

In-Vitro Activity of Saponins of Bauhinia Purpurea, Madhuca Longifolia, Celastrus Paniculatus and Semecarpus Anacardium on Selected Oral Pathogens

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Abstract

Objective: Dental caries, periodontitis and other mucosal diseases are caused by a complex community of microorganisms. This study aimed to investigate the antimicrobial properties of saponins of four important oil yielding medicinal plant extracts on selected oral pathogens that are involved in such diseases.

Materials and Methods: Saponins were extracted from Bauhinia purpurea, Madhuca longifolia, Celastrus paniculatus and Semecarpus anacardium and purified. Antimicrobial properties of these saponins against Streptococcus mutans, Streptococcus mitis, Streptococcus salivarius, Staphylococcus aureus and Lactobacillus acidophilus were determined using well diffusion method. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of saponins inhibiting bacterial growth after 14 h of incubation at 37°C. The bactericidal activity was evaluated using the viable cell count method.

Results: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Madhuca longifolia saponin on Streptococcus mutans MTCC 890, Streptococcus mitis and Staphylococcus aureus was $18.3 \pm 0.15/34.4 \pm 0.24$ µg/ml, $19.0 \pm 0.05/32.2 \pm 0.0$ µg/ml and $21.2 \pm 0.35/39.0 \pm 0.30$ µg/ml, respectively and Bauhinia purpurea saponin on Streptococcus mutans MTCC 890, Staphylococcus aureus and Lactobacillus acidophilus was $26.4 \pm 0.20/43.0 \pm 0.40$ µg/ml, $29.0 \pm 0.30/39.6 \pm 0.12$ µg/ml and $20.2 \pm 0.05/36.8 \pm 0.23$ µg/ml, respectively.

Conclusion: The strong antimicrobial activity of Madhuca longifolia and Bauhinia purpurea may be due to the presence of complex triterpenoid saponins, oleanane type triterpenoid glycosides or atypical pentacyclic triterpenoid saponin. Hence, these extracted saponins may be used in food and oral products to prevent and control oral diseases.

Key Words: Antimicrobial Agents; Caries, Dental; Madhuca Longifolia; Plants, Medicinal; Streptococcus Mutans

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INTRODUCTION

Oral diseases such as caries and periodontitis begin with the formation of plaque, which

plays a major role in the initiation of dental caries [1]. Dental plaque is a complex community of micro-organisms originated on the sur-

face of the tooth as a biofilm, fixed in an extracellular matrix comprising polymers of host and microbial origin [2]. Plaque formation involves two stages; the initial reversible stage is formed by various oral bacteria, the later is formed by glucosyltransferase activity which forms insoluble glucan layer, where adhesion of *Streptococcus mutans* occurs [3]. Dental caries and periodontal disease starts by plaque accumulation on the soft tissue and on the surface of the tooth [4] which may later result in loss of teeth and also gum problems. In the mouth, multispecies biofilms are associated not only with dental plaque and tooth decay, but also with other soft tissue diseases which affect the buccal cavity and periodontium [5]. A consequence of such biofilm growth that has profound implications for their control in the oral environment and in medicine is an increased resistance to chemical antimicrobial agents and antibiotics [5].

Antibiotics have been used to treat the existing oral diseases, but prevention has mainly been influenced by diet, oral hygiene procedures and an awareness of oral health. The destruction of normal oral and intestinal flora and undesirable side-effects caused by these synthetic drugs has resulted in the search for safer and more easily available plant sources which may have other therapeutic effects too. We rely heavily on bacterial and fungal sources for their ability to fight major diseases because manufacture and supply on a large scale is required to meet the demand for pharmaceutical drugs. This leaves the plant resources which are easily available inadequately utilized [6]. Plants produce secondary metabolites for their protection against pathogens. These secondary metabolites are divided into several categories such as phenolics, alkaloids, steroids, terpenes and saponins. These phytochemicals exhibit a wide range of biological activities such as anticarcinogenic, antioxidant, antimicrobial and other properties [7].

