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Studies on the antimicrobial activity of Tamarind (*Tamarindus indica*) and its potential as food bio-preservative

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Abstract

Tamarind (*Tamarindus indica*) is used in Indian spices as a souring agent to provide the desired acidity in the various food recipes. In this era of increased concern on safety of chemical food additives, natural methods of preservation and natural preservatives are receiving increased attention. The antimicrobial activity of tamarind extract (50% ethanol) was tested against ten bacterial strains (7 Gram-positive and 3 Gram-negative) and seven fungi known to cause food spoilage by agar well diffusion assays. The aqueous-ethanolic extract exhibited a broad spectrum of anti-bacterial activity inhibiting both the groups of bacteria. Tamarind extract was active against all the test Gram-positive bacteria isolates but was highly effective against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Listeria monocytogenes* with an inhibition zone of 18 mm, 19 mm, 16 mm and 16 mm, respectively. It also inhibited the growth of all Gram-negative bacteria isolates but produced an inhibition zone greater than 15 mm only in the case of *Pseudomonas aeruginosa*, *Pseudomonas* sp. and *Salmonella* sp. However, the extract was found to be ineffective against majority of test fungal species. Only *Aspergillus* sp. and *Penicillium* sp. were found to be partially sensitive to the extract. The phytochemical analysis of tamarind extract revealed the presence of tannins, terpenoids and citric acid. This study shows the potential for replacement of synthetic food grade preservatives with the use of natural extracts of tamarind.

Introduction

Many food products are perishable by nature and require protection from spoilage during their preparation, storage and distribution to give them desired shelf-life. Food preservation is a continuous fight against microorganisms spoiling the food or making it unsafe. The most common classical preservative agents used are weak organic acids, like acetic, lactic, benzoic and sorbic acid. These chemical preservatives can be harmful because of chemical residue in foods. An increasing number of consumers prefer minimally processed foods, prepared without chemical preservatives. A renewed interest in 'natural preservation' appears to be stimulated by present food safety concerns, growing problems with microbial resistance and rise in the production of minimal processed food joined with 'green' image policies of food industries (Chen *et al*., 2013). Environmental groups and health conscious people are further putting pressure on governments to reduce and stop the used chemical preservatives. Numerous research studies have also shown that many natural substances of plant origin including spices may play a fundamental role in the host-pathogen relationship (Gupta *et al*., 2008a; Gupta *et al*., 2008b; Gupta *et al*., 2010). Phytochemicals from different plant genera are reported to be biologically active, endowed with antimicrobial, allelopathic and antioxidant properties.

Tamarind (*Tamarindus indica*) is a leguminous tree in the family Fabaceae that is indigenous to tropical Africa. The tamarind tree produces edible, pod-like fruits which are used extensively in cuisines around the world. Tamarind seeds are traditionally used to treat diabetes, fevers and intestinal infections. They are also used in the treatment of diarrhoea and as a laxative. The leaves have a proven hepato-protective activity associated with the presence of poly-hydroxylated compounds, with many of them flavonolic in nature (El-Siddig *et al*., 2004; Meléndez and Carriles, 2006). The seeds and the bark also have medicinal properties. Due to their antimicrobial, antifungal and antiseptic effects; tamarind leaves have an extensive ethnobotanical use (Escalona-Arranz *et al*., 2010; Lans, 2007). Tamarind has been used for centuries as a medicinal plant; its fruits are the most valuable part which has often been reported as curative in several pharmacopoeias. In this context, studies on the antimicrobial effect of extracts of tamarind were carried out against ten bacterial strains (7 Gram-positive and 3 Gram-negative) and seven

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fungi known to cause food spoilage.

Materials and Methods

Materials

All chemicals used were of analytical-reagent grade and obtained from E. Merck (Mumbai, India). Tamarind (*Tamarindus indica*) was collected from local market of Delhi NCR, India) and identified by Dr. C.M Govil, Botany Department, C.C.S University, Meerut, India.

