In vitro bactericidal activity of lyophilized ethanolic extract of Indian almond (Terminalia catappa Linn.) fruit pulp on two pathogenic bacteria from subgingival plaques

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The present study is on antibacterial activity of lyophilized ethanolic extract of unripe fresh fruit pulp (mesocarp part) of Terminalia catappa Linn. on two subgingival pathogenic bacteria (Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis) isolated from the outpatients reporting to a Government-aided hospital, at Chennai, India. These bacteria were isolated from a pooled blood sample collected from infected gingival, using a sterile paper point. The sample was inoculated into a test tube containing transport media (Brain Heart Infusion Broth) and kept at 4°C. Tryptic Soy Serum Broth (TSBV) was used to cultivate A. actinomycetemcomitans and blood agar supplemented with haemin and vitamin was the enriched-cum-selective medium used to cultivate P. gingivalis. Sensitivity of the isolated bacteria to standard antibiotic namely Amoxicillin, Penicillin, Gentamycin, Norfloxacin, Metranidazole, Novobiocin, Nalidixic acid and Vancomycin was estimated. The MIC of Seitz-filtered lyophilized ethanolic extract for both the anaerobic bacteria was estimated as 500 µg/ml.

Keywords: Indian almond, Terminalia catappa, Antibiotics, Oral anaerobes, Subgingivitis, Lyophilized extract.

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Introduction
Growing worldwide interest on utilization of medicinal plants for treatment and cure of microbial diseases unifies all the countries to evaluate potential medicinal plants described in traditional system of medicine by modern assays. Terminalia catappa Linn. belongs to the family of Combretaceae which is found in wild in the Andaman Islands and in the Malay Peninsula along the coastal forests. It is extensively grown tree in India and Myanmar as an avenue tree. Indian almond, Bengal almond, Singapore almond, Malabar almond, Tropical almond, Sea almond, Talisay tree, Umbrella tree are the common English names of the tree. The tree has corky, light fruit and the nut within the fruit is edible when fully ripe, tasting almost like an almond but underutilized by humans however, fruits are eaten by birds and bats. The fruit pulp is sweet, acrid, cooling astringent to the bowels and is useful in biliousness and bronchitis1. The juice of young leaves is used in South India to prepare an ointment for scabies, leprosy and other cutaneous diseases2.

The potential medicinal use of this tree has not been fully studied and hence it is an underutilized plant compared to its related species Terminalia chebula Retz. Kluck et al studied the antibacterial activity of ethanolic extract from nine Peruvian medicinal plants among which Phyllanthus amarus Schum. and T. catappa had been found to possess appreciable antibacterial property. The MIC values of the extract ranged from 0.25 to 16 mg/ml3.

Periodontal diseases are one of the causes of early loss of teeth and represent a worldwide socioeconomic and public health problem. Two bacteria, Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis, are frequently found in large numbers in inflammation of gingival and connective tissue at the root of the tooth. Statistical analysis showed Actinobacillus actinomycetemcomitans was detected in 40.3% of healthy subjects, 68% of patients with chronic periodontitis, 92.86% of patients with aggressive periodontitis and 40.14% of children with gingivitis4. These bacteria when organizing themselves into a community on tooth surface, a soft layer of biofilms is formed. More than 200 species of microorganisms colonise the oral cavity, but only a few of these are considered as

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periodontal pathogens. P. gingivalis, Provetella intermedia, and A. actinomycetemcomitans were isolated from subgingival lesions in periodontitis of adult patients. Later Esenmann, et al reported the isolation of A. actinomycetemcomitans from 60% of the patients suffering from periodontitis. P. gingivalis are frequently isolated from subgingival infections. P. gingivalis have been frequently identified in several oral abscesses and periodontitis and it requires nutritionally complex media for primary isolation. Blood agar and serum has been the base for the selective media.