Higher plants are sources of saponins which are high molecular weight glycosides depend-

ing upon their molecular structure and may be named as tri-terpene or steroid glycosides. They have the potential to affect resistance to plant diseases [8]. Antimicrobial activities of different types of saponins from a few plant extracts were conducted and proved that they have bactericidal and fungicidal activity [9]. *Bauhinia purpurea* L. belonging to the family leguminosae, is a small evergreen medicinal and avenue tree. Several parts of this tree have been used in Ayurveda to prepare indigenous medicines and to treat common disease conditions such as cough and jaundice. Detailed analysis of fatty acid composition and bioactive molecules has been reported [10]. Different types of triterpenoid saponins have also been detected and were isolated from the leguminosae family [11].

Madhuca longifolia is a large tree famous for its flowers, fruits and seeds. Extracts of flower and leaves are used to treat various diseases and the oil extracted from the seeds is used to cook food. The bark extract is used to treat dental problems. Detailed investigations of fatty acids and bio-active constituents of *madhuca* seeds have also been reported. It has also been reported that different types of saponins triterpenoids, saponins, steroids and flavonoids are present [12]. *Celastrus paniculatus*, the plant known from centuries as "elixir of life" has got many medicinal properties. Almost all parts of the plant are used in Ayurveda to treat various diseases like gout, dyspepsia, rheumatism, insomnia, epilepsy and the oil acts as a powerful stimulant used in the treatment of scabies, body and rheumatic pain and it is even used in the treatment of wound eczema [13].

The fruit of *Semecarpus anacardium* is used as an anthelmintic, in asthma, neuralgia and psoriasis. The fruit is also used in the treatment of neurological disorders, ulcers, corn, leprosy and leucoderma. The nut of *Semecarpus anacardium* contains different types of phenolics and biflavonoids. More importantly, the presence of main constituents such as trihydroxyf-

lavone, semicarpol, anacardoside and bhilawanol has been reported. Many medicinal properties like antimicrobial, antirheumatic, anti-tumor and even in some studies anticancer potency were also reported [14].

This study aimed to investigate the activities of saponins of four important oil yielding medicinal plant extracts on selected oral pathogens, which have been known to be involved in oral and dental diseases.

MATERIALS AND METHODS

Plant materials:

The mature *Bauhinia purpurea* L seeds were collected from Gulbarga University campus, the ripened seeds of *Madhuca longifolia* were collected from Konchavaram forest, *Celastrus paniculatus* seeds were collected from Khanapur forest, Bidar and *Semecarpus anacardium* Linn seeds were collected from Karakpalli forest Bidar and all the plants were identified by the flora of Gulbarga District [15] and a voucher specimen copy was deposited in the department of Botany, Gulbarga University, Gulbarga.

Bacterial strains:

The microorganisms used in this study *Streptococcus mitis* MTCC 2696, *Streptococcus salivarius* MTCC 1938, *Lactobacillus acidophilus* MTCC 447, *Streptococcus mutans* MTCC 497, *Streptococcus mutans* MTCC 890 and *Staphylococcus aureus* MTCC 96 were obtained from the Institute of Microbial Technology Sector, Chandigarh. Bacterial cultures were maintained in Brain Heart Infusion broth (BHI) at 37°C for 24 hours.

Isolation and purification of saponins:

About 1 kg of *Bauhinia purpurea*, *Madhuca longifolia*, *Celastrus paniculatus* and *Semecarpus anacardium* seeds were milled into fine powder, the size of particles were ca. 0.2 mm and Soxhlet extracted exhaustively five times for *Bauhinia purpurea*, six times for *Madhuca*

longifolia, with 4 l of 80% methanol and seven times for *Celastrus paniculatus* and *Semecarpus anacardium* with 5 l of 80% methanol. The solvent was evaporated and a brown-color syrupy liquid (162 g, 210 g, 112 g and 65 g residue, respectively) was obtained and 30 g of each extract was separated by column chromatography (25 mm × 500 mm) on Sephadex LH-20 with gradient CHCl_3 -MeOH (methanol) solvent system. About nine fractions for *Bauhinia purpurea*, fourteen fractions for *Madhuca longifolia*, six fractions for *Celastrus paniculatus* and two fractions for *Semecarpus anacardium* were collected. Thin-layer chromatography (TLC) analysis of saponin was carried out on silica 60 F₂₅₄ (Merck, Germany) pre-coated plates, mobile phase CHCl_3 :MeOH = 95:5 and detection of saponin was done by spraying the plates with anisaldehyde-sulfuric acid reagent, saponin gives green spots. Saponin from TLC plates were scraped and extracted with 80% MeOH.

