ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 8, Issue 4, 2015

ISSN - 0974-2441

Research Article

ANTIBACTERIAL ACTIVITY OF *ANNONA SQUAMOSA* L. AND *ANNONA RETICULATA* L. AGAINST CLINICAL ISOLATES OF MUTANS STREPTOCOCCI THE CAUSATIVE AGENTS OF DENTAL CARIES

HAMZAH ABDULRAHMAN SALMAN*, SENTHILKUMAR R

Department of Microbiology, JJ College of Arts and Science, Pudukkottai, affiliated to Bharathidasan University, Tamil Nadu, India.
Email: hamza.alayash@gmail.com

Received: 16 April 2015, Revised and Accepted: 12 May 2015

ABSTRACT

Objective: Evaluation of antibacterial activity of *Annona squamosa* L and *Annona reticulata* L against *Streptococcus mutans* and *Streptococcus sobrinus* (mutans streptococci MS) the causative agent of dental caries.

Methods: Leaves and bark of *A. squamosa* and *A. reticulata* were collected and extracted by methanol solvent using soxhlet apparatus. Clinical samples were isolated from dental caries subjects, and identified by 16S rDNA sequencing. The isolates were tested to study the antibacterial activity of *A. squamosa* and *A. reticulata* leaves and bark by disc diffusion method. 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, and 100 mg concentration of the extract were used to study the antibacteial activity of *S. mutans* and *S. sobrinus*. Ampicillin (AMP) 10 µg was used as a positive control in all the species.

Results: The isolates were identified based on 16S rDNA sequencing as two *Streptococcus mutans* and two *Streptococcus sobrinus*. *A. squamosa* bark showed antibacterial activity against MS species, while, at 100 mg concentration leaves extract of *A. squamosa* did not show any inhibition against MS species. However, no inhibitory activity being shown in *A. reticulata* (leaves and bark) against MS species. The result indicates that *S. sobrinus* is more susceptible to the *A. squamosa* bark extract than *S. mutans*. Not much difference showed between the species in regards of different concentration.

Conclusion: *A. squamosa* bark showed antibacterial activity against MS species, further investigation of the *A. squamosa* bark should be studied to determine the anti-caries properties.

Keywords: *Annona squamosa*, *Annona reticulata*, Plant extract, Dental caries, Mutans Streptococci, Antibacterial susceptibility testing, 16S rDNA sequencing.

INTRODUCTION

Dental caries is one of the most infectious diseases in human being and is still a main public health concern in many nations [1]. Mutans streptococci (MS), *Streptococcus mutans,* and *Streptococcus sobrinus* are the most common bacteria isolated from human dental caries and it's considered the major etiologic agents of caries disease. Many studies have reported that *S. sobrinus* is less prevalent than *S. mutans* in dental caries [1,2]. However, the prevalence of *S. sobrinus* is more likely connected with a high dental caries [3].

Plants have been one of the essential sources of medicines from the start of human development. There is a developing interest in plant based medicines, health items, pharmaceuticals, nutrient supplements, beautifying agents, and so forth. As per the WHO review 80% populations living in the third world countries depend solely on conventional medication for their essential human needs [4].

The plant *Annona squamosa* and *Annona reticulata* belong to genus Annona and family Annoanaceae. *A. squamosa* commonly known as Custard apple in English and Sharifa in Hindi is cultivated all over India and other tropical countries [5]. *Annona reticulata* is likewise known as Ramphal, Bullock's heart and Custard apple [6]. Different parts of *A. reticulata* like leaves, bark, seed, and root are therapeutically helpful and they indicate numerous remedial activities as anticancer, central nervous system depressant, pain relieving, antihyperglycemic, anti-inflammatory, antiproliferative, wound healing, and antiulcer activity [7,8].

Various parts of *A. squamosa* are used in conventional remedies for the treatment of several disorders and useful for heart ailments, diabetes, hyperthyroidism, and tumor [9]. *A. squamosa* is traditionally used for the treatment of epilepsy, diarrhea, worm infestation, constipation, hemorrhage, dysuria, fever, thirst, ulcers, and also as an abortion agent [10-12].

The leaves of *A. squamosa* is reported to contain glycoside, alkaloids, saponins, flavonoids, tannins, carbohydrates, proteins, phenolic compounds, phytosterols, amino acids [13,14]. The volatile constituents of *A. squamosa* bark contains annonaine, and alkaloid which is found to have many benefits [15]. An ethanol extract of *A. squamosa* and bark is reported to have anticancer activity [16,17]. Methanolic extract of *A. squamosa* bark possesses antimicrobial activity against gram-positive and gram-negative bacteria [18]. Methanolic leaves extract of *A. reticulata* showed significant activity against *Bacillus subtilis*, *Staphylococcus aureus,* and *Vibrio alginolyticus*. In comparison to the extracts of *A. reticulata*, *A. squamosa* had strong antibacterial activity [19].