Bacterial and fungal strains

Ten bacterial strains (7 Gram positive and 3 Gram negative), mostly food borne including pathogens, were selected for the study. Gram positives were *Bacillus cereus*, *B. mycoides*, *B. subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *S. epidermidis* and *Listeria monocytogenes* while Gram negatives were *Escherichia coli*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*. The fungal species used in the present study were *Alternaria* sp., *Aspergillus fumigatus*, *A. niger*, *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp. and *Rhizomucor* sp. The standard bacterial and fungal stock cultures were obtained from the culture collection of Institute of Microbial Technology (IMTECH), Chandigarh, India. The viability tests for each isolate were carried out by resuscitating the organism in nutrient agar medium and Sabouraud's dextrose agar (SDA) medium respectively. The stock on nutrient agar medium (Hi Media, Mumbai, India) and potato dextrose agar medium was incubated for 24 h at 37°C (bacteria) and 28°C for 3 days (fungi) respectively following refrigeration storage at 4°C until required for sensitivity testing.

Preparation of Tamarind extract

The tamarind fruits were obtained from the local market. The pod like fruits were cleaned, descaled when necessary, and washed in sterile distilled water. The herbal extract was prepared by dissolving 1 g of tamarind pulp in 5 ml of solvent (50% aqueous-ethanol) in a 250 mL Erlenmeyer flasks. The aqueous-ethanol (50%) was found to be the best solvent for extracting the biologically active phytochemical constituents from tamarind pulp. The other solvents were also used including chloroform, petroleum ether, isopropanol, ethanol and water. The flasks were closed with cotton plug and aluminium foil. The tamarind fruit pulp was soaked in 50% aqueous-ethanol for 48 h at room temperature with intermittent shaking. The mixture was centrifuged at 3500 g for 20 min and finally filtered through Whatmann filter paper No.1 (Azoro, 2000). The pellet was discarded and the supernatant was collected and concentrated under reduced pressure in a rotary vacuum evaporator (Buchi Type) until semisolid residue was obtained. This was dried inside the crucible under a controlled temperature (45ºC) to obtain solid powder (Jonathan and Fasidi, 2003). The process of extraction was repeated until the weight of 500 mg was obtained. The powder was weighed and reconstituted in dimethyl sulfoxide (DMSO) and were sieved through a fine mesh cloth and sterilized using a membrane filter (0.45-micron sterile filter). This extract was considered as the 100% concentration. These were stored in the refrigerator at 4ºC for testing antimicrobial sensitivity.

Antibacterial activity testing using agar well method (cup plate method)

The antimicrobial activity of tamarind extract was determined by agar well diffusion method (Okeke *et al.*, 2001). Pure isolate of each bacterium was first sub cultured in nutrient broth at 37ºC for 24 h. One hundred microlitres (100 µL) of standardized inoculum (10^6 CFU/mL; 0.5 Mac-Farland) of each test bacterium was spread with the help of sterile spreader on to a sterile Muller-Hinton Agar plate (Hi Media, Mumbai, India) so as to achieve a confluent growth. The plates were allowed to dry and a sterile cork borer (6.0 mm diameter) was used to bore wells in the agar. Subsequently, a 50 µL volume of the extract was introduced in triplicate wells of the agar plates. Sterile DMSO and sodium propionate (standard food preservative) served as negative and positive control respectively. The plates were allowed to stand for 1h or more for diffusion to take place and then incubated at 37ºC for 24 h. The zone of inhibition was recorded to the nearest size in mm (NCCLS, 1999). All the experiments were performed in triplicate.

Antifungal assay

For determining the antifungal activity of the tamarind extract, the fungal isolates were sub-cultured on SDA at 28°C for 3-5 days. Sterilized Sabouraud's Dextrose Agar plates were taken and a sterile cork borer (6-mm diameter) was used to bore wells in the agar. 50 µL volume of the extract(s) was introduced into each of the peripheral wells while a fungal disc was inoculated into the central well. A negative control (sterilized DMSO) was also included in one of the peripheral wells to compare the activity. The plates were then incubated at 28°C. The evaluations were carried out by means of daily measurement of colony diameter, starting 24 h after the experiment began and finishing when 2/3rd the plate surface of the

control treatment was covered by the fungus (Fiori *et al*., 2000). The appearance of zones of inhibition was regarded as the presence of antimicrobial action in the test substance. All the experiments were performed in triplicate. The results were expressed in terms of the diameter of the inhibition zone: <9 mm, inactive; 9-12 mm, partially active; 13-18 mm, active; >18 mm, very active (Junior and Zanil, 2000).

