India, from Rohilkund, eastward and southwards to Travancore, Penang and the Andaman Islands [5] (Hooker, 1886).

The pharmacognostical studies of its stem bark as well as its root bark have been carried out [6, 7] (Iyengar and Pandey, 1963; Chaudhari, 1968). It finds place in the Ayurvedic Pharmacopoeia of India (2001) [8] and has also been described in some monographs [9] (Gupta *et al*., 2005), but none have described the complete chemistry and pharmacology of this important ethnomedicinal plant. Therefore, we aimed to compile an up-to-date and comprehensive review of *S. asper* that covers its traditional and folk medicinal uses, phytochemistry and pharmacology.

Streblus asper is a well known ethnomedicinal plant which is also used in Ayurveda [10, 11, 12, and 13] (*Chopra et al*.,1956; Jain, 1991; Singh and Singh, 1976; Singh and Ram, 1988). Its use in the Indian traditional folk medicine is also well documented.

Streblus asper is a rich source of cardiac glycosides. Reichstein and co-workers [14, 15] (Khare *et al*., 1962; Manzetti *et al*., 1964) have isolated more than 20 cardiac glycosides from the root bark of *S. asper* and were able to structurally characterize ∼15 such compounds, mainly as a result of the application of degradative techniques, namely kamloside, asperoside, strebloside, indroside, cannodimemoside, strophalloside, strophanolloside,16-*O*-acetyl-glucogitomethoside, glucogitodimethoside, glucogitodimethoside, glucokamloside, sarmethoside and glucostrebloside. The other glycosides reported from the roots include β-sitosterol-3-*O*-β-D-arabinofuranosyl-*O*-α-L-rhamnopyranosyl-*O*-β-D-glucopyranoside [16] (Chaturvedi and Saxena, 1984), lupanol-3-*O*-β-D-glucopyranosyl-[1-5]-*O*-β-D-xylofuranoside [17] (Chaturvedi and Saxena, 1985) and vijaloside, i.e. periplogenin-3-*O*-β-D-glucopyranosyl-[1-5]-*O*-β-D-xylopyranoside [18] (Saxena, 1985).

From the stem bark of this plant, α-amyrin acetate, lupeol acetate, β-sitosterol, α-amyrin, lupeol and diol [19] (Barua *et al*., 1968), strebloside and mansonin [20] (Fiebig *et al*., 1985) have been isolated. A pregnane glycoside named sioraside [21] (Prakash *et al*., 1992) has also been isolated. *n*-Triacontane, tetraiacontan-3-one, β-sitosterol, stigmasterol, betulin and oleanolic acid were identified from the aerial parts [22] (Chawala *et al*., 1990). An unidentified cardenolide [23] (Fernandes *et al*., 1961), β-sitosterol, α-amyrin and lupeol were isolated from root bark and leaves [24] (Mukherjee and Roy, 1983).

The volatile oil [25] (Phutdhawong *et al*., 2004) from fresh leaves of *S. asper* was obtained in 0.005% yield as a brown liquid. The major constituents of the volatile oil were phytol (45.1%), α-farnesene (6.4%), *trans*-farnesyl acetate (5.8%), caryophyllene (4.9%) and *trans*-*trans*-α-farnesene (2.0%). The other constituents were α-copaene, β-elemene, caryophyllene, geranyl acetone, germacrene, δ-cadinene, caryophyllene oxide and 8-heptadecene.

Several workers have reported the different biological activities of *S. asper* in various *in vitro* and *in vivo* test models. Different parts of his plant have been found to exhibit cardiotonic [26] (Gaitonde *et al*., 1964), antifilarial [27,28, 29, 30, 31, 32] (Chatterjee *et al*., 1992; Pandey and Das, 1990; Hashmi and Singh, 2002; Nazneen *et al*., 1989; Singh et al. 1994; Baranwal et al. 1978), anticancer[33, 34, 25] (Rastogi and Dhawan, 1996; Fiebig et al. 1985; Phutdhawong *et al*., 2004), antimicrobial [35, 36, 37, 38, 39, 41] (Triratana and Thaweboon, 1987; Wongkham *et al*., 2001; Taweechaisupapong *et al*., 2005; Taweechaisupapong *et al*., 2000; Limson *et al*., 2004; Taweechaisupapong *et al*., 2002, 2005), anti-allergic[42] (Amarnath *et al*., 2002), insecticidal [43, 44] (Atal, 1969; Hashim and Devi, 2003) and antiparasitic [45] (Das and Beuria, 1991) activities. Besides these, *S. aspera* is most useful in oral hygiene because its leaf extract is active against *Streptococcus mutans* which is associated with dental caries [46, 47, 48, 49, 50, 51, 52] (Triratana and Thaweboon, 1987; Wongkham *et al*., 2001; Taweechaisupapong *et al*., 2005; Taweechaisupapong *et al*., 2000; Limson et al. 2004; Taweechaisupapong *et al*., 2002, 2005).