Materials and Methods

Preparation of lyophilized ethanolic extract

The unripe fruits of T. catappa were collected from the medicinal garden of the Presidency College (Autonomous), Chennai, Tamil Nadu, India. The healthy fruits were collected in a separate python bag and the surface of the fruit was washed in double distilled water. The fresh ripe mesocarp pulp was ground with 100 ml of 75% ethanol in the ratio 1:3 W/V and stored overnight at 4°C. The stored mixture was filtered through sterile gauze and the filtrate was collected by hand pressure. Further, it was centrifuged at 3000 rpm for 10 min. The supernatant was filtered through Seitz filter (0.2 nm). Nutrient Agar and Sabouraud’s Dextrose Agar plates were inoculated with the extract for sterility checking. Seitz-filtered extract was poured into special lyophilizing flask. The flask was connected to a vacuum pump and evacuated till drying. Lyophilisation enables concentration of plant components by evaporation under pressure and till drying. Lyophilisation enables concentration of plant components by evaporation under pressure and it requires nutritionally complex media for primary isolation. Blood agar and serum has been the base for the selective media.

Collection of clinical samples and isolation of bacteria

Samples were collected from patients suffering mainly from chronic generalized gingivitis and chronic generalized periodontitis in the age group ranging from 22 to 65 years. Four deepest bleeding sites were selected; one side in each quadrant was chosen from subgingival plaque. Sample was taken from these pockets using two sterile paper points per pocket. The paper points were inserted into a depth of the subgingival pocket and kept in position for 60 seconds. Then the samples are transported in BHI broth and stored at 4°C. TSBV was used for isolation and presumptive identification of A. actinomycetemcomitans as described by Holm et al. Blood agar, supplemented with haemin and vitamin, was used as enriched and selective medium, to isolate P. gingivalis. The plates were incubated in a tightly packed anaerobic jar at 37°C for 5-7 days. Identification of species was done on the characteristics of Gram staining, Culture and Biochemical reactions (Insole, Methyl red, Voges praskauer, Citrate, Triple Sugar Ion test, Urease). Standardisation of inoculum of test bacteria was done by comparing it with of 0.5 McFarland’s standard solution as described in Working Party Report.

In vitro susceptibility of bacteria

Disc diffusion assay

Disc diffusion assay of Bauer et al was adopted to compare the in vitro susceptibility of bacterial isolates from periodontitis patients to standard antibiotics. Amoxicillin (30 µg/ml), Penicillin (30 µg/ml), Gentamycin (10 µg/ml), Norfloxacin (10 µg/ml), Novobiocin (30 µg/ml), Metranidazole (30 µg/ml), Nalidixic acid (30 µg/ml), Vancomycin (30 µg/ml) were the antibiotics used in antibiotic sensitivity tests. The zone of inhibition around each disc in the experiment was measured after 24 h of incubation at 37°C.

Microdilution assay

The inhibitory effect of lyophilized ethanolic extract was estimated by modified serial two-fold dilution technique. The principle of the assay method was to estimate the lowest concentration among the serial dilution at which the visual growth of microbes was absent. Hence, the in vitro susceptibility of these bacteria of the plant extract was carried out by adopting NCCLS method. A serial two-fold dilution of the extract was performed by dissolving lyophilized extract in distilled water and serially diluted. One thousand microgram (1000 µg) of lyophilized extract is dissolved in 250 µl of double distilled water out of which 1 ml was taken in a series of 8 sterile test tubes (1.2 cm in diam.). The lyophilized extract was dissolved in distilled water and added to the first tube and then serially diluted till the eighth tube containing 1ml of distilled water. The final concentration were of 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 µg/ml in the wells 1 to 8, respectively. The microtitre plates were sealed and incubated overnight at 37°C in a moisturized incubator.
Results

Two different bacterial colonies were isolated from the seven blood samples of subgingival patients and processed with detailed laboratory procedure. The colonies were observed on both the selective medium TSBV and blood agar supplemented with haemin and vitamin. The morphological colony observation on TSBV agar was circular, convex, translucent, glistening 0.5 mm in diam. pinpoint, with central 4-6 pointed star like configuration with a tinch of non-diffusible orange pigment. On Gram staining, Gram negative coccobacilli were seen. The organisms were found to be non-motile, catalase positive and oxidase negative. Further results on biochemical test (IMVIC Test) and sugar fermentation results had confirmed that the isolated colony on the TSBV agar was A. actinomycetemcomitans.