The procedure was repeated for many times till the pure samples of saponin (yellowish brown colour amorphous powder) about 4-5 g from each extract was collected. The pure saponin fractions were stored at 4°C for further experiment.

Antibacterial assay:

Agar well diffusion assay method [16] was applied to find out the zone of inhibition for the saponins of all four extracts. Microorganisms were incubated in Brain Heart Infusion (BHI) medium at 37°C for 24 h and the cultures were used to determine the zone of inhibition. Stock solution was prepared by dissolving 10 mg/ml of pure saponin and the final concentration was made to 500 µg/ml. The medium was poured into the petriplates and about 1 ml of 24 h incubated old culture was inoculated; then the plates were set at room temperature for 10 minutes. With a sterile cork borer 6 mm wells were made, the wells were filled with 100 µl of test samples. Standard

antibiotics such as tetracycline and amoxicillin, 3 µg/ml each, were used as positive and 1% dimethyl sulfoxide (DOMS) used as negative control. All the plates were incubated at 37°C for 24 hours. The zone of inhibition was measured and expressed in millimetres. All the tests were repeated thrice.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC):

Saponins extracted from *Bauhinia purpurea* and *Madhuca longifolia* were subjected to MIC and MBC; whereas, *Celastrus paniculatus* and *Semecarpus anacardium* did not show any considerable zone of inhibition; hence, the saponins of these two plants were not considered for MIC and MBC activity. The saponins of *Bauhinia purpurea* and *Madhuca longifolia* were dissolved in 1% dimethyl sulfoxide (DOMS) and transferred to the tube containing 1 ml of BHI broth and serially diluted by the two-fold dilution method resulting in the range of 1000-4 µg/ml [9, 17]. The bacterial suspension of 0.1 ml containing 2×10^5 colony forming units (CFU) per ml of test bacteria was added to each tube.

MIC values were recorded, as the lowest concentration of saponins that is required for complete inhibition of bacterial growth after 14 h of incubation at 37°C. The control was only with growth medium with no test compounds and the positive control was tetracycline and amoxicillin. All the experiments were performed in triplicate.

Bactericidal activity:

The viable cell count method was used to measure the bactericidal activity for both saponin samples. All the bacterial cultures were washed twice with 1 ml of phosphate buffer and final concentration of cells were kept at 2×10^5 CFU/ml. The saponin samples of *Bauhinia purpurea* and *Madhuca longifolia* were mixed with the bacterial culture in the ratio of 4:1 and all the tubes were incubated at 37°C for 36 h and the viability of the cells in the mixture were determined using the pore plate technique. The procedure was repeated thrice.

STATISTICAL ANALYSIS

All the experiments were done in three times. The mean and standard error of mean (SEM) were calculated for each variable.

Table 1. Antimicrobial Activity of Saponins of the Four Plant Extracts Against Oral Pathogens

Standard Drugs/Plant names	Bacterial Strains					
	Streptococcus mitis MTCC 2696	Streptococcus salivarius MTCC 1938	Streptococcus mutans MTCC 890	Staphylococcus aureus MTCC 96	Streptococcus mutans MTCC 497	Lactobacillus acidophilus MTCC 447
Tetracycline	23.0 ± 0.57	24.0 ± 0.57	22.0 ± 1.0	20.0 ± 1.15	17.0 ± 0.57	21.0 ± 0.57
Amoxicillin	21.0 ± 0.57	26.0 ± 0.0	21.0 ± 0.57	20.0 ± 0.57	19.0 ± 0.57	22.0 ± 0.57
Bauhinia purpurea	15.0 ± 1.0	18.0 ± 0.0	16.0 ± 0.57	14.0 ± 0.57	15.0 ± 0.0	20.0 ± 0.0
Madhuca longifolia	16.0 ± 0.57	21.0 ± 0.0	19.0 ± 0.0	17.0 ± 0.57	16.0 ± 0.57	22.0 ± 0.0
Celastrus paniculatus	8.0 ± 0.57	11.0 ± 0.57	6.0 ± 1.53	7.0 ± 0.57	4.0 ± 0.0	10.0 ± 1.00
Semecarpus anacardium	6.0 ± 1.0	8.0 ± 1.0	5.0 ± 1.15	6.0 ± 1.53	5.0 ± 0.57	8.0 ± 0.57

Diameter of zone of inhibition in mm. Values are mean ± SEM (standard error of mean)

The values were analyzed by one-way ANOVA, post hoc Tukey's t-test. A P-value of <0.05 was considered statistically significant.

RESULT

The antimicrobial activity of saponins of all four extracts were investigated in terms of zone of inhibition and the results are represented in Table 1. The saponins of Bauhinia purpurea and Madhuca longifolia have shown a good zone of inhibition. Madhuca longifolia saponins have shown a significant zone of inhibition against lactobacillus species (22.0 ± 0.0 mm) followed by Streptococcus salivarius (21.0 ± 0.0 mm) and Streptococcus mutans MTCC 890 (19.0 ± 0.0 mm). Bauhinia purpurea showed good inhibition on oral pathogens, but when compared to Madhuca longifolia it was a little less. Celastrus paniculatus showed some antibacterial activity against Streptococcus salivarius and Lactobacillus acidophilus.

The saponins of Semecarpus anacardium did not show any considerable antibacterial activity. This may indicate the absence of triterpenoid saponins or oleanane type triterpene in them. The MIC and MBC values of the saponins extracted from Bauhinia purpurea and Madhuca longifolia have been shown in Table 2. The least MIC/MBC values for Madhuca longifolia was against Lactobacillus acidophilus ($16.8 \pm 0.05/30.2 \pm 0.10$ µg/ml), Streptococcus salivarius ($17.2 \pm 0.10/31.4 \pm 0.25$ µg/ml), Streptococcus mutans MTCC 890 ($18.3 \pm 0.15/34.4 \pm 0.24$ µg/ml), Streptococcus mitis ($19.0 \pm 0.05/32.2 \pm 0.0$ µg/ml), Staphylococcus aureus ($21.2 \pm 0.35/39.0 \pm 0.30$ µg/ml), but Streptococcus mutans MTCC 497 showed higher MIC and MBC values. Whereas, in Bauhinia purpurea, the least MIC and MBC values were shown by Lactobacillus acidophilus ($20.2 \pm 0.05/36.8 \pm 0.23$ µg/ml), Streptococcus salivarius ($24.8 \pm 0.11/40.0 \pm 0.57$ µg/ml).

Table 2. Mean, Standard Error of Mean (SEM) Values of MIC and MBC of Bauhinia Purpurea, Madhuca Longifolia Saponins and of Tetracycline and Amoxicillin Against Oral Microorganisms

Bacterial strains	Tetracycline		Amoxicillin		Bauhinia purpurea		Madhuca longifolia	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Streptococcus mitis</i> MTCC 2696	1.0 ± 0.10	1.20 ± 0.05	0.90 ± 0.05	1.60 ± 0.10	$29.6 \pm 0.13^*$	$48.0 \pm 0.0^*$	$19.0 \pm 0.05^*$	$32.2 \pm 0.0^*$
<i>Streptococcus salivarius</i> MTCC 1938	0.8 ± 0.0	1.0 ± 0.20	0.68 ± 0.01	1.10 ± 0.05	$24.8 \pm 0.11^*$	$40.0 \pm 0.57^*$	$17.2 \pm 0.10^*$	$31.4 \pm 0.25^*$
<i>Streptococcus mutans</i> MTCC 890	1.0 ± 0.15	1.10 ± 0.10	0.60 ± 0.05	1.80 ± 0.11	$26.4 \pm 0.20^*$	$43.0 \pm 0.40^*$	$18.3 \pm 0.15^*$	$34.4 \pm 0.24^*$
<i>Staphylococcus aureus</i> MTCC 96	1.0 ± 0.20	1.23 ± 0.13	0.90 ± 0.05	2.0 ± 0.15	$29.0 \pm 0.30^*$	39.6 ± 0.12	$21.2 \pm 0.35^*$	39.0 ± 0.30
<i>Streptococcus mutans</i> MTCC 497	1.0 ± 0.20	1.20 ± 0.10	0.70 ± 0.05	1.80 ± 0.10	$26.3 \pm 0.42^*$	$38.0 \pm 0.11^*$	$23.6 \pm 0.32^*$	$39.6 \pm 0.32^*$
<i>Lactobacillus acidophilus</i> MTCC 447	1.0 ± 0.10	1.0 ± 0.05	0.50 ± 0.05	1.0 ± 0.10	$20.2 \pm 0.05^*$	$36.8 \pm 0.23^*$	$16.8 \pm 0.05^*$	$30.2 \pm 0.10^*$