Dental caries (cavities) is one of the most common chronic infectious diseases in the world [53, 54] (Anusavice, 2000; WHO, 2002). There are three major hypotheses for the etiology of dental caries: the specific plaque hypothesis, the nonspecific plaque hypothesis, and the ecological plaque hypothesis [55, 56, 57] (Loesche, 1992; Marsh, 1994; Theilade, 1986).The specific plaque hypothesis has proposed that only a few specific species, such as *Streptococcus mutans* and *Streptococcus sobrinus*, are actively involved in the disease. On the other hand, the nonspecific plaque hypothesis maintains that caries is the outcome of the overall activity of the total plaque microflora, which is comprised of many bacterial species [57] (Theilade, 1986). The ecological plaque hypothesis suggests that caries is a result of a shift in the balance of the resident microflora driven by changes in local environmental conditions [56] (Marsh, 1994).

The subjects suffering from dental caries experience pain, problem with eating, chewing, smiling and communication due to missing, discolored or spoiled teeth [58] (Yadav and Prakash, 2016). Low self-esteem, adverse pregnancy outcomes, increased risk of myocardial infarction, cardiovascular disease, respiratory, erectile, diabetes complications, cavernous sinus thrombosis and Ludwig angina are associated with dental caries which can be crucial to human beings [59, 60, 61] (Yadav and Prakash, 2015; Hartman and Richard, 2008; Cvitkovitch, 2001). Moreover, oral diseases hamper activities at school, at occupation and at residence causing millions of school and employment hours to be vanished each year throughout the globe [62, 58] (Petersen *et al*., 2005; Yadav and Prakash, 2016). As a consequence, the treatment need is enhanced nowadays.

Streptococcus mutans and *Streptococcus sobrinus* are the two principal agents of enamel caries [59, 57, 63, 64, 65] (Yadav and Prakash, 2015; Theilade, 1990; Bowden, 1991; van Houte, 1994; Loesche, 1986). *Lactobacillus* and *Actinomyces* are also associated with caries. *Actinomyces odontolyticus* colonizes infants before eruption of teeth [66] (Nyvad and Kilian, 1990). Some root caries lesions are dominated by *Actinomyces naeslundii, A. israelii* and *A. gerencseriae* [67, 68, 69, 70, 71] (Sarkonen *et al*., 2000; Bowden *et al*., 1990; Bowden, 1990; Schupbach *et al*., 1995; Schupbach *et al*., 1996). The other significant species involved in caries

includes *Streptococcus mitis, Bifidobacterium* and *Actinomyces*, a group of 'low pH' aciduric isolates which have been isolated from white spot lesions in humans [58, 72, 73] (Yadav and Prakash, 2015; Sarkonen *et al*., 2000; Brailsford and Lyach, 1998).

Dental plaque is a multifaceted biofilm community where bacterial populations exist as separated micro-colonies in physiologically diverse environments. Biofilm cells exhibit different characteristics from the same cells growing in suspended culture [74] (Segal and Ron, 1998).

The pathogens responsible for dental caries can be classified into following groups:

• **Gram positive cocci:** *Streptococcus mutans, S. mitis, S. salivarus, S. sanguis, S. intermedius, S. vestibularis, Staphylococcus aureus, Atopobium* spp, *Peptostreptococcus* spp, *Enterococcus fecalis* .

• **Gram positive rods:** *Actinomyces odontolyticus, A. naeslundii, A. viscosus, A. israelii, Lactobacillus fermentum, L. acidophillus, Bifiodobacterium dentium, Propionibacterium* spp.

• **Gram negative cocci:** *Veillonella parvula, Nesseria* spp.

