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Antibaterial activity of the extracts of pineapple and pomelo against five different pathogenic bacterial isolates

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To combat the infections caused by antibiotic resistant bacteria, natural candidates are being studied to find out antibacterial activity against the drug-resistant microorganisms. Among the variety of natural candidates of plant origin, many fruits have been proved to have potent antibacterial activity. In the current study, we chose pineapple (*Ananas comosus*), and pomelo (*Citrus maxima*) to determine their efficacy against some clinical isolates. Fruit samples were subjected to prepare crude, ethanol, methanol and aqueous extract to determine their antibacterial potency. Clinical isolates were used to determine the antibacterial activity of the extracts against them. The isolates were found to be multi-drug resistant. Out of twenty-eight antibiotics, *Pseudomonas aeruginosa* was resistant to ten antibiotics and *Salmonella* spp. was resistant to nine antibiotics. Rather than the crude extracts of the fruits, ethanol and methanol extracts showed antibacterial activity towards multi-drug resistant pathogenic bacteria. Aqueous extract did not show any significant antibacterial activity at all. Extracts of pomelo fruit exhibited the highest results whereas pomelo skin and pineapple peel crude extracts were the least effective compared to the other extracts. Ethanol extract of pineapple fruit (against all isolates but *Staphylococcus aureus*) and methanol extract of pomelo fruit (against all isolates) showed the lowest MIC (minimum inhibitory concentration) of 187.5 µg/ml. MBC (minimum bactericidal concentration) was found (within the range of 500 µg/ml to 1000 µg/ml) only with ethanol and methanol extracts of pomelo and pineapple. As the clinical isolates were found to be inhibited by the extracts, they can be used as an alternative for treating infections caused by these bacteria.

Keywords: Antibacterial activity, Antibiotic resistance, Extracts, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration.

INTRODUCTION

Antibiotic resistance has become a common scenario worldwide which is responsible for the higher rate of morbidity and mortality due to infections caused by resistant microorganisms (1-4). *Pseudomonas aeruginosa* isolated from the patients of tertiary health care facilities in Bangladesh showed decreased sensitivity towards antibiotics (5). Other microbes like *Escherichia coli* and *Klebsiella pneumoniae* also showed different degrees of resistance against antibiotics (ceftriaxone, levofloxacin, ciprofloxacin, ampicillin, amoxicillin) (6), *Shigella sonnei* (to ciprofloxacin, mecillinam, ampicillin, nalidixic acid, trimethoprime-sulfamethoxazole), *Acinetobacter* spp. (against gentamicin, ceftriaxone, amikacin, imipenem) (6-9). Moreover, multidrug resistant *Staphylococcus aureus*, *Streptococcus* spp., *Listeria monocytogenes*, *Salmonella* spp., *Vibrio* spp. etc. have also been reported in different countries including Bangladesh (10, 11). Antibiotics are losing their activity due to the development of drug resistance in the pathogens and also antibiotics possess some side effects which reinforced the need to search for alternate chemotherapeutic agents that can be effective for killing or inhibiting these resistant microfloras and will exhibit no side effects (12).

Many people still prefer herbal medicines prepared from plant origin to treat different kinds of diseases (infectious diseases, cancers, etc.) (13). Plant parts (leaves, fruits, seeds, bark, etc.) are used for treatment purposes due to the presence of many antimicrobial components like alkaloids, flavonoids, steroids, terpenoids, phenolic compounds, antioxidants, etc. (14-17). Among 5700 species of plants, almost about 700 have been listed as the therapeutic plant in Bangladesh (18). Several studies have found the antibacterial activity of some phytochemicals against antibiotic-resistant bacteria. Some of these phytochemicals include tannin (active against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Pseudomonas* spp.), favatin and circulin (against *E. coli*, *Pseudomonas* spp.), catechin (against *Staphylococcus* spp.), leaves and bark extracts (*Pseudomonas* spp., *Klebsiella* spp., *Yersinia* spp., *Salmonella* spp., *Bacillus* spp. etc.) (19, 20). Many micro and macronutrients of fruits work as immunostimulants capable to increase immune response after infection in patients of impaired immunity (21).

The aim of the study was to detect the antibacterial potency of pineapple (*Ananas comosus*), and pomelo

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(*Citrus Maxima*). Besides using the crude samples, ethanolic, methanolic, and aqueous extracts of both the fruits and their peel were used to determine their antibacterial activity. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were also determined after confirming the antibacterial traits of these extracts against five different pathogenic multi-drug resistant bacteria collected from clinical samples.