In an earlier study, there have been observed increases in antibiotic resistant strains of *S. mutans*, which have led to the emergence of new strains with a multidrug-resistant [20]. Plant products have been tested in an attempt to prevent dental caries [21,22]. However, most studies on dental caries had been performed using the strains of MS derived from Westerns countries. It is unclear if the plant products previously used would have a similar effect on the MS of Indian population. In order to test the anti-cariogenic effect of plant extracts, it would be important to evaluate the antibacterial activity against clinical strains of the MS isolated from the dental plaque obtained from Indian population.

To best of our knowledge, there is no antibacterial activity study of *A. squamosa* and *A. reticulata* against MS species been reported. Hence,

the objective of the study was to test the antibacterial effectiveness of *A. squamosa* and *A. reticulata* (leaves and bark) against MS species the causative agents of dental caries.

METHODS

Collection of plant

Leaves and bark of *A. squamosa* and *A. reticulata* were collected in the month of February from UAS university, GKVK Bangalore and authenticated by Dr. Vasundhara M. Professor of the Horticulture Department, UAS university, GKVK Bangalore, India. With an authentication number as A no. 29 and A no. 30, respectively. The leaves of both the species were separately rinsed with distilled water. Leaves and bark were kept separately in hot air oven at 50°C for complete drying. The leaves and the bark were powdered by using an electrical blender.

Extract preparation

A total of 40 g of powder was extracted with 600 ml of 100% v/v methanol using a soxhlet apparatus, the soxhlet was ran for 30 hrs at 20°C, methanolic extract then concentrated by rotary vacuum.

Bacterial isolates

The Ethical Approval of this study was taken from the Institutional Ethics Committee of PMNM Dental College, Bagalkot, Karnataka, India. Clinical isolates were isolated from dental caries subjects. Reference cultures used were *S. mutans* ATCC 25175, *S. mutans* MTCC 497, and *S. sobrinus* ATCC 33478. Dental plaques were collected from the patients and placed in sterile phosphate buffered saline (PBS) (HiMedia, India). The samples were diluted by 100-fold in 1× PBS and streaked on mitis salivarius agar (HiMedia, India) supplemented with 15% sucrose and 0.2 units of bacitracin (HiMedia, India) (MSB agar) the plates then incubated anaerobically at 37°C for 48 hrs. Out of 65 clinical samples, four isolates were selected randomly for the present study.

Genomic DNA isolation

CTAB method [23] was used for bacterial genomic DNA isolation. DNA concentration was determined by measuring the OD at 260 and 280 nm using an UV spectrophotometer (Sartorius stedim biotech, Germany).

16S rDNA sequencing

16S rDNA sequencing was done to identify the species of the isolates, the PCR was performed using universal primer 16S FP (AGA GTT TGA TCC TGG CTC AG), 16S RP (AAG GAG GTG ATC CAG CCG CA), and the following conditions: Initial denaturation 94°C for 2 minutes, denaturation 94°C for 50 seconds, annealing 48°C for 30 seconds, extension 72°C for 1 minutes 30 seconds, and final extension 72°C for 6 mintes. The sequences were submitted to National Centre for Biotechnology Information (NCBI) database to obtain GenBank accession numbers.

Disc diffusion method

5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg and 100 mg of the extract were dissolved. The sterile discs (HiMedia, India) were aseptically soaked overnight in different concentrations of plant extract. Bacterial cultures were grown in brain heart infusion broth (BHI) (HiMedia, India) at 37°C for 48 hrs, then diluted to obtain 0.5 (Marc-Farland). The cultures were swabbed on BHI agar and allowed to dry for 10 minutes, the disc containing the extract was placed on the media aseptically. The plates were anaerobically incubated at 37°C for 24 hrs. Ampicillin (AMP) 10 µg (HiMedia, India) was used as a positive control in all the species. All the experiments were performed in triplicate in order to confirm reproducibility and reliability.

RESULTS

Among the four clinical isolates, two were *S. mutans* and the other two were *S. sobrinus*, the NCBI GenBank accession numbers are KP975192, KP975193, KP975179, and KP975203, respectively.

A. squamosa bark showed an inhibition zone in both MS species in different concentrations (5-50 mg/ml), while leaf extracts of *A. squamosa* did not show any inhibition even at 100 mg/ml as shown in Fig. 1. *A. reticulata (leaves and bark)* did not possess antibacterial activity against both MS species even with higher concentration, such as 100 mg/ml as shown in Fig. 2.

Ten different concentrations of the *A. squamosa* bark were tested against MS species as shown in Table 1, Fig. 3 and Fig. 4.

DISCUSSION

A. squamosa is a multipurpose tree with edible fruits and is a source of the medicinal and industrial products. The antibacterial activities of the leaves and bark extract of *A. squamosa* and *A. reticulata* has been evaluated against MS (*S. mutans* and *S. sobrinus*).

The result indicates that 5-50 mg/ml concentration of the bark extract of *A. squamosa* showed a significant antibacterial activity against *S. mutans* and *S. sobrinus*. However, in this study, 100 mg/ml concentration of methanolic leaves extract of *A. squamosa* and *A. reticulata* did not show any inhibition zone to any isolates. No antibacterial activity of *A. reticulata* bark against *S. mutans* and *S. sobrinus* been observed either.

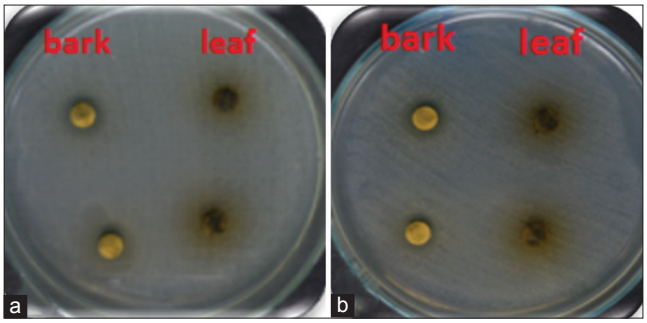

Fig. 1: Antibacterial susceptibility testing of *A. squamosa* (leaves and bark) against (a) *S. mutans* ATCC 25175, (b) *S. sobrinus ATCC 33478*

Table 1: Antibacterial activity of different concentration of *A. squamosa* bark against MS species, the zone of inhibition measured by millimeter in diameter mm

Bacterial sample number	*A. squamosa* bark (mg/ml)										Amp 10 µg
	5	10	15	20	25	30	35	40	45	50	
S. mutans MTCC 497	9	10	10	10	14	14	14	14	14	14	52
S. mutans ATCC 25175	9	9	10	11	11	11	12	12	12	12	50
S. mutans KP975192	11	12	12	12	13	13	14	14	14	14	55
S. mutans KP975193	10	10	10	10	11	11	11	11	11	11	42
S. sobrinus ATCC 33478	9	9	10	11	12	14	14	15	14	15	48
S. sobrinus KP975179	10	10	11	12	14	14	15	17	15	17	56
S. sobrinus KP975203	10	10	11	12	14	15	15	16	15	16	50

Amp: Ampicillin antibiotic

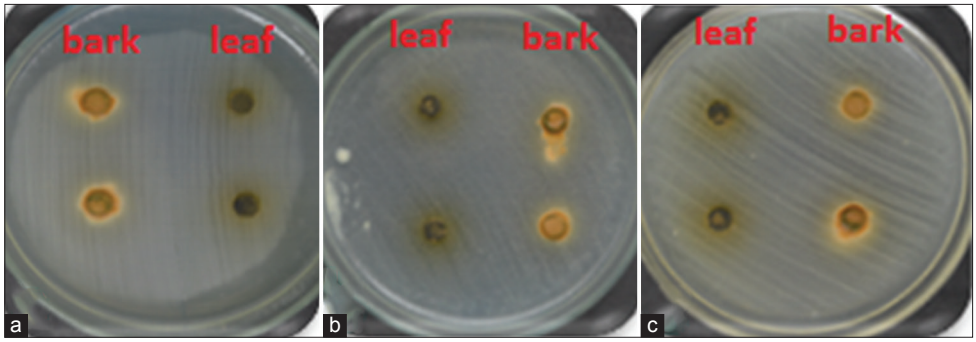

Fig. 2: Antibacterial susceptibility testing of *A. reticulata* (leaves and bark) against (a) *S. mutans* MTCC 497, (b) *S. mutans* KP975192 and (c) *S. sobrinus* ATCC 33478

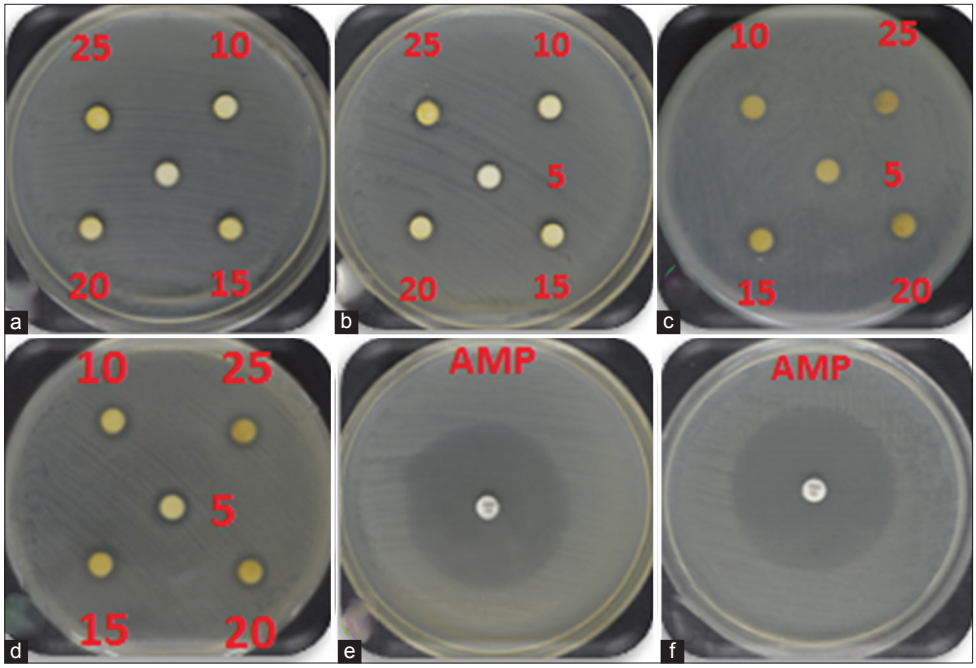

Fig. 3: Antibacterial susceptibility testing of *A. squamosa* bark against (a) *S. mutans* ATCC 25175, (b) *S. mutans* MTCC 497, (c) *S. sobrinus* ATCC 33478, (d) *S. mutans* KP975193, (e) *S. sobrinus* ATCC 33478, (f) *S. mutans* MTCC 497. AMP: ampicillin 10 unit

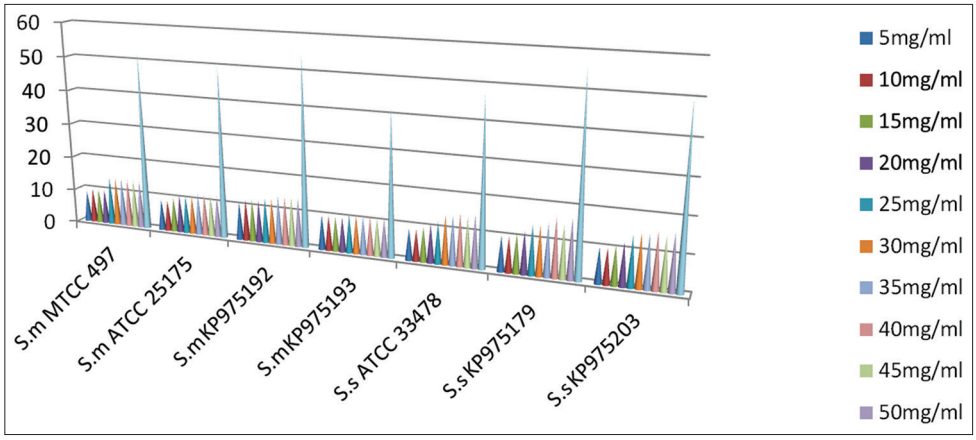

Fig. 4: Distribution of antibacterial activity values into mutans Streptococci species. S.m: S. mutans, S.s: S. sobrinus

There were differences in the susceptibility between the reference strains and the clinical isolates of MS. In addition, the antibacterial effectiveness of the bark-extract from *A. squamosa* differed among the clinical isolates, this is a similar finding with Lim *et al.* 2003 [24]. Our study revealed, *S. sobrinus* is more susceptible to the extract than *S. mutans* as shown in Table 1. Not much difference showed between the species in regards of different concentration. In contrast to an old study, the methanolic extract of *A. squamosa* and *A. reticulata* leaf showed high inhibition zone against *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. aureus*, and *V. alginolyticus* [19]. While in this study no activity been shown against *S. mutans* and *S. sobrinus*.

CONCLUSION

This study reveals the presence of antibacterial activity of *A. squamosa* bark against *S. mutans* and *S. sobrinus*. These results suggest that *A. squamosa* bark could be employed as a potent antibacterial agent for preventing dental caries. These extracts might be added in toothpaste or mouth wash to prevent its decay. The results suggest that traditional remedies may lead to treatment of dental caries.

ACKNOWLEDMENTS

The authors like to thank Dr. Vasundhara M. and Mrs. Ashwini Jayaram, Horticulture Department, UAS university, GKVK Bangalore, India, for their help.

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