The characterisation of the colony morphology on the blood agar showed minute, opaque, circular and black pigmented isolates. On Gram staining, non-spore forming anaerobic rods, catalase and oxidase negative Gram-negative rods were observed. Further biochemical and sugar fermentation tests confirmed the identification of the bacterium as P. gingivalis.

Antibiogram

The bacterial isolates, A. actinomycetemcomitans and P. gingivalis, were subjected to in vitro susceptibility test. The observation of antibiotic sensitivity test were recorded as follows: A. actinomycetemcomitans exhibited sensitivity to Gentamycin (22 mm), Norfloxacin (27 mm), Novobiocin (6 mm), Nalidixic acid (20 mm), Vancomycin(15 mm) but showed resistance towards Amoxicillin and Metranidazole whereas P. gingivalis showed sensitivity of Gentamycin (23 mm), Norfloxacin (27 mm), Novobiocin (6 mm), Vancomycin (14 mm). However, both the bacteria showed resistance to Amoxicillin, Nalidixic acid, Pencillin and Metranidazole.

Estimation of the MIC extract

The ethanolic extract of T. catappa showed inhibitory activity at first and second dilutions, (1000 and 500 µg/ml). Hence, the minimum inhibitory concentration of extract on both the bacteria was determined as 500 µg/ml. Though the desirable MIC for a potential drug is below 100 µg/ml for a pure compound, the MIC of a lyophilized extract of T. catappa is 500 µg/ml, which is not considered as high because the lyophilized extract is not a pure compound but also contains other organic and inorganic materials that are inactive on microbes.

Discussion

Kluck et al reported that Phyllanthus amarus Schum. and T. catappa showed the most promising antibacterial properties on many Gram negative and gastrointestinal bacteria at an MIC ranging from 0.25 to 16 mg/ml. More et al reported antibacterial activity in ten South African plants on P. gingivalis and A. actinomycetemcomitans at an MIC ranging from 2500 to 800µg/ml. Iauk et al showed antibacterial activity of ethanolic extract from roots of Althacea officinalis Linn. on P. gingivalis at a MIC of 2048 mg/l.

In the present study, the lyophilized ethanolic extracts of Terminalia catappa was found completely inhibiting the presence of A. actinomycetemcomitans and P. gingivalis at an MIC of 500 µg/ml of the isolated organism. Standard antibiotics to organism were also studied for comparison. Hence, this study is the foremost on the antibacterial activity of Indian almond on the two subgingival bacteria. Inspite of the improvement prevailing on the oral hygiene, gingival infection of bacterial origin seems to be increasing, based on the patients reporting to Government and Government-aided hospitals. This could be attributed partially to the lack of knowledge on oral-cum-dental hygiene. In the view of microbiologists, this is due to bad food habits, which make normal microbial flora in gingivitis. Isolation and characterization of the active principle by appropriate phytochemical studies is suggested to ensure the standardization in vitro efficacy of the pure drug as an active principle.

Conclusion

The present study is a pioneering work on the usefulness of lyophilized ethanolic extract of Indian almond fruit on the antibacterial activity on two subgingival bacteria. The MIC of the extract has been estimated as 500 µg/ml adopting modified NCCLS serial two-fold dilution method using the lyophilized extract of unripe fruit pulp. The results of this study have indicated an alternative and a potential plant, which is under-utilized for an effective treatment of subgingivitis.

References

2 Nadkarni KM and Nadkarni AK, Indian Materia Medica, Popular Prakashan Pvt Ltd, Bombay, 1976, 2, 1205