MATERIALS AND METHODS

Study area and sampling. For detection of antibacterial activity of some natural products, five clinical bacterial isolates (*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella* spp.) were selected. Two local fruit samples pineapple (*Ananas comosus*), and pomelo. (*Citrus maxima*), were collected from different markets of Dhaka city, Bangladesh for detection of antibacterial activity against the selected clinical bacterial isolates. Both fruit and the peel of the fruit have been used for this study. The experiment was carried out during the time span of September 2020 to December 2020 in the microbiology laboratory of the Department of Microbiology, Stamford University Bangladesh.

Antibiotic susceptibility test of the pathogenic isolates. For the detection of antibiotic resistance traits of the clinical isolates, twenty-eight antibiotics commonly in use were selected. Meropenum (MME 10µg), Ceftazidime (CAZ 30µg), Cefuroxime (CMX 30 µg), Amoxyclav (AMC 30 µg), Amoxicillin (AX 25 µg), Azithromycin (AZM 15 µg)), Cefixime (CFM 30 µg), Ciprofloxin (CIP 5 µg), Colistin (CO 30 µg), Doripenum (DOR 10 µg), Doxycycline (DO 30 µg), Fusidic acid (10 µg), Gentamycin (GN 10 µg), Amikacin (AK 30 µg), Cephradine (CE 30 µg), Vancomycin (VA 30 µg), Teicoplanin (TEC 30 µg), Cotrimazole (COT 30 µg), Piperocillin/Tazobactam (PTZ, PIT 30 µg), Nitrofurantion (F 300 µg), Nalidixic acid (NAL 30 µg) Impenem (IPM 10 µg), Levofloxacin (LE 5 µg), Linezolid (LZD 30 µg), Clindamycin (CN 10 µg), Cefepime (CPM 30 µg), Tigecycline (TGC 15 µg) and Ceftriaxone (CRO 30 µg). Kirby Bauer disc diffusion method (22) was followed for the antibiotic drug resistance test. Using CLSI guidelines (23) the zone sizes were measured and determined the strains as sensitive or resistant.

Sample processing. The fruit samples were washed vigorously first with tap water and then with distilled water several times to wash out all kinds of impurities. Crude extracts were prepared by blending 10 g of the raw fruits and fruit peel separately with 90 ml saline (24). Before extraction, raw samples were shed dried for a week after cutting into small portions to make it all dry followed by blending to get a fine powder. The dried powder samples were then further processed for extract preparation (25).

Preparation of solvent extracts. About 20 g of each dried and powdered fruit and peel samples were mixed with 80 ml of 95% ethanol, methanol, and water separately in sterilized glass bottles followed by incubation at 37^{o}C for 48 hours in shaking condition. After 48 hours, the ethanol, methanol, and aqueous extracts of all of these fruit and peel extract samples were filtered through sterilized cheesecloth and then through Whatman filter paper. Extracts were then concentrated by keeping them in evaporator and kept at 4^{o}C until use as stock solution (25).

Determination of antibacterial activity of the extracts (crude, ethanolic, methanolic , and aqueous extracts). Bacterial suspensions were prepared until they reach McFarland turbidity standard (10^8 CFU/ml) and bacterial lawn was made using sterile cotton swab on the Muller Hinton agar media (26). Crude, ethanol, methanol, and aqueous extracts (100 µl each) of pineapple, pineapple peel, pomelo, and pomelo peel were placed into the well made in the media. Plates were then kept in the refrigerator in an upright position for better absorption for 20 to 30 minutes and then incubated at 37^{o}C for 24 hours (25). Plates were observed for the presence of zone of inhibition after incubation and measured in mm.

Determination of MIC and MBC. Extracts of the samples were diluted in the concentrations of 500 µg/ml, 250 mg/ml, and 125 mg/ml with sterile nutrient broth followed by addition of 0.2 ml bacterial suspensions in each tube. After incubated at 37^{o}C for 24 hours, tubes with no visible growth will be considered for determining MIC using the following equation,
MIC=(lowest concentration of extract inhibiting growth+highest con. that allow growth)/2 (27). To detect the concentration of extracts, loop fool samples from the visibly clear tubes were inoculated onto fresh nutrient agar plates where no bacterial growth occurs (25). The complete absence of visible growth on the agar plate after streaking onto the medium was determined as the MBC (27).

