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Original Article

Dentistry Section

Anti-microbial Efficacy of Soursop Leaf Extract (*Annona muricata*) on Oral Pathogens: An In-vitro Study

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ABSTRACT

Introduction: *Annona muricata* also called as Soursop is a, flowering evergreen tree native to Mexico, Cuba, Central America and parts of India. The miracle tree as it is widely known as a natural cancer killer that is 10,000 times stronger than chemotherapy. Based on these miraculous claims, the leaves of these plants were used as an extract at varying concentrations as an antibacterial agent against oral pathogens.

Aim: The aim of the study was to assess antimicrobial efficacy of Soursop leaf extarct (*Annona muricata*) on *Streptococcus mutans*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Candida albicans* using disc diffusion method.

Materials and Methods: Extracts of *Annona muricata* leaves of concentrations of 1%, 5%, 10%, 15% and 20% were prepared. The anti-microbial efficacy was evaluated using disc diffusion method against *Streptococcus mutans*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Candida albicans* on agar plates.

Results: All concentrations of extracts were effective on the microbiota except for the *P. Intermedia*. The Soursop extract was highly effective on *Candida* species, with all concentrations exhibiting bactericidal and fungicidal property. The extracts at different concentration were effective when compared to the gold standard controls and the effect was statistically significant ($p<0.05$). Data obtained was analysed using one way analysis of variance (ANOVA) and Tukey's post-hoc test.

Conclusion: The Soursop extracts were efficient for all test organisms expect *P. intermedia*. The present study demonstrated the in-vitro efficacy of Soursop was highest against *S. mutans* followed by *C. albicans* and least on *P. intermedia*. Hence, this study proves to an extent that the Soursop extract when used against oral microbiota has sufficient anti-microbial and fungicidal property.

Keywords: Dental caries, Periodontal disease, *Streptococcus mutans*

INTRODUCTION

The use of plants as medicine is a worldwide phenomenon; plants not only provide safe and cost effective remedies, they are also available and accessible at affordable prices. The use of resources already available, forms the basic core of any public health practice and what is better than plants as medicine as they are associated with fewer side effects and no known resistance to microorganisms [1]. Ethno medicine may be broadly defined as the use of plants by humans as medicines but can be more accurately called ethnobotanic medicine [2].

Ethno medicine has its roots in treatment of dental caries and periodontal disease. This has been well practiced in traditional medicine of various civilizations like Indian, Egyptian, Greek and Chinese [3]. The number of higher plant species on this planet is estimated at 250,000 with a lower level at 215,000 and an upper level as high as 500,000. Of these, only about 6% have been screened for biologic activity and a reported 15% have been evaluated phytochemically [2]. This provides an avenue for newer search among plant kingdom for alternatives to traditional therapies and *Annona muricata* commonly called as Soursop is gaining world wide acclaim for being a miracle tree in the field of cancer research and can pave way for research in many fields, including dentistry.

Annona muricata is a flowering evergreen tree native to Mexico, Cuba, Central America and parts of India. The miracle tree as it is widely known as a natural cancer killer that is 10,000 times stronger than chemotherapy, based on these miraculous claims, the leaves of this plant were used as an extract at varying concentrations as an antibacterial agent against oral pathogens [4]. The use of this plant in medicine has again come to fore as researchers are claiming it to have potential against common pathogen [5]. However, historically ethnomedicine, to an extent has been providing solutions to problems related to human diseases. Hence, Soursop with its miraculous properties was used in this study with an intention to find newer use of these miracle plants.

Oral diseases are multifactorial. There is a strong association between oral diseases and microbial involvement. More than 700 phylotypes of bacterial species, of which over 50% have not been cultivated, of which many are associated with oral and paraoral diseases [6]. Accordingly, the levels of periodontal pathogens *P. gingivalis*, *P. intermedia* are increased in periodontitis where as *P. gingivalis* is present in severe adult periodontitis, causing failure of guided tissue regeneration and acute periodontal abscesses [7]. *Prevotella intermedia*, gram-negative anaerobic bacteria, has been implicated as a conjectural periodontal pathogen present in patients with early periodontitis, advanced periodontitis, and Acute Necrotizing Ulcerative Gingivitis (ANUG). The disease of the periodontium spell a greater health risk than assumed earlier as, clear associations between periodontal disease are presently considered as a risk factor for many systemic diseases important among them being coronary heart disease [8]. *Streptococcus* species have an association and plays a vital role in initiation and progression of caries [9]. *Streptococcus* species have been evaluated mainly for their role in dental diseases and various phyto extracts have been evaluated for their microbial properties [10,11]. Various studies have shown that *C. albicans* has capacity

to demineralise enamel similar to that of *S. mutans* and has been implicated in several periodontal diseases [11]. Polyphenols and tannins extracted from different naturally available products help in prevention of oral diseases, particularly those related to dental biofilms [11,12].

The literature presently has inadequate proof on the use of Soursop leaf extract (*Annona muricata*) on systemic pathogens, let alone oral pathogens. Hence, the present study is an archetypal report of the anti-microbial potential of Soursop on the common oral pathogens using an in-vitro study model, with the objective to asses anti-microbial efficacy of Soursop leaf extarct (*Annona muricata*) on *Streptococcus mutans*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Candida albicans* using disc diffusion method at concentrations of 1%, 5%, 10%, 15% and 20% and to compare the anti-microbial efficacy of different concentrations of Soursop leaf extract (*Annona muricata*) with chlorhexidine.

MATERIALS AND METHODS

The present in-vitro study was conducted in the Department of Public Health Dentistry, Manipal College of Dental Sciences, Mangaluru, Karnataka, India, after obtaining the ethical approval from the Institutional Ethics Committee (Ref. No. 13102).

Collection of materials: The Soursop leaf (*Annona muricata*) was obtained from the trees from the surroundings of Mangaluru city in the month of November. After washing the leaf they were separated from the twigs and dried in shade for further catharsis. The leaves were then grounded to dry coarse powder using a homogenizer and about 500 grams of dry course powder was obtained. Specimens were identified by a pharmacognosist and a botanist for their authenticity. The study was conducted over a period of two months in the month of November 2015 and December 2015.

Pure cultures of *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. mitis*, and *C. albicans* were obtained from the Department of Microbiology, Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belgaum, Karnataka, India.

Preparation of extracts: Aqueous extracts of the Soursop leaf were prepared in the Department of Pharmacology, Kasturba Medical College, Mangaluru, Karnataka, India. The procedure of aqueous decoction followed to prepare the extracts of Soursop leaf was based on a previous study by Pai et al., [11]. The aqueous decoctions were prepared by adding 10 grams of Soursop leaf in 100ml sterile distilled water and boiled over a low flame for 15 minutes. The flasks were then plugged, removed from heat and allowed to cool for 45 minutes. After cooling, the contents of the flasks were filtered with double filter paper and sterile filters to remove any impurities [3,11].

Concentrations of 1%, 5%, 10%, 15% and 20% of extract were prepared using sterile distilled water. The extracts were stored separately in sterile air tight containers and labeled accordingly. These containers were stored in refrigerator and transported for microbiological assays. A positive control Chlorhexidine Gluconate (0.2%) was used, with benchmark controls of standard anti-microbials in disc diffusion for comparison. All the samples were stored in a refrigerator at 4ºC until the analyses were accomplished.

Anti-microbial tests: Agar disc diffusion method was used to determine the in-vitro antimicrobial activity of Soursop extract. The following media were used to determine anti-microbial property of Soursop leaf extracts.

i. For *P. gingivalis* and *P. intermedia* - Blood agar
ii. For *S. mutans* and *S. mitis* - Brain Heart Infusion agar
iii. For *C. albicans* - Sabouraud's Dextrose Agar (SDA)

The Swab method was used to transfer the colonies on to the agar plates. A visually adjusted turbidity with the broth to equal that of a 0.5 McFarland turbidity standard was vortexed. The inoculum was taken, a sterile cotton swab was dipped and rotated against the tube wall to remove excess inoculum, within 15 minutes. Uniform distribution was obtained by swabbing the entire surface of agar plate thrice in order to ensure uniform distribution. Previously, prepared extract impregnated disc (6mm in diameter) at the concentrations of 30µg/ml for bacterial and 30µg/ml for fungal strains were placed aseptically. Plates were incubated at 37 ºC in an incubator for 24 hours for aerobic bacteria. *P. gingivalis* and *P. intermedia* (anaerobic bacteria), the blood agar plates were incubated in the anaerobic jar and the jar was kept in the incubator for 48 hours [13,14].

After the incubation period, plates were read only if the lawn of growth was coalescent or nearly coalescent. The diameter of inhibition zone was measured to the nearest whole millimetre by using a Vernier's calliper. The cultures were done multiple times (a minimum of five mean values obtained) for all concentrations of extract on all the four bacteria and the mean values were obtained. These means of every extract were compared with the values of their respective benchmark controls (Ciprofloxacin/ Fluconazole) and with the positive control (Chlorhexidine 0.2%). A single calibrated examiner performed all measurements and a high kappa value was obtained.

STATISTICAL ANALYSIS

SPSS software was used for statistical analysis (version 16). The disc diffusion values of all the different concentrations were entered in the SPSS software for statistical analysis. Descriptive statistics were retrieved and data was analysed using one way analysis of variance (ANOVA) and Tukey's post-hoc test. Statistical significance level was established at $p<0.05$.

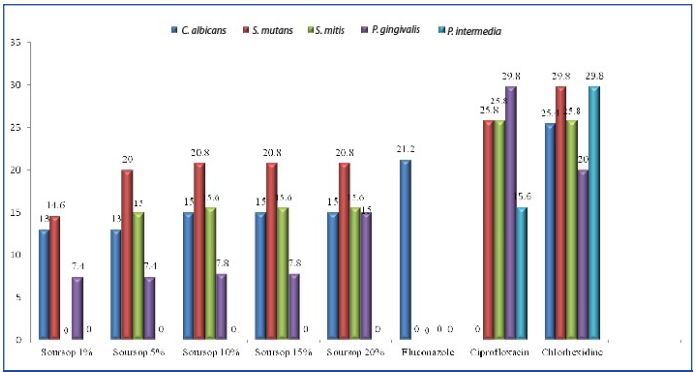
RESULTS

The results of the various concentration aqueous extracts of the Soursop leaf on the test microorganisms have been tabulated in [Table/Fig-1].

All concentrations of extracts were effective on the microbiota except on *P. intermedia*. The Soursop extract was highly effective on *Candida* species, with all concentrations exhibiting fungicidal property. The Soursop extracts showed moderate efficacy against the cariogenic bacteria *S. mutans* and *S. mitis* ($p = 0.01$). The best efficacy was demonstrated against *S. mutans* that is the most commonly associated bacterium with caries. But the overall effect of the extracts was underwhelming on perio-pathogens.

Concentrations	*C. albicans*	*S. mutans*	*S. mitis*	*P. gingivalis*	*P. intermedia*
Soursop 1%	13 [0.21]	14.6 [1.51]	R	7.4 [1.14]	R
Soursop 5%	13 [0.23]	20 [1.58]	15 [0.00]	7.4 [1.14]	R
Soursop 10%	15 [1.22]	20.8 [2.38]	15.6 [2.07]	7.8 [1.64]	R
Soursop 15%	15 [1.22]	20.8 [2.38]	15.6 [2.07]	7.8 [1.64]	R
Soursop 20%	15 [1.22]	20.8 [2.38]	15.6 [2.07]	15 [1.22]	R
Fluconazole	21.2 [1.64]*	NA	NA	NA	NA
Ciprofloxacin	NA	25.8 [2.77]*	25.8 [2.77]*	29.8 [3.27]*	15.6 [0.54]*
Chlorhexidine	25.4 [5.02]*	29.8 [3.27]*	25.8 [2.77]*	20 [3.67]*	29.8 [3.27]*

[Table/Fig-1]: Mean values of zones of inhibition of Soursop extracts, 0.2% Chlorhexidine, Gold standard (fluconazole/ciprofloxacin) in millimetres (standard deviation in parenthesis).
* Statistically significant (p value< 0.05). R- resistant; † NA- not applicable;



[Table/Fig-2]: Mean efficacy values of Soursop extracts, respective Gold standard and positive control (Chlorhexidine 0.2%) on oral pathogens.

Soursop extract showed sufficient activity against *P. gingivalis* with maximum activity demonstrated at twenty percent concentration. *P. Intermedia* were resistant to the extracts at all concentrations. However, it was observed that the antibacterial efficacy of all the extracts increased with an increase in its concentration. Comparison of mean efficacy values of 20% extracts with chlorhexidine 0.2% and the respective gold standards (ciprofloxacin/fluconazole) has been shown in [Table/Fig-2]. The extracts at different concentration were effective when compared to the gold standard controls and the effect was statistically significant ($p<0.05$).

DISCUSSION

The 'magic bullet' introduced by Paul Ehrlich, due to pioneered the so called anti-microbial era, but with time and due to illogical use of this anti-microbial therapy the microorganisms are slowly getting resistant to these agents [15]. The required effect of an antibiotic is to expunge or prevent the growth of pathogenic microorganisms, but these drugs also impact the host in a deleterious manner. Generalized adverse events are common to most antibiotics, paving way to other modes of treatment and therapies.

Plants as medicines are being used from time immemorial. The main advantages of using plants as alternative medicine include its diversity and flexibility of use, their availability and affordability in the region and mainly to reduce adverse reactions. The widespread acceptance of plants in low- and middle-income countries, its comparatively low cost and the relatively low level of technological input required, make them the ideal alternative to costly therapies. Hence, plant extracts may prove to be better and safer alternatives if they are supported by scientifically based evidence [16,17].

Many commonly used natural products like neem stick, pomegranate and tulsi have been tested for their antibacterial properties. Most of these plants used for traditional medicines are grown locally and have been used for centuries as medicine in those regional areas. Also, resistance of pathogenic bacteria to currently used antibiotics and chemotherapeutics has increased the global requirement for alternative safe, efficacious and cost-effective treatment options for infections, particularly in developing countries [3].

The use of Soursop extract on microorganisms has a strong traditional foundation; many countries in the world use this extract for treatment of various diseases. In countries like Peru, Brazil and Togo the extracts have been used for various treatments such as liver disorders, diarrhoea, dysentery, fevers, hypertension, sores, internal ulcers and diabetes. These various curative properties and its effect against cancer prompted the authors of the present study to investigate into the effect of Soursop extract, on oral microbiota [17].

The anti-microbial efficacy of the extracts of Soursop have been investigated in the past but to a very less extent, very few studies have been found on the effect of these extracts. The microorganisms on which these extracts were evaluated by Vieira et al., *Staphylococcus aureus, Vibrio cholerae, Escherichia coli* and *Salmonella spp*., were susceptible to the extracts and gram positive bacteria showed higher zones of inhibition than that of gram negative bacteria, this may form the basis of the present study too where the extracts were less effective on *P. gingivalis* and *P. Intermedia* was completely resistant to these extracts which may be due to cell wall in gram negative bacteria that act as barrier for diffusion of the extracts into them thus, rendering them ineffective [18].

The anti-fungal efficacy of Soursop extracts have been evaluated on a much larger degree than its antibacterial properties. The anti-fungal property of Soursop extract is comparatively higher than its antibacterial efficacy, as at all concentrations the extract showed potent anti-fungal property against *Candida albicans*, which substantiates the previous findings of Jonny et al., and Donati M et al., [19,20].

The anti-microbial activity of Soursop extracts have never been evaluated on oral microorganisms; hence, it is difficult to compare its efficacy to that of any other extract; therefore, a positive control Chlorhexidine Gluconate was used, as an effective scale to compare the same. The most effective concentration of Soursop extract was 20%, the effects of this extract was much less than that of the gold standard control in both the cases (i.e., Ciprofloxacin for bacteria and fluconozole for *Candida*), but were nearly similar to that of Chlorhexidine Gluconate, which paves way for further analysis of the same at higher concentration.

The mode of action of Soursop extract against microorganisms is presently unknown but the common mechanism as to how they act against microbes, insects, and herbivores in their natural environment might prevail.

Biologically as to what makes Soursop potent against micro-organisms is the presence of acetogenins. These are bioactive compounds found in the annonacea family, these acetogenins, are known to have tumoricidal, anti-malarial, anti-helmintic, anti-viral, and anti-microbial effects, suggesting many potentially useful application. Of the annonaceous-acetogenins, bullatacin, an acetogenin is a powerful tumoricidal and antibacterial agent [21].

The current concentrations of extracts used were just anecdotal. For, it remains a matter of consensus whether an increase in concentration would prove to be better than chlorhexidine 0.2% and/or gold standard or the 'ceiling effect' as seen in most of the therapeutic drugs, might come into focus. Hence, it is difficult to conclude without investigating the efficacy of higher concentrations of these extracts, that *Annona muricata* would make any significant difference in oral microbial activity. Assuming that an increase in concentration improves efficacy, most of the problems encountered with the use of synthetic drugs and chemicals might tend to taper [3]. Hence, it might open new avenues for research in treating patients who might be undergoing chemotherapy and radiotherapy for oral cancer as it shows both anti-cancer and antimicrobial activity.

LIMITATION

The study has following limitations; the following is an in-vitro study and as it is an exploratory study the concentration of extracts were anecdotal. The suggested dose and response in humans may vary. This study was done to see if a line of research is on Soursop on oral pathogens was worth pursuing, hence, studies with higher evidence can be pursued to prove the that Soursop is really miraculous.

CONCLUSION

The Soursop extracts were efficient for all test organisms expect *P. intermedia*. The present study demonstrated the in-vitro efficacy

of Soursop was highest against *S. mutans* followed by *C. albicans* and least on *P. intermedia*. Hence, this study proves to an extent that the Soursop extract when used against oral microbiota has sufficient anti-microbial and fungicidal property. Thus, going to nature for finding cure now has become an essential part of medicine, natural products that are also available at homes of individuals might be an adjuvant to empirical therapy, as best use of resources that are already available might be the solutions going forward.

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