Role of Aqueous Extract of *Morinda Citrifolia* (Indian Noni) Ripe Fruits in Inhibiting Dental Caries-Causing Streptococcus Mutans and Streptococcus Mitis

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Abstract

**Objectives:** Use of alternative medicine to control oral streptococci is a new topic worthy of further investigation. This study aimed to elucidate the dose-dependent anti-bacterial activity of crude aqueous extract of ripe *Morinda citrifolia* L. (Family: Rubiaceae) fruits against oral streptococci i.e. *Streptococcus mutans* and *Streptococcus mitis*, that cause dental caries in humans.

**Methods:** Fresh ripe *M. citrifolia* fruits (750g) were ground in an electronic blender with sterile water (500ml). The crude aqueous extract was lyophilized to yield a brown colored powder. Various concentrations (1000-100µg/ml) of the extract were tested for its anti-bacterial activity (Kirby and Bauer method) against whole cells of *S. mutans* and *S. mitis*. Minimum Inhibitory Concentration (MIC) was determined by micro-dilution method, using serially diluted (2 folds) fruit extract, according to the National Committee for Clinical Laboratory Standards (NCCLS).

**Results:** Crude aqueous extract (1000µg/ml) of ripe *M. citrifolia* fruits effectively inhibited the growth of *S. mutans* (19±0.5 mm) and *S. mitis* (18.6±0.3 mm) compared to the streptomycin control (21.6±0.3 mm). The growth inhibition was clearly evident with “nil” bacteriostasis, even after 48 hours of incubation at 37°C. The MIC of the extract for *S. mutans* and *S. mitis* was 125 µg and 62.5 µg, respectively.

**Conclusion:** Our results suggest that phytochemicals naturally synthesized by *M. citrifolia* have an inhibitory effect on oral streptococci. Furthermore, purification and molecular characterization of the “bioactive principle” would enable us to formulate a sustainable oral hygiene product.

**Key Words:** Morinda citrifolia L.; Streptococcus mutans; Streptococcus; mitis; Anti-bacterial activity

INTRODUCTION

Infections of the oral cavity result from the loss of equilibrium between the hosts’ immune response and virulence factors of the indigenous microbiota [1, 2]. Irrespective of the advancements in medicine, these infections continue to pose a threat to public health, and put a heavy burden on health care services global-
ly. This is particularly true in developing countries [3, 4].

Despite general advances in the overall health, including oral and dental health of the people living in industrialized countries, the prevalence of dental caries in school aged children is close to 90% and majority of adults are also affected [5]. There is evidence linking poor oral health and systemic diseases, such as cardiovascular diseases, rheumatoid arthritis, and osteoporosis [6], while periodontal diseases may also contribute to the risk of pregnancy complications such as preterm low-birth weight [7]. Furthermore, tooth loss caused by poor periodontal health can lead to significant morbidity and premature death [8]. The link between oral infections and the activities of microbial species that form part of the microbiota of the oral cavity is well established [9]. Around 750 bacterial species colonize the oral cavity, out of which 50% are yet to be identified. Interestingly many of these bacteria are associated with oral diseases. The progression of dental caries is governed by acidogenic and aciduric gram-positive bacteria like S. mutans, lactobacilli and actinomycetes, which convert sucrose to organic acids, particularly lactic acid, that dissolve the calcium phosphate present in teeth and eventually lead to decalcification and tooth decay [10]. Several agents are available that can alter the profile of oral microflora but can cause undesirable contraindications such as vomiting, diarrhea and staining [11, 12]. Since ancient times medicinal plants have been utilized for oral hygiene. Scientific evaluation of several herbs has been undertaken against oral streptococci. Crude aqueous twig extract (50%) of Mangifera indica L. effectively inhibits the growth of S. mutans and S. mitis [13]. Among the plants in Rubiaceae family, Isertia laevis inhibits S. mutans and S. sobrinus growth with a MIC of 2mg/ ml [14]. Similarly, the adherence of S. mutans ATCC 35688 to dental enamel and dentine was greatly reduced after treatment with boiled aqueous extract of Coffea arabica L. [15]. Morinda citrifolia, popularly known as noni, has been an important medicinal plant for many centuries throughout the south pacific and has been used in folk remedies by Polynesians for over 2000 years [16]. It is a small shrub and its potential therapeutic properties remain vastly unknown [17]. It is reported to have anti-microbial, analgesic, hypotensive, anti-inflammatory and immunomodulatory properties [18, 19]. In traditional medicine, M. citrifolia fruit juice has been used to treat various illnesses including arthritis, diabetes, muscular aches, menstrual cramps, cardiac diseases, cancers, gastric ulcers, vascular diseases, and drug addiction. Nevertheless, the inhibitory activity of M. citrifolia fruits against predominant caries causing bacteria, S. mutans and S. mitis, has not been evaluated. Therefore the objective of this study was to evaluate the inhibitory effect of M. citrifolia fruits on development of dental caries caused by S. mutans and S. mitis.