RESULTS

Enterococcus spp., *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa,* all of the clinical isolates used in the study showed different degrees of resistance. The highest resistant bacteria were *Pseudomonas aeruginosa* which showed resistance to ten antibiotics out of twenty one tested antibiotics. *Salmonella* spp. was resistant towards nine antibiotics whereas *Klebsiella pneumoniae* showed resistance to one antibiotic (Amoxyclav) followed by intermediate results for three antibiotics (Azithomycin, Ciprofloxacin, Cefixime). *Staphylococcus aureus* was resistant to three antibiotics as well (Levofloxacin, Ciprofloxacin, Cefixime). *Escherichia coli* was resistant to three antibiotics (Azithromycin, Cotrimazole, Doxycycline) and intermediate resistance to one antibiotic (Amoxyclav). So, all of these isolates showed multi-drug resistance (mostly to azithromycin, cotrimazole, cefixime). While *Pseudomonas aeruginosa* and *Salmonella* spp. were found to be resistant against many antibiotics already, the other three bacteria still have many other antibiotics as drugs of choice (Table 1).

Crude extracts of pineapple, pineapple peel, pomelo, and pomelo peel showed very minimal antibacterial activity against the selected drug-resistant bacterial isolates. *Staphylococcus aureus* did not show any inhibition of growth with any of the crude extracts. Pineapple fruit extract showed the highest activity here against *Klebsiella pneumoniae* (8 mm). Pineapple peel and pomelo peel crude extracts were both only active against *Escherichia coli* (6 mm and 5 mm, respectively) (Table 2).

Pineapple fruit and peel, pomelo fruit and peel all of them showed some antibacterial activity in lower degree with ethanol, methanol, and aqueous extracts. Highest antibacterial activity was found with pineapple and pomelo fruits whereas the ethanol and methanol extracts peel of pineapple and pomelo showed antibacterial activity only against *Pseudomonas aeruginosa* and *Escherichia coli,* respectively. All extracts of both fruits were able to show zone of inhibition against *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* spp. (Table 3).

Ethanol extracts of pineapple showed antimicrobial activity against *Pseudomonas* spp. and *Salmonella* spp. (at 250 µg/ml). *Escherichia coli* showed inhibition with 500 µg/ml concentration. Ethanol extracts of pineapple peel showed positive results against *Pseudomonas aeruginosa* and *Salmonella* spp. The antimicrobial activity of pomelo fruit extract was constantly similar for all of these bacteria at 500 µg/ml concentration. *Escherichia coli* and *Pseudomonas aeruginosa* were inhibited by pomelo peel. Other isolates showed no inhibition of growth with pomelo peel ethanol extract (Table 4).

Table 1. Antibiotic susceptibility of the clinical isolates.

Antibiotics	Isolates				
	Escherichia coli	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Salmonella* spp.**
Amoxicillin	ND	ND	ND	ND	S
Azithromycin	R	R	S	I	R
Meropenum	S	S	S	S	R
Ceftazidime	S	S	ND	S	S
Ciprofloxin	S	S	R	I	ND
Gentamycin	S	S	S	S	R
Amikacin	S	S	S	S	R
Cefixime	S	S	R	I	R
Cefuroxime	S	R	S	S	R
Cephradine	ND	ND	S	ND	ND
Nitrofurantion	S	R	ND	ND	S
Vancomycin	ND	ND	S	ND	ND
Teicoplanin	ND	ND	S	ND	S
Cotrimazole	R	R	S	S	R
Piperocillin/ Tazobactam	S	S	ND	S	S
Colistin	S	S	ND	S	S
Doxycycline	R	R	S	S	S
Fusidic acid	ND	ND	S	ND	ND
Amoxyclav	I	R	S	R	S
Impenem	S	S	ND	S	ND
Linezolid	ND	ND	S	ND	ND
Doripenum	S	S	ND	S	S
Tigecycline	S	R	S	S	R
Clindamycin	ND	ND	S	ND	ND
Levofloxacin	S	S	R	S	S
Cefepime	S	R	ND	S	ND
Nalidixic acid	S	R	ND	ND	R
Ceftriaxone	S	R	S	S	ND

Note: R=Resistant, S= Sensitive/Susceptible, I= Intermediate, ND= Not Done.

Table 2. Antibacterial activity of crude extracts of pineapple fruit, pineapple peel, pomelo fruit, and pomelo peel samples against pathogenic bacteria (Zone of inhibition was measured in mm).