Assessment of minimum inhibitory concentration (MIC)

The method of Thongson *et al.* (2004) was applied. The MIC for the crude extract was determined by agar-well diffusion method. A two-fold serial dilution of the test extracts was prepared by first reconstituting it in DMSO. It was then diluted in sterile DMSO to achieve a decreasing concentration range of 1000-31.25 mg/mL. A 50µL volume of each dilution was added aseptically into Mueller Hinton agar plates that were already seeded with the standardized inoculum (10^6 CFU/mL) of the test bacterial cells. Sodium propionate was used as positive control. All the experiments were performed in triplicate. The same procedure was used for fungi, except that SDA plates were used and the plates were incubated at 28ºC. The lowest concentration of tamarind extract showing a clear zone of inhibition was considered as the MIC.

Preliminary phytochemical analysis of tamarind pulp

The aqueous-ethanol extract of tamarind pulp was subjected to several phytochemical screening tests and were found to contain tannins and glycosides (Harbone, 1973).

Tannins

Five gram powdered dried tamarind pulp was boiled in 100 mL water for 3 minutes. The extract was filtered and 5 mL of filtrate was added with 2 mL of 2% gelatin solution. A curdy white precipitate indicated the presence of tannins.

Terpenoids

Two millilitre of chloroform and concentrated sulphuric acid was added to 1 mL of extract. An appearance of reddish brown colour indicated the presence of terpenoids.

Citric acid

Add a few milligrams powdered dried tamarind pulp to a solution containing 15 mL of pyridine and 5 mL of acetic anhydride. A bright red color indicates the presence of citric acid.

Results and Discussions

A total of ten bacterial species and seven fungal species have been used in the present study to assess the antimicrobial activity of aqueous-ethanolic extract of tamarind. The antimicrobial activity of the extract was determined by agar well diffusion method (Figure 1). Table 1 shows the antimicrobial activity of the tamarind extract against seven Gram positive bacteria and three Gram negative bacteria. The extract was effective against both Gram-positive and Gram-negative bacteria that were isolated from the spoiled food products. However the aqueous-ethanolic extract was most effective against Staphylococcus epidermidis and *S. aureus* with diameter of zone of inhibition 19.0 mm and 18.0 mm respectively followed by *Bacillus subtilis* and *Listeria monocytogenes*. Sodium propionate (commonly used food preservative) was used as a positive control in the study and it was less effective producing an inhibition zone of diameter of 15.0, 12.0, 14.0 and 13.0 mm, respectively (Figure 1).

Amongst the Gram negative bacteria, the extract showed highest activity against *Pseudomonas aeruginosa* with diameter of zone of inhibition 16.0 mm and was least effective against *Escherichia coli* and *Enterobacter aerogenes* with diameter of zone of inhibition of 13.0 mm each. Antifungal effects of the tamarind extract have also been investigated as shown in Table 2. All the seven test fungi were isolated from the spoiled food products. The tamarind extract was only partially effective against *Aspergillus* sp. and *Penicillium* sp. producing an inhibition zone of 20.0 and 10.0 mm, respectively while the other test fungi were resistant to it. The MIC for the fungal species was therefore not determined. In contrast, sodium propionate which is used as a standard food preservative inhibited all the test fungal species. Table 3 depicts the minimum inhibitory concentration (MIC) of the aqueous-ethanolic extract of tamarind. It ranged from 250 to 500 mg/mL (Figure 2). *Listeria monocytogenes* and *Pseudomonas aeruginosa* was found to be highly sensitive to the extract exhibiting lowest MIC of 250 mg/mL each followed by the other bacterial species (MIC = 500 mg/mL each).

From these studies, it was observed that although tamarind is effective against both groups of bacteria but its activity was high in Gram positive bacteria as compared to Gram-negative bacteria. These observations are in accordance with the earlier observations reported by Escalona-Arranz *et al.* (2010) and Desta (1993) who also found that Gram-negative organisms were less susceptible to the herbal extracts than Gram-positive isolates. It may possibly

Table1. Zone of inhibition (mm) of ethanolic extract of tamarind (*Tamarindus indica*) on selected bacteria that cause food spoilage