MATERIALS AND METHODS

Plant Collection and Extraction

Fresh ripe M. citrifolia fruits were collected in December (Temperature: 95˚F, Humidity: 35%). Seven hundred fifty grams of the fruit were ground in an electronic blender that was sterilized with 70% ethanol. Five hundred milliliters of sterile water were added to this pulp and the mixture was soaked for 48 hours in vitro, at room temperature. The slurry was filtered using filter paper (Whatman®, need to insert the location of the manufacturer here) and the extract was condensed in a lyophilizer (Martin Christ-alpha 1-2 LD plus, location of the manufacturer) at -55°C under 0.25 mbar pressure for 72 hours. The powdered extract was stored at -20°C till usage for up to 3 months.

Microorganism Source

The microbial strains used for this study were procured from the Institute of Microbial Technology, Chandigarh (Streptococcus mutans...
Preparation of Inoculum
Stock cultures of the bacterial pathogens were maintained at 4°C on nutrient rich agar slants. Inoculum culture for the bioassay was prepared by transferring a loop full of cells from the stock culture to test tubes containing sterile Mueller-Hinton broth (HiMedia®, insert the location of the manufacturer here) that was incubated in an incubator at 37ºC for 24 hours. Prior to the assay, the turbidity of the cultures was adjusted as per McFarland standard (0.5), using sterile Mueller-Hinton broth.

Fortification of discs
Sterile Whatman filter paper discs (HiMedia®, insert the location of the manufacturer here) were segregated in a pre-sterilized petri dish. Different concentrations of the extract, including 1000, 500, 250 and 100µg/20µl were prepared with DMSO (Dimethyl sulfoxide) and loaded on the discs and dried in a vertical air draft for three hours to remove residual solvent.

Disc-diffusion Bioassay Method (20)
Sterile Muller Hinton Agar (MHA) medium was prepared and dispensed in sterile petri dishes (30ml/ dish). After solidification, 100 µl of the bacterial inoculum was added to the plate using a micropipette (Finnpipette®, insert the location of the manufacturer), and was evenly spread with the aid of a sterilized glass spreader. Extract-fortified discs were placed on the plate using sterile forceps. The plates were incubated for 24 hours at 37°C. Growth inhibition was determined by measuring the diameter of the zones of inhibition using a meter scale. This procedure was repeated three times for both organisms (S. mutans, S. mitis) and the average values were calculated. Bacteriostasis (emergence of resistant colonies) was observed by continued incubation of the assay plates for another 24 hours at 37°C.

Results
Zone of inhibition
Different concentrations of the M. citrifolia ripe fruit extract including 1000, 500, 250 and 100 µg were analysed for their action against S. mutans and S. mitis and were compared with the standard antibiotic streptomycin. Crude aqueous extract of the ripe M. citrifolia fruits at 1000 µg/ml concentration effectively inhibited the growth of S. mutans (19±0.5 mm) and S. mitis (18.6±0.3 mm) compared to the positive control streptomycin (21.6±0.3 mm). Extracts at 100 µg/ml, 250 µg/ml and 500 µg/ml showed zones of inhibition of 8.3 mm, 9.6 mm, 15.3 mm for S. mutans and 7.3 mm, 9.6 mm, 14 mm for S. mitis, respectively. Thus, the inhibition pattern was found to be dose-dependent and increased with increased concentration of the extract against both organisms (Table 1).
The bar graph depicted for the different concentrations of M. citrifolia extract and the control shows this pattern clearly (Figure 1).

**Minimum inhibitory concentration**

Growth inhibition was clearly evident with "nil" bacteriostasis, even after 48 hours of incubation at 37°C (Figure 2).

**Table 1.** Anti-microbial activity of aqueous fruit (ripe) extract of *M. citrifolia* L.

| S. No | Microorganism      | Concentration of the Extract | Zone of Inhibition (in mm) | Standard Deviation | Standard Error |
|-------|---------------------|------------------------------|---------------------------|--------------------|----------------|
|       |                     | 1000 µg                      | 18.6±0.3                  | 0.58               | 0.33           |
|       |                     | 500 µg                       | 14.0±0.5                  | 1.00               | 0.58           |
|       |                     | 250 µg                       | 9.6±0.3                   | 0.58               | 0.33           |
|       |                     | 100 µg                       | 7.3±0.3                   | 0.58               | 0.33           |
| 1     | *Streptococcus mitis* | Streptomycin (100µg)         | 21.6±0.3                  | 0.58               | 0.33           |
|       |                     | DMSO Control                 | -                         | -                  | -              |
|       |                     | 1000 µg                      | 19.0±0.5                  | 1.00               | 0.58           |
|       |                     | 500 µg                       | 15.3±0.6                  | 1.15               | 0.67           |
|       |                     | 250 µg                       | 9.6±0.3                   | 0.58               | 0.33           |
|       |                     | 100 µg                       | 8.3±0.3                   | 0.58               | 0.33           |
| 2     | *Streptococcus mutans* | Streptomycin (100µg)         | 21.0±0.5                  | 1.00               | 0.58           |
|       |                     | DMSO Control                 | -                         | -                  | -              |

*The average of triplicates is measured for the zone of inhibition*
The MIC was checked with different concentrations of the extract including 1 mg, 500 µg, 250 µg, 125 µg, 62.5 µg, 31.25 µg, 15.62 µg and 7.81 µg/ml. The MIC values of the extract for S. mutans and S. mitis were found to be 125 µg and 62.5 µg, respectively. An independent t-test was done to compare the action of M. citrifolia extract and streptomycin against S. mutans. The P-value was found to be 0.64, proving that there was no significant difference in mean value between M. citrifolia extracts and streptomycin against S. mutans.