• **Gram negative rods:** *Bacteriodes denticola, B. melaninogenicus, Fusobacterium necrophorum, F. mortiferum, Escherishia coli, Klebsiella pneumoniae, Enterobacter aerogens, Citrobacter freundi, Pseudomonas fluorescence, Haemophilus spp, Prevotella* spp, *Leptotrichia* spp.

• **Yeasts:** *Candida albicans, C. tropicalis, C. glabrata*.

In the present investigation the antimicrobial activity of leaf extract of *streblus asper* has been assayed *in vitro* against Gram positive *Streptococcus mutans, Streptococcus salivarus, Actinomyces odontolyticus, Bifidobacterium dentium*, and Gram negative *Escherichia coli* and *Enterobacter aerogens.*

II. Materials and Methods

Test Bacteria: The test organisms viz. Gram positive *Streptococcus mutans, Streptococcus salivarus, Actinomyces odontolyticus, Bifidobacterium dentium and Staphylococcus aureus*, and Gram negative *Escherichia coli* and *Enterobacter aerogens* were clinical isolates obtained from Microbiology research laboratory, IGIMS, Patna.

Preparation of Plant extract: The leaves of *Streblus asper* were oven dried and ground into coarse powder using high capacity grinding machine. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place. About 900 gm of powder was taken in clean, round bottomed flask and macerated at room temperature in 3 liters of methanol for 10 days with occasional shaking for better extraction. The whole mixture was then filtered through Whatman No.1 filter paper. After filtration the filtrate was concentrated at 40°C with a Heidolph rotary evaporation. The concentrated extract was then air dried to solid residue. The weight of the crude methanolic extract of leaves obtained was 56 gm.

The crude extract (35g) of *S. asper* was dissolved in a solvent containing 90% methanol 10% distilled water to obtain aqueous methanol and partitioned between n-hexane, Di-Choloro Methane (DCM) and Ethyl Acetate fractions. All the four fractions were evaporated to dryness. After evaporation the weight of different fractions obtained was as follows:

Crude Methanol extract:	35.0 gm
n-Hexane fraction:	6.57 gm
Ethyl acetate fraction:	4.567 gm
Methanol fraction:	2.94 gm
Dichloro methane fraction:	5.77 gm

Solutions of known concentration (µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates were then kept at low temperature (4^0C) for 24 hours to allow maximum diffusion. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter.

The test microbes were grown in nutrient agar medium consisted of following ingredients:
Bacto peptone: 0.5 g/l; Sodium chloride: 0.5 g/l; Bacto yeast extract: 1.0 g/l; Bacto agar: 2.0 g/l; pH 7.2

The test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop under aseptic condition. The cultures were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial suspension was immediately transferred to the sterilized petridishes. Three types of discs were prepared for antimicrobial screening.

Standard discs: These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, kanamycin (30μg/disc) disc was used as the reference.

Blank discs

These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

Preparation of sample discs with test samples

Measured amount of each test sample was as follow:

- Dichloromethane fraction of crude extraction (DCM)
- n-Hexane fraction of crude extraction (NHE)
- Ethylacetade fraction of crude extraction (EAE)
- Methanol fraction of crude extraction (MEL)

The amount of test sample taken was as follows:

Sample	Dose (μg/disc)	Required amount for disc (mg)
Dichloromethane	200	4.0
n-Hexane	200	4.0
Ethylacetate	200	4.0
Methanol	200	4.0

Standard Kanamycin (30 mg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents.

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

Determination of antimicrobial activity

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

III. Results

The antimicrobial activity of the four solvent extracts of *Streblus asper* has been presented in Table-1; Figure-1, 2 and 3.

Figure-1: Clear zone of Inhibition

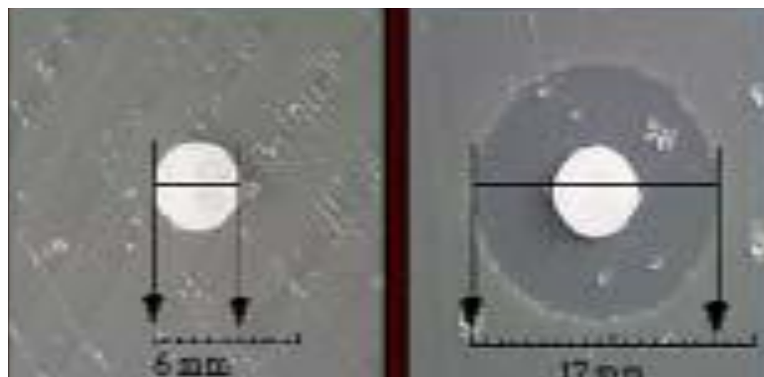

Figure-2: Measurement of clear zone of inhibition

Table-1: Antimicrobial activity of bacteria against four different solvent extracts of *Streblus asper*