Bacterial species	Tamarind extract	Sodium propionate (+ve C)	DMSO (-ve C)
Bacillus cereus	14.0	12.0	0.0
Bacillus subtilis	16.0	14.0	0.0
Bacillus mycoides	13.0	10.0	0.0
Staphylococcus aureus	18.0	12.0	0.0
Staphylococcus epidermidis	19.0	15.0	0.0
Listeria monocytogenes	16.0	13.0	0.0
Micrococcus luteus	12.0	11.0	0.0
Escherichia coli	13.0	9.0	0.0
Enterobacter aerogenes	13.0	9.0	0.0
Pseudomonas aeruginosa	16.0	12.0	0.0

Table 2. Zone of inhibition (mm) of ethanolic extract of tamarind (*Tamarindus indica*) against common food spoilage fungi on SDA medium

Fungal species	Tamarind extract	Sodium Propionate (+ve C)	DMSO (-ve C)
Aspergillus niger	0.0	10.0	0.0
Aspergillus fumigates	0.0	11.0	0.0
Aspergillus sp.	20.0	15.0	0.0
Alternaria sp.	0.0	12.0	0.0
Rhizomucor sp.	0.0	10.0	0.0
Rhizopus sp.	0.0	11.0	0.0
Penicillium sp.	10.0	9.0	0.0

Table 3. The MIC (Minimum inhibitory concentration) values of tamarind extract (mg/mL) against different bacteria on Mueller- Hinton Agar Medium

Bacterial species	Tamarind extract (mg/mL)	Sodium Propionate (mg/mL)
Bacillus cereus	500	125
Bacillus subtilis	500	250
Bacillus mycoides	500	125
Staphylococcus aureus	500	250
Staphylococcus epidermidis	500	250
Listeria monocytogenes	250	250
Micrococcus luteus	1000	1000
Escherichia coli	500	250
Enterobacter aerogenes	500	250
Pseudomonas aeruginosa	250	125

be due to the presence of high lipid content in the cell walls of Gram negative bacteria. Gram-positive bacteria like *Staphylococcus epidermidis* and *S. aureus* contains teichoic acid in the peptidoglycan layer and is therefore inhibited by tamarind extracts (Shan *et al.*, 2007). Furthermore, the outer membrane of Gram-negative bacteria is known to present barrier to penetration of numerous antibiotic molecules, and the periplasmic space contains enzymes, which are capable of breaking down foreign molecules introduced from outside thus providing greater resistance to them (Duffy and Power, 2001).

The analysis of bioactive phytochemical constituents of the tamarind fruit pulp revealed the presence of tannins, terpenoids and citric acid while alkaloids and flavonoids were absent. The pulp of the young fruit is very sour and acidic and due to the change in pH of the medium to acidic range; tamarind exhibits antimicrobial activity. pH is known to control the growth, development and sporulation of all microbes including bacteria. It also contains small amount of terpenes (limonene, geraniol), phenyl propanoids (safrole, cinnamic acid, ethyl-cinnamate), methyl-salicylate, pyrazine and alkylthiazoles (Srinivasan *et al*., 2001; Doughari, 2006). The mechanism of action of terpenoids involves membrane disruption by the lipophilic compounds (Ahmed and Beg, 2001; Gupta *et al*., 2010). The demonstration of antibacterial activity against both gram positive and gram negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds. The mode of antimicrobial action of tannins present in tamarind pulp is related to their ability to inactivate microbial adhesions, enzymes and cell envelope proteins. They also form complex with microbial polysaccharides (Cowan, 1999). The mode of action of citric acid is that it gives away protons in solution thereby decreasing the pH of the solution, which directly co-relates with the antimicrobial properties. The main effect is due to the prevention of absorption of essential nutrients by the microorganisms due to disruption of the protein motive force, which provides energy for active absorption of nutrients. It alters the permeability of cell wall causing damage and hence cell death, especially in Gram negative bacteria. It is also capable of chelating metal ions present in the cell wall thereby causing damage (Cowan, 1999). Thus tamarind pulp will be of immense advantage in fighting the menace of antibiotic refractive pathogens that are so prevalent in recent times.

Conclusions

The foregoing study suggests that tamarind extract is a much better antagonistic agent, exhibiting broad range of antibacterial activity against common bacteria than sodium propionate. It is therefore, conceivable that it represents an inexpensive source of food preserving agents. Further research on the use of other botanical extracts can be rewarding to pursue in hunt for new herbal therapeutic agent. The effect of this plant on more pathogenic organisms and toxicological investigations and further purification however, needs to be carried out.

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