DISCUSSION
Dental caries are a major health concern throughout the world due to common issues such as socioeconomic factors, immigration, lack of preventive efforts and dietary changes. Therefore, we are in need of new and renewed efforts to cut down the drastic increases in dental caries [21]. Our aim was to find an herbal anti-caries agent which would effectively replace the commercially available agents. M. citrifolia was chosen because of its well-known antimicrobial and therapeutic properties.

It is said to be an underutilised miracle plant that grows naturally in most geographical conditions, even without proper care and it is now being cultivated by the farmers as a crop in different parts of India [22]. Recent studies have shown that M. citrifolia has a wide array of biologically active compounds. Around 160 phytochemical compounds have been isolated from the M. citrifolia plant, the majority of which are organic acids, phenolic compounds, and alkaloids. Among the phenolic compounds, the most important ones are anthraquinones, aucubin, asperuloside, and scopoletin [23]. M. citrifolia is reported to have antibacterial, antiviral, antifungal, antitumor, antihelmintic, analgesic, hypotensive, anti-inflammatory, and immune enhancing properties [23-25]. Therefore, M. citrifolia can be regarded as a valuable medicinal plant and a possible source for modern drug development.

Morinda citrifolia has also been demonstrated to be effective in removing smear layers in endodontically treated teeth. In a trial by Murray, M. citrifolia was more effective than chlorhexidine in removing the smear layer.
The efficacy of M. citrifolia was similar to sodium hypochloride (NaOCl) in conjunction with EDTA as an intracanal irrigant. Morinda citrifolia appears to be one of the first fruits to be identified as a possible alternative to NaOCl as an intracanal irrigant [26]. A recent small trial in eleven patients has shown that the combination of good oral hygiene and administration of M. citrifolia juice is a promising treatment for reducing bleeding caused by probing. Similarly, additional treatment with M. citrifolia juice significantly alleviated the gingival inflammation [27]. Morinda citrifolia has also been shown to have in vitro antibacterial activity against different strains of several oral bacterial pathogens isolated from different sources [28]. However, scientific studies on the anti-cariogenic properties of M. citrifolia remain scarce.

Crude mixtures of phytochemicals from natural products have been routinely evaluated against oral streptococci, specifically S. mutans and S. mitis. Ethanol and diethyl ether extracts (30mg/ml) of Nigella sativa L. (Black seed) inhibited the growth of S. mutans (12.7 ±2.1 and 6.3±0.6 mm) and S. mitis (10.4 ±0.9 and 5.1±0.6 mm), respectively [29]. Chava et al. proved that crude aqueous extract of Azadirachta indica L. twigs at 50% concentration inhibited the growth of S. mutans (4.6mm), S. mitis (3.1mm), S. salivarius (2.3mm) and S. sanguis (3.2mm) at 48 hours respectively [30]. In the present study, 1000 µg (1mg/disc) of M. citrifolia fruit extract demonstrated superior inhibitory activity against S. mutans that is five times (19.0±0.5mm) as effective as that of A. indica extract. It has been reported that M. citrifolia L. inhibits pathogenic bacteria such as S. aureus, Pseudomonas aeruginosa, Proteus morgaii, Bacillus subtilis, E. coli, Helicobacter pylori, Salmonella species and Shigella species. This might be attributed to the presence of secondary metabolic phenolic compounds such as acubin, L-asperuloside, alizarin and anthraquinones including scopoletin [31]. Another study demonstrated that crude acetonitrile extract of the dried fruit was bactericidal against P. aeruginosa, Bacillus subtilis, E.coli, and S. pyogenes [32]. Ethanol and hexane extracts of M. citrifolia L. inhibit 89-95% of Mycobacterium tuberculosis species [33]. Other studies have reported a significant antimicrobial effect on various strains of Salmonella, Shigella, and E. coli [34]. Nevertheless, the inhibitory activity against cariogenic S. mutans and S. mitis has not been proven previously. Furthermore, reports have shown that this anti-microbial activity is highly dependent on the stage of ripeness of the fruit and its processing, showing greater activity when the fruit is ripe and undried. This is in agreement with our observation in oral streptococci.

Base on our results, further purification and formulation of the extract would certainly pave the way for an antibacterial mouth wash/rinse that is easily available and could be used safely. Chloride, chlorhexidine, fluoride, and fluoride-containing agents can cause tooth staining, and ethanol, the main ingredient of the common mouthwash, can cause oral cancer [35]. Hence, the search for alternative products still continues and phytochemicals used in traditional medicine should be considered as viable alternatives to synthetic products.

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