Test bacteria	Diameter if zone of inhibition in mm				
	Solvent fractions				
Gram positive bacteria	DCM	EAE	NHE	MEL	Kanamycin
Streptococcus mutans	-	-	17.5 ±0.17	12.25 ±1.12	-
Streptococcus salivarus	-	-	18.5 ±0.23	13.35 0.65±	-
Actinomyces odontolyticus	11.35 ±1.17	9.7 ±0.24	-	10.25 ±0.35	34.60 ±0.23
Bifidobacteriumn dentium	-	19.5 ±0.19	16.5 ±0.23	-	-
Staphyloccocus aureus	-	14.35 ±1.05	-	19.5 ±0.21	35.5 ±0.35
Gram negative bacteria	DCM	EAE	NHE	MEL	Kanamycin
Eschericia coli	19.71 ±0.31	19.35 ±0.32	17.25 ±0.27	-	36.50 ±0.21
Enterobacter aerogens	19.80 ±0.18	18.25 ±0.24	16.75 ±0.29	17.35 ±0.15	36.50 ±0.41

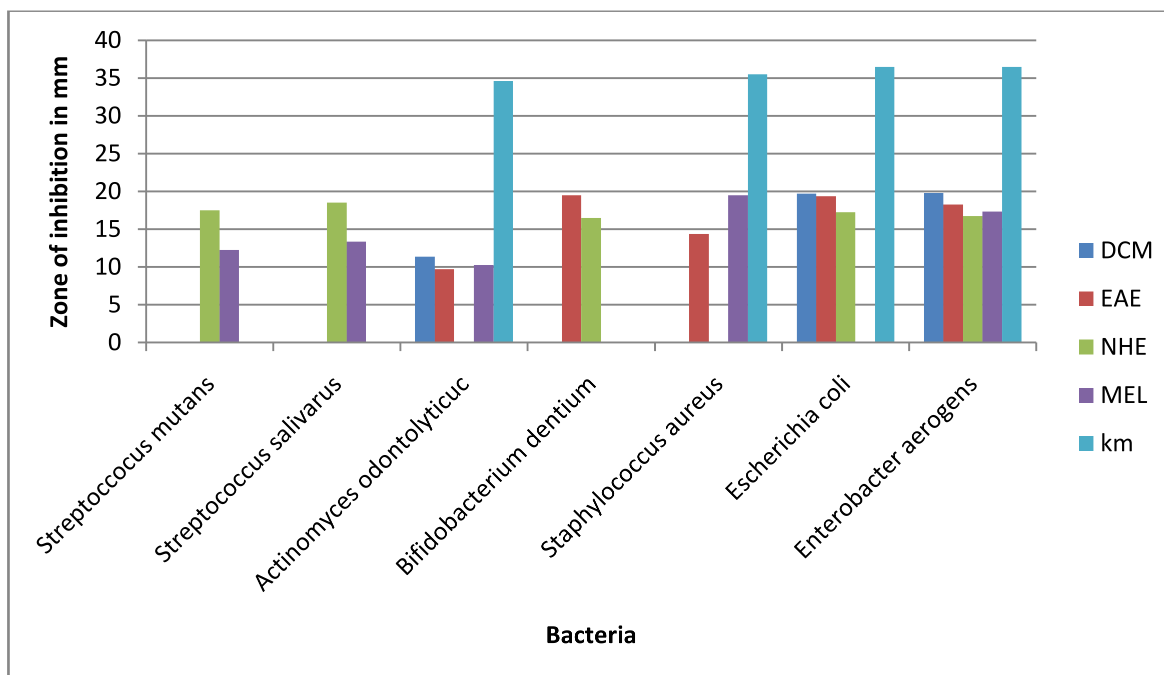

Figure-3: Growth inhibition of Bacteria in four different solvent fractions of *streblus asper* and kanamycin (Km)

From the results it is evident that the four solvent extract fractions were not equally antimicrobial against Gram positive and Garam negative bacteria. The solvent fractions are found to be more inhibitory to Gram negative bacteria in comparison to Gram positive bacteria. Among four solvent fractions Dichloromethane (DCM) and ethyl acetate (EAE) did not cause any growth inhibition to *Streptococcus mutans* and *Streptococcus salivarus*. The n-hexane (NHE) and Methanol (MEL) caused 17.50 mm and 12.25 mm growth inhibition in *S. mutans* respectively; and 18.50 mm and 13.35 mm growth inhibition in *S. salivarus* respectively. The n-hexane fraction was not inhibitory to *Actinomyces odontolyticus*, but dichloromethane

(DCM), ethyl acetate (EAE) and methanol (MEL) fractions were less inhibitory to *A. odontolyticus*. DCM, EAE and MEL fractions of *S. asper* caused 11.35 mm, 9.7 mm and 10.25 mm inhibition in this Gram positive bacterium. The growth of *Bifidobacterium dentium* was inhibited by EAE and NHE fractions, but ineffective to DCM and MEL fractions. Similarly, the growth of *Staphylococcus aureus* was inhibited by EAE and MEL fractions in the order of 14.35 mm and 19.5 mm respectively (Table-1; Figure-3).

Among Gram negative bacteria *Enterobacter aerogens* was inhibited by all the four solvent fractions viz. DCM, EAE, NHE and MEL in order of 19.80 mm, 18.25 mm, 16.75 mm and 17.35 mm respectively. Similarly, the growth of *Escherichia coli* was inhibited by DCM, EAE, NHE in the order of 19.71 mm, 19.35 mm and 17.25 mm, but not by MEL fraction (Table-1; Figure-3).

IV. Discussion

The antimicrobial potential of leaves of *S. asper* has been demonstrated by several workers viz.Triratana *et al*., (1987) [46], Wongkham *et al*., (2001) [47], Taweechaisupapong *et al*., (2005) [48], Taweechaisupapong *et al*., (2000), Taweechaisupapong *et al*., (2000) [49], Limsong *et al*., (2004) [50], Taweechaisupapong *et al*., (2002) [51], Taweechaisupapong *et al*., (2005) [48] and found a more or less similar results. Ethanol extracts from the sticks and leaves of *S. asper* have been shown to inhibit the growth of *Streptococcus mutans* (Triratana *et al*., 1987) [46].

The antimicrobial activity of *S. asper* leaf extract has been studied upon various microorganisms involving oral and nasopharyngeal infections, especially *S. mutans*. Bactericidal activity was found in the 50% ethanol (v/v) extract of *S. asper* leaves. The extract possessed a selective bactericidal activity towards *Streptococcus*, especially to *S. mutans* which has been shown to be strongly associated with dental caries. In the present investigation the antimicrobial activity of four different solvent extract of S. asper has been studied and found to be inhibitory to *Streptococcus mutans, S. salivarus, Actinomyces odontolyticus, Bifidobacterium dentium, Staphylococcus aureus, Escherichia coli* and *Enterobacter aerogens*. This is in conformity with the work of Wongkham *et al*., (2001) [47], Taweechaisupapong *et al*., (2000) [49], Limsong *et al*., (2004) 50], Taweechaisupapong *et al*., (2002) [51].

In vitro study of leaf extract of *S. asper* against the six anaerobic bacteria viz *Porphyromonas gingivalis* W50, *Prevotella intermedia*, *Actinomyces naeslundii* (T14V), *Peptostreptococcus micros*, *Actinobacillus actinomycetemcomitans* ATCC 43717 and ATCC 43718 has been demonstrated by Taweechaisupapong *et al*., (2005) [52] and found that 15 µl of the leaf extract at 250 and 500 mg ml^{-1}had inhibitory effects towards all bacterial strains tested except *A. actinomycetemcomitans* ATCC 43717. The extract had no bactericidal activity against *P. intermedia* and *A. naeslundii* (T14V). Although the extract did not show inhibitory effect towards *A. actinomycetemcomitans* ATCC 43717 by disc diffusion method, but it did inhibit growth of *A. actinomycetemcomitans* ATCC 43717 by using broth microdilution method.

V. Conclusion

From the results it can be concluded that the mouthrinse containing *S. asper* leaf extract can reduce the microbial count to safe level without changing an oral ecology. The leaf extract of *Streblus asper* found to be antibacterial and might be useful in controlling Gram positive and Gram negative aerobic and anaerobic bacteria that cause dental caries.

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