Isolates	Crude extracts			
	Pineapple fruit	**Pineapple peel**	**Pomelo fruit**	**Pomelo peel**
Escherichia coli	-	6 mm	-	5 mm
Pseudomonas aeruginosa	5 mm	-	6 mm	-
Staphylococcus aureus	-	-	-	-
Klebsiella pneumoniae	8 mm	-	-	-
Salmonella spp.	-	-	6 mm	-

Table 3. Antibacterial activity of ethanol, methanol, and aqueous extracts (100 µl) of the fruit samples against pathogenic bacteria (Zone of inhibition was measured in mm).

Isolates	Samples											
	Pineapple Fruit			**Pineapple Peel**			**Pomelo Fruit**			**Pomelo Peel**		
	Ethanol	**Methanol**	**Aquaous**	**Ethanol**	**Methanol**	**Aquaous**	**Ethanol**	**Methanol**	**Aquaous**	**Ethanol**	**Methanol**	**Aquaous**
Escherichia coli	15	10	7	-	-	-	10	10	7	10	10	-
Pseudomonas aeruginosa	15	10	6	10	10	-	10	10	8	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	8	10	-	-	-	-
Klebsiella pneumoniae	10	10	-	-	-	-	8	8	-	-	-	-
Salmonella spp.	15	12	7	-	-	-	10	10	6	-	-	-

Methanol extracts of pineapple fruit showed activity with all isolates (at 500 µg/ml concentration) except *Staphylococcus aureus*. Methanol extract of pineapple fruit was able to inhibit *Pseudomonas aeruginosa* and *Salmonella* spp. at 500 µg/ml and 1000 µg/ml concentrations, respectively. On the other hand, pomelo fruit extract showed the best result here. It was able to inhibit visible bacterial growth for all of the five isolates at varying concentrations. Pomelo peel showed minimal activity only against *Pseudomonas aeruginosa* at 500 µg/ml concentration (Table 5). Aqueous extracts of pineapple peel and pomelo peel both showed no antibacterial activity up to 1000 µg/ml concentration against any of the five clinical bacterial isolates. Pineapple fruit and pomelo fruit both showed activity towards *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* spp. at 1000 µg/ml concentration (Table 6).

After detecting the growth inhibition visually, the MIC was calculated. Lowest MIC (187.5 mg/ml) was counted for ethanol extract of pineapple fruit (against *Salmonella* spp.), and methanol extract of pomelo fruit (against *Escherichia coli, Staphylococcus aureus* and *Salmonella* spp.). Aqueous extract of pineapple peel and pomelo peel showed no MIC against any of the clinical isolates. Pomelo fruit showed the highest MIC concentration compared to pineapple (Table 7).

Finally, the minimum bactericidal concentration was determined for ethanol, methanol and aqueous extracts against the multi-drug resistant bacterial isolates. No MBC was found for the aqueous extracts of pineapple fruit, pineapple peel, and pomelo peel. Best result was found with ethanol and methanol extract of pomelo fruit against all of the five isolates at 500 µg/ml concentration (*Staphylococcus aureus*-ethanol and methanol extract, *Klebsiella pneumoniae*-ethanol and methanol extract, *Salmonella* spp.-methanol extract, *Escherichia coli*- methanol extract) and 750 µg/ml concentration (*Escherichia coli*-ethanol extract, *Pseudomonas aeruginosa*- ethanol and methanol extract, *Salmonella* spp.- ethanol extract). Highest concentration was 1000 µg/ml which was found against *Klebsiella pneumoniae* (pineapple fruit- methanol extract), *Salmonella* spp. (pineapple peel-ethanol and methanol extract) (Table 8).

Table 4. Determination of MIC (minimum inhibitory concentration) of the ethanol extracts against five different pathogens (concentrations in µg/ml).

Isolates	**Estract samples**											
	Pineapple Fruit			**Pineapple Peel**			**Pomelo Fruit**			**Pomelo Peel**		
	Ethanol	**Methanol**	**Aqueous**	**Ethanol**	**Methanol**	**Aqueous**	**Ethanol**	**Methanol**	**Aqueous**	**Ethanol**	**Methanol**	**Aqueous**
Escherichia coli	375	375	750	-	-	-	375	187.5	375	375	-	-
Pseudomonas aeruginosa	187.5	375	750	375	375	-	375	375	750	375	375	-
Staphylococcus aureus	-	-	-	-	-	-	375	187.5	-	-	-	-
Klebsiella pneumoniae	750	375	-	-	-	-	375	375	-	-	-	-
***Salmonella* spp.**	187.5	375	750	750	750	-	375	187.5	750	-	-	-

Note: (-) = not found upto 1000 µg/ml concentration.

Table 5. Determination of MBC (minimal bactericidal concentration) of the extracts (concentrations in µg/ml).

