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Antimicrobial Activity and Toxicity of *Punica granatum L.*, Peel

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Abstract

Methanols extract of the *Punica granatum* L. (Punicaceae) peel was screened for antimicrobial activity against enteric pathogenic bacteria. Antimicrobial tests were carried out using the disk diffusion assay and broth dilution method. The extract showed favorable antimicrobial activity against enteric pathogenic bacteria tested with a minimum inhibitory concentration (MIC) value of 12.50 mg/ml. Furthermore, the extract was also tested against brine shrimp for toxicity. The extract exhibited no significant toxicity (LC_{50} = 1.42 mg/ml) against *Artemia salina*. The study confirms the possible antimicrobial potentiality of the peel extract of *P. granatum*. The study shows that the extract is a favorable antimicrobial agent with potential applications in public health against diseases.

Key Words: Antimicrobial activity, Enteric bacteria, *Punica granatum*

INTRODUCTION

Punica granatum L., belongs to the family Punicaceae which includes only one genus and two species, the other one, little-known, being *P. protopunica* Balf, peculiar to the island of Socotra. *P. granatum* originating from the Middle East, extending throughout the Mediterranean, eastward to China and India, and on to the American Southwest, California and Mexico in the New World [1]. While the pomegranate plant is considered either a small tree or a large shrub, its fruit is often deemed to be a large berry. The fruit is delimited by a leathery pericarp, contained within are numerous *arils*, each a single seed surrounded by a translucent juice-containing sac. Thin acrid-tasting membranes extend into the interior of the fruit from the pericarp, providing a latticework for suspending the arils. Thus, the fruit itself gives rise to three parts: the seeds, about 3% of the weight of the fruit, and themselves containing about 20% oil, the juice, about 30% of the fruit weight, and the peels (pericarp) which also include the interior network of membranes. Other useful parts of the plant include the roots, bark, leaves, and flowers [1].

Bacteria have evolved numerous defenses against antimicrobial agents, and drug-resistant pathogens are on the rise. In the recent years, incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented [2]. Therefore, search for new antimicrobials to combat infectious diseases caused by multidrug-resistant bacteria including fast-spreading ESβL-producing enteric bacteria is urgently needed. In this study, we examined the toxicity and antibacterial activity methanolic extract of *P. granatum* peel against several enteric bacteria.

MATERIALS and METHODS

Materials

Peel of *P. granatum,* was collected from Bandar Laguna Merbok. It was identified and classified by a botanist at the Biotechnology Department of Asian Institute of Medicine, Science and Technology (AIMST University), Kedah, Malaysia.

Preparation of the crude extract

The pulverized sun dried peel of *P. granatum* (60 g) was boiled in a Soxhlet with 400 ml of methanol (v/v) for 72 h. The entire extract of *P. granatum* peel was evaporated to dryness in a rotary evaporator. