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Antimicrobial and Phytochemical Studies of Fresh Ripe Pulp and Dried Unripe Pulp of *Mangifera indica* (AMCHUR)

C. Gupta, A.P. Garg and S. Gupta

[1]Amity Institute for Herbal Research and Studies, Amity University, Noida, India
[2]Department of Microbiology, C.C.S university, Meerut- 250005, India
[3]Department of Zoology, R.G.P.G. college, Meerut, India

Abstract: The antimicrobial activity of amchur (dried pulp of unripe *Mangifera indica*) extract (50% ethanol) was tested against ten bacterial strains (7 Gram-positive and 3 Gram-negative) and seven fungi by agar well diffusion assays. The crude extract exhibited a broad spectrum of antibacterial activity inhibiting both the groups of bacteria. The extract was most effective against *Staphylococcus aureus* (26.0mm). *Bacillus mycoides* was found to be the most sensitive, with the lowest MIC of 62.5mg/mL, followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (125mg/mL each), whereas *Micrococcus luteus* was found to be the most resistant surviving up to 500mg/mL. However, the extract was found to be ineffective against majority of test fungal species. Only *Alternaria sp.* and *Penicillium sp.* was found to be partially sensitive to the extract. The dried unripe pulp of *M. indica* was found to be more inhibitory as compared to its fresh ripe pulp. The phytochemical analysis of amchur extract revealed the presence of tannins and terpenes. This study shows the potential for replacement of synthetic preservatives by the use of natural extracts.

Key words: *A*mchur · *Mangifera indica* · Antibacterial · Minimal inhibitory concentration · Food spoilage

INTRODUCTION

The problem of food spoilage has plagued humans since ancient times. Spices and herbs are used in foods mainly for their flavour and aroma but it is recognized that they may fulfill more than one function in foods to which they are added. In addition to imparting flavour, certain spices prolong the storage life of food by preventing rancidity through their bacteriostatic or bactericidal activity [1]. Being natural foodstuff, they appeal to consumers who tend to question the safety of synthetic food additives. Many natural substances of plant origin may play a fundamental role in the host-pathogen relationship and products from different plant genera are reported to be biologically active, endowed with antimicrobial, allelopathic and antioxidant properties.

Amchur (dried pulp of unripe *Mangifera indica*) is used in Indian spices as a souring agent to provide the desired acidity in the various food recipes.

Mango is considered as a king of fruits in Indian delicacy. The roots and bark of mango *Mangifera indica* (Anacardiaceae) are astringent, acrid, anti-inflammatory and constipating. The leaves and flowers are refrigerant, styptic, vulnerary and constipating. Amchur (dried or dehydrated product of unripe mango flesh in the form of peeled slices or powder) is used as an acidulant or a souring agent for curries. Amchur is rich in citric acid.

Very limited literature is available on the antimicrobial activity of amchur extract. In the present study, we have investigated the antibacterial activity and phytochemical study of dried pulp of unripe *Mangifera indica* for the first time.

MATERIALS AND METHODS

Materials: All chemicals used were of analytical-reagent grade and obtained from E. Merck (Mumbai, India). Amchur (*Mangifera indica*) was collected from local market of Meerut (Uttar Pradesh, India). Dr. C.M Govil, Botany Department, C.C.S University, Meerut, India confirmed the species.

Bacterial and Fungal Strains: Ten bacterial strains (7 Gram positive and 3 Gram negative), mostly food borne

Corresponding Author: Charu Gupta, Lecturer, Amity Institute for Herbal Research and Studies, E- 3 block, Amity University, Sector 125, Noida

including pathogens, were selected for the study. Gram positives were *Bacillus cereus, Bacillus mycoides, Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Staphylococcus 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, Aspergillus niger, Aspergillus sp., Penicillium sp., Rhizopus sp.* and *Rhizomucor sp.* The bacterial and fungal stock cultures were obtained from the Department of Microbiology of this University. 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 24h at 37°C (bacteria) and 28°C for 3 days (fungi) respectively following refrigeration storage at 4°C until required for sensitivity testing.

Extraction: The extraction was performed by two different modes: (a) Extraction of fresh mango (ripe) without drying and (b) Extraction after drying the pulp of unripe mango.

Extraction of Fresh Mango (ripe) Without Drying (aqueous Extraction): 100g of the ripe pulp of fresh mango was macerated in mortar and pestle with 100mL distilled water at room temperature for 48h with intermittent shaking. The mixture was centrifuged at 3500xg for 20 min and finally filtered through Whatmann filter paper No.1 [2]. The pellet was discarded and the supernatant was collected and concentrated under reduced pressure in a rotary vacuum evaporator (Buchi Type) until semisolid substance was obtained. This was dried inside the crucible under a controlled temperature (45°C) to obtain solid powder [3]. The process of extraction was repeated until the weight of 500mg was obtained.

Extraction after Drying the Pulp of Unripe Mango: The pulp of unripe mango (*Mangifera indica*) was air-dried and powdered in milling machine (Inalsa Mixer Grinder) to obtain fine dry powder called amchur. The powder was weighed using single pan electronic weighing balance (Ohaus model). The powder was then subjected to organic solvent extraction protocols as described below.

Organic Solvent Extraction: The herbal extract was prepared at the rate of 1g (amchur powder)/5ml of solvent (50% ethanol) in a 250mL Erlenmeyer flasks. The flasks were closed with cotton plug and aluminium foil. The amchur powder was soaked in 50% ethanol for 48h at room temperature with intermittent shaking. The mixture was centrifuged at 3500xg for 20 min and finally filtered through Whatmann filter paper No.1 [2]. The pellet was discarded and the supernatant was collected and concentrated under reduced pressure in a rotary vacuum evaporator (Buchi Type) until semisolid substance was obtained. This was dried inside the crucible under a controlled temperature (45°C) to obtain solid powder [3]. The process of extraction was repeated until the weight of 500mg was obtained. The powder was weighed and reconstituted in dimethyl sulfoxide (DMSO). These were stored in the refrigerator at 4°C for testing antimicrobial sensitivity. Once the extracts are dissolved in pure DMSO, these are also sterilized and thus, a very costly and time consuming step of membrane filtration sterilization was omitted [4].

Antibacterial Susceptibility Assay: The antimicrobial activity of amchur extract (aqueous and organic solvent) was determined by agar well diffusion method [5]. Pure isolate of each bacterium was first subcultured in nutrient broth at 37°C for 24h. One hundred microlitres (100µL) of standardized inoculum (106CFU/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 (6.0mm diameter) was used to bore wells in the agar. Subsequently, 50µL volume of the extract(s) 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 37°C for 24h. The zone of inhibition was recorded to the nearest size in mm [6].

Antifungal Susceptibility Assay: For determining the antifungal activity of the oil, the fungal isolates were subcultured 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. 50µL 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 24h after the experiment began and finishing when 2/3rd the plate surface of the control treatment was covered by the fungus [7]. The appearance of zones of inhibition was regarded as the presence of antimicrobial action in the test substance.

The results were expressed in terms of the diameter of the inhibition zone: <9mm, inactive; 9-12mm, partially active; 13-18mm, active; >18mm, very active [8].

Assessment of Minimum Inhibitory Concentration (MIC) of amchur extract: The method of Thongson *et al.,* 2004 [9] 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- 31.25mg/mL. 50µL volume of each dilution was added aseptically into Mueller Hinton agar plates that were already seeded with the standardized inoculum (106CFU/mL) of the test bacterial cells. Sodium propionate only served 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 28°C. The lowest concentration of amchur extract showing a clear zone of inhibition was considered as the MIC.

Preliminary Phytochemical Analysis of Amchur Extract: The ethanolic extract of the amchur powder was subjected to phytochemical tests for the presence of tannin, alkaloid, saponin, cardiac glycoside, steroid, flavanoid and. terpenoid.

Tannins: (200 mg plant material in 10 ml distilled water, filtered); a 2 ml filtrate + 2 ml FeCl3, blue-black precipitate indicated the presence of Tannins.

Alkaloids: (200 mg plant material in 10 ml methanol, filtered); a 2ml filtrate + 1% HCl + steam, 1 ml filtrate + 6 drops of Mayor's reagents/Wagner's reagent/ Dragendroff reagent, creamish precipitate/brownish-red precipitate/orange precipitate indicated the presence of respective alkaloids.

Saponin: (frothing test: 0.5 ml filtrate + 5 ml distilled water); frothing persistence indicated presence of saponins.

Cardiac Glycosides: (Keller-Kiliani test) 2 ml filtrate + 1 ml glacial acetic acid + $FeCl_3$ + conc. $H_2{}^{SO}{}_4$); green-blue color indicated the presence of cardiac glycosides.

Steroids: (Liebermann-Burchard reaction) 200 mg plant material in 10 ml chloroform, filtered); a 2 ml filtrate + 2 ml acetic anhydride + conc. $H_2{}^{SO}{}_4$. Blue-green ring indicated the presence of terpenoids.

Flavonoids: (200 mg plant material in 10 ml ethanol, filtered); a 2 ml filtrate + conc. HCl + magnesium ribbon pink-tomato red color indicated the presence of flavonoids

Terpenoid: 2mL of chloroform and concentrated sulphuric acid was added to 1mg of extract and observed for reddish brown colour (10).

RESULTS

Following the extraction of the fresh ripe pulp and dried unripe pulp of *Mangifera indica* (Amchur) using distilled water and 50% ethanol by maceration method, the antimicrobial activity of the extract was determined. Table 1 shows the antimicrobial activity of the fresh ripe pulp of mango extract (aqueous and 50% ethanol) on the selected food borne bacteria that cause spoilage. The aqueous extract was ineffective against all the test bacteria (both Gram positive and Gram negative). However the ethanolic extract from the fresh pulp was slightly more effective than its aqueous extract but the activity was very low. The widest inhibition zone diameter (IZD) was produced against *Bacillus mycoides* with an IZD of only 13mm.

Table 2 shows the antimicrobial activity of the dried unripe pulp of *Mangifera indica* extract (aqueous and 50% ethanol) on the test bacteria. On comparing the antibacterial activity of both the extracts, it was found that the ethanolic extract was more potent than its aqueous counterpart. 50% ethanol was found to be a more suitable solvent for the maximum extraction of active metabolites than distilled water. Gram positive bacteria were found to be more susceptible as compared to Gram negative species. *Staphylococcus aureus* was found most susceptible, as it was inhibited by both the solvents (aqueous and ethanolic; unripe and ripe pulp of mango). Other Gram positive bacteria (*Staphylococcus epidermidis, Bacillus subtilis, Listeria monocytogenes*) were shown to have moderate to mild resistance,

Table 1: Zone of inhibition (mm) of fresh mango (*Mangifera indica*) extract on selected bacteria

S.No.	Test bacterial species	Aqueous extract	Ethanolic extract (50%)	Positive control	Negative Control
1.	*Bacillus cereus*	0.0	12.0	20.0	0.0
2.	Bacillus subtilis	0.0	11.0	14.0	0.0
3.	Bacillus mycoides	0.0	13.0	14.0	0.0
4.	Staphylococcus aureus	0.0	12.0	17.0	0.0
5.	*Staphylococcus epidermidis*	0.0	10.0	14.0	0.0
6.	*Listeria monocytogenes*	0.0	9.0	12.0	0.0
7.	*Micrococcus luteus*	0.0	10.0	14.0	0.0
8.	*Escherichia coli*	0.0	8.0	12.0	0.0
9.	*Enterobacter aerogenes*	0.0	9.0	12.0	0.0
10.	*Pseudomonas aeruginosa*	0.0	8.0	11.0	0.0

Incubation temperature: 37°C; Incubation period: 24h

Positive control- Sodium propionate; Negative control- Dimethyl sulfoxide

Each value is the average of three independent replicates.

Table 2: Zone of inhibition (mm) of amchur (*Mangifera indica*) extracts (unripe pulp) on selected bacteria

S.No.	Test bacterial species	Aqueous extract	Ethanolic extract (50%)	Positive control	Negative control
1.	*Bacillus cereus*	11.0	15.0	20.0	0.0
2.	Bacillus subtilis	10.0	17.0	14.0	0.0
3.	Bacillus mycoides	12.0	16.0	14.0	0.0
4.	Staphylococcus aureus	13.0	26.0	17.0	0.0
5.	*Staphylococcus epidermidis*	12.0	19.0	14.0	0.0
6.	*Listeria monocytogenes*	11.0	16.0	12.0	0.0
7.	*Micrococcus luteus*	10.0	13.0	14.0	0.0
8.	*Escherichia coli*	9.0	15.0	12.0	0.0
9.	*Enterobacter aerogenes*	9.0	14.0	12.0	0.0
10.	*Pseudomonas aeruginosa*	8.0	17.0	11.0	0.0

Incubation temperature: 37°C; Incubation period: 24h

Positive control- Sodium propionate; Negative control- Dimethyl sulfoxide

Each value is the average of three independent replicates.

Table 3: The MIC (Minimum inhibitory concentration) values of amchur (50% ethanolic extract (mg/mL) against different bacteria on Mueller- Hinton Agar Medium

S.No.	Test bacterial species	Amchur extract (mg/mL)	Sodium propionate (mg/mL)
1.	*Bacillus cereus*	250	125
2.	Bacillus subtilis	250	250
3.	Bacillus mycoides	62.5	62.5
4.	Staphylococcus aureus	125	125
5.	*Staphylococcus epidermidis*	250	500
6.	*Listeria monocytogenes*	250	500
7.	*Micrococcus luteus*	500	500
8.	*Escherichia coli*	250	500
9.	*Enterobacter aerogenes*	250	500
10.	*Pseudomonas aeruginosa*	125	1000

Incubation temperature: 37°C; Incubation period: 24h

Negative control- Dimethyl sulfoxide

Each value is the average of three independent replicates.

Table 4: Zone of inhibition (mm) of ethanolic extract of amchur (*Mangifera indica*) against common food spoilage fungi on SDA medium

S.No.	Fungal strains	Amchur extract	Positive control	Negative control
1.	*Aspergillus niger*	0.0	10.0	0.0
2.	*Aspergillus fumigatus*	0.0	11.0	0.0
3.	*Aspergillus sp.*	12.0	12.0	0.0
4.	*Alternaria sp.*	13.0	14.0	0.0
5.	*Rhizomucor sp.*	0.0	12.0	0.0
6.	*Rhizopus sp.*	0.0	14.0	0.0
7.	*Penicillium sp.*	13.0	13.0	0.0

Positive control- Sodium propionate; Negative control- Dimethyl sulfoxide

Each value is the average of three independent replicates.

Table 5: Results of phytochemical tests for mango and amchur extract

S.No.	Phytochemical compounds	Fresh ripe pulp	Dried unripe pulp
1.	Tannin	+	+
2.	Saponin	-	-
3.	Flavanoid	-	-
4.	Steroid	-	-
5.	Terpenoid	+	+
6.	Alkaloid	-	-
7.	Cardiac glycosides	-	-

as they were not inhibited to such an extent. *A*mongst the Gram negative bacteria, *Pseudomonas aeruginosa* was found to be most susceptible to the ethanolic extract of unripe pulp. Sodium propionate (positive control, standard food preservative) exhibited lower activity compared to the amchur extract.

Extracts found to have inhibitory effects were further tested for determination of minimum inhibitory concentration (MIC) by two-fold macro-dilution method against the test bacterial species (Table 3). Data indicated that the MIC of ethanolic extract of amchur ranged from 500mg/mL for *Micrococcus luteus* to 62.5mg/mL for Bacillus mycoides.

Antifungal effects of the amchur extract against some fungi have also been investigated as shown in Table 4. The ethanolic extract was only partially active against *Alternaria sp.* and *Penicillium sp.* 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. The highest zone of inhibition was observed in *Alternaria sp.* and *Rhizopus sp.* with an IZD each of 14mm.

The phytochemical analysis of the ethanolic extract of amchur powder was also carried out. The study revealed the presence of tannins and terpenes in both the fresh ripe and dried unripe pulp of mango (amchur powder) (Table 5).

DISCUSSION

The present study reveals the antimicrobial potential of crude extracts of fresh and dried pulp of both ripe and unripe *Mangifera indica.* It was clearly observed that extracts prepared from dried unripe pulp of mango (amchur powder) revealed better antimicrobial activity than those extracts prepared from the fresh ripe pulp against both groups of bacteria. Amchur (dried unripe pulp of *Mangifera indica*) contains large amount of citric acid related compounds which is responsible for its sour taste. It is due to the change in pH of the medium due to amchur which cause the pH to bring down in acidic range. pH is known to control the growth, development and sporulation of all microbes including bacteria [11].

The antimicrobial activity of amchur extract is also due to presence of tannins and terpenes. It has been well documented that several terpenes (ocimene, myrcene, limonene), aldehydes and esters occur in dried unripe mango fruit [12]. Moreover, investigations into the effect of terpenoids upon isolated bacterial membrane have suggested that their activity is a function of the lipophilic properties, the potency of their functional groups and their aqueous solubility [13, 14]. Their site of action is at the phospholipid bilayer, caused by biochemical mechanisms catalysed by the phospholipid bilayers of the cell. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme – dependent reactions [13].

Furthermore, Gram positive bacteria were found to be more susceptible as compared to Gram negative bacterial species. This is probably due to the differences in chemical composition and structure of cell wall of both types of microorganisms.

The present study also confirms the use of organic solvents in the preparation of plant extracts as compared to aqueous extracts. The polarity of antimicrobial compounds make them more readily extracted by organic solvents and using organic solvents does not negatively affect their bioactivity against both bacterial and fungal species. The findings also showed that some antimicrobial substances could only be extracted by organic solvents, suggesting that organic solvents are clearly better solvents of antimicrobial agents [9].

CONCLUSION

In conclusion, amchur (dried unripe pulp of *Mangifera indica*) extract was found to be 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.

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