Isolates	**Extract samples**											
	Pineapple Fruit			**Pineapple Peel**			**Pomelo Fruit**			**Pomelo Peel**		
	Ethanol	**Methanol**	**Aquaous**	**Ethanol**	**Methanol**	**Aquaous**	**Ethanol**	**Methanol**	**Aquaous**	**Ethanol**	**Methanol**	**Aquaous**
Escherichia coli	750	750	-	-	-	-	750	500	500	500	-	-
Pseudomonas aeruginosa	500	750	-	500	500	-	750	750	-	750	750	-
Staphylococcus aureus	-	-	-	-	-	-	500	500	-	-	-	-
Klebsiella pneumoniae	-	1000	-	-	-	-	500	500	-	-	-	-
***Salmonella* spp.**	500	500	-	1000	1000	-	750	500	-	-	-	-

Note: (-) = not found upto 1000 µg/ml concentration.

DISCUSSION

Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and *Salmonella* spp. all are responsible for different health issues. *Escherichia coli* is responsible for urinary tract infections (28). *Pseudomonas aeruginosa* can cause a wide magnitude of problems in immune-compromised people. Some of the diseases include urinary tract infection, cystic fibrosis, pneumonia, surgical site infection, bloodstream infection, etc. (29). *Klebsiella pneumoniae* can be the reason for pyogenic liver abscesses and meningitis (30). *Staphylococcus aureus* is capable to initiate pleuropulmonary infection, bacteremia, endocarditis, etc. (31). *Salmonella* spp. has been reported to cause enteric fever, gastroenteritis, bacteremia, extra-intestinal complications, etc. (32). In all of the above mentioned disease conditions, antibiotics are prescribed but the development of multi-drug resistance is a challenge in combating the infections. As the days are going by, more resistance is showing up. There are many reasons for antibiotic resistance (33). In many developing countries antibiotics are randomly used in food and water as a part of preliminary treatment. Patients are often taking antibiotics without even consulting with the doctor. Antibiotics are sold without valid prescriptions as well. Bacteria often get used to with the presence of antibiotics and evolve to withstand the antibiotic presence. Bacteria can produce enzymes to inactivate the antibiotics, can bypass the metabolic pathway, which was aimed by the drug to stop, modifying the antibiotic binding site, etc. (34-36).

As pathogenic bacteria are becoming more and more resistant to antibiotics, it is necessary to be ready with alternated effective drugs to fight the diseases caused by the resistant bacteria. In this study, we used two different fruits (pineapple, pineapple peel, pomelo, pomelo peel) to find out if they possess any antibacterial activity against these bacteria. Though they showed incredibly low potency as the crude extract, the ethanol and methanol extracts showed quite effective results. Aqueous extracts did not show any satisfactory result at all to any of the isolates. Except for pomelo fruit, crude extracts showed potency against one or two bacteria only. But the effectivity showed much higher after extraction with ethanol and methanol compared to crude extract. Highest activity was found by ethanol and methanol extracts of pomelo fruit against five bacterial pathogens. The fruits used in the study are very popular among people due to their taste and nutritional value (37). Overall, all the isolates showed sensitivity towards one extract or another (ethanol/methanol extract of fruit/peel) out of four extracts. *Pseudomonas aeruginosa* showed the best susceptibility towards the extracts compared to other isolates. Other researchers also found antibacterial activity of these fruits against pathogenic bacteria (38). Pineapple has been previously been found to have antibacterial activity against *Staphylococcus aureus* (38). Similarly, pomelo showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (39).

Most of the cases showed MBC within 1000 µg/ml for all kinds of extracts used in the study. Other extracts with which we did not find MBC, might show the MBC with higher concentration. The lowest concentration of extract showing MBC was 500 µg/ml. Antbacterial activity of some other fruits (like lychee, date palm, black palm, jackfruit etc.) besides pineapple and pomello have also been reported by other researches (40). As we have found the MBC from some of the extracts of the fruits, they might be a valuable alternative source for treating the antibiotic-resistant pathogenic bacteria.

CONCLUSION

Natural candidates are potent alternate sources for searching antibacterial activity which can be used against antibiotic-resistant bacteria. Many plants, fruits, leaves, barks have been proved to be effective in this way to treat infections. Pineapple fruit, pineapple peel, pomelo fruit, and pomelo peel also possess such antibacterial activity which was found against some multidrug resistant clinical isolates. Identification of the specific phytochemical would be the next step in determining the way to use these chemicals as therapeutic agents.

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