All the values of MIC and MBC are given in µg/ml. Values are mean \pm SEM. *P < 0.05 is statistically significant

All other strains MIC and MBC values lie between $26.3 \pm 0.42/38.0 \pm 0.11$ $\mu\text{g/ml}$ to $29.6 \pm 0.13/48.0 \pm 0.0$ $\mu\text{g/ml}$.

DISCUSSION

There are many reports on *Streptococcus salivarius* and *Lactobacillus acidophilus* that use sugars available in the oral cavity and produce acids which cause demineralization of the tooth [18]. Antibiotics are being used continuously to avoid caries and periodontal disease which leads to overdosing of penicillin, chlorhexidine, tetracycline, amoxicillin or other antibiotics too, which cause damage to the oral flora [19, 20]. Saponins extracted from *Madhuca longifolia* and *Bauhinia purpurea* have shown a strong antibacterial activity against *Streptococcus mutans*, *Streptococcus mitis*, *Staphylococcus aureus* and *Lactobacillus acidophilus*, hence formation of plaque and caries may be effectively controlled by using these natural plant sources. More interestingly an oleanane triterpene saponin isolated from the Vietnamese plant *Maesa balansae* showed significant inhibition against drug resistant visceral *Leishmania* strains. It was also confirmed that saponins can be one of the potential natural molecules used as antileishmanial drugs [21]. When the anti-fungal activity of steroidal saponins and steroidal sapogenins isolated from several monocotyledonous plants were studied, the results proved that steroidal saponins are active against fungus even in low concentrations [22].

In a recent study when an oleanane type triterpenoid glycoside was isolated from the seeds of *Tephrosia purpurea* and antimicrobial activity was conducted against human and plant pathogenic bacteria and fungi, the MIC value was less than 50 $\mu\text{g/ml}$ which clearly indicated that saponins are one of the best natural drugs [23]. The saponins extracted from *Acacia auriculiformis* were tested on bacterial and fungal strains; the results were almost comparable [24]. The antimicrobial activity of crude and pure saponin extract from *Gymnema sylvestre*

and *Eclipta prostrata* leaves against pathogenic bacteria and fungi showed that the pure fractions of saponin have more effective bactericidal and fungicidal activity than crude extracts [25].

The strong antibacterial activity of *Madhuca longifolia* may be due to the presence of more amounts of complex triterpenoid saponins or oleanane type triterpenoid glycosides present in the saponin extract. A pentacyclic triterpenoid saponin (madhushazone), an untypical isoflavone (madhusalmone) and bis (isoflavone) were also reported [26]. In another study, a new oleanane-type triterpene glycoside (madlongisides A-D) was isolated [27]. Four new remarkable compounds (dibenz[b,f]oxepins) which are cancer cell growth inhibitors were designated as Bauhiniastatins 1-4 and extracted from leaves, stems, roots and pods of *Bauhinia purpurea* [28].

Presence of triterpenoids in the leguminosae family has been already reported. The presence of these complex molecules in the saponin extracts of *Madhuca longifolia* and *Bauhinia purpurea* may have high antibacterial activity. The antimicrobial activity of pure saponins are further supported by a pure saponin extracted from gaur meal which showed hemolytic and antibacterial activity against *Staphylococcus aureus*, *Escheria coli*, *Lactobacillus* species and *Salmonella typhimurium* [29]. Hence, it is very much evident that the complex saponin molecules are potent antibacterial agents.

CONCLUSION

Within the limits of this research, it may be concluded that the saponins extracted from *Bauhinia purpurea* and *Madhuca longifolia* have potential antibacterial activity against oral pathogens. Hence, the saponins of these plants can be used as an ingredient in chewing sticks, toothpaste or in any oral care product which may reduce the incidence of caries, periodontal problems and may help sustain a healthy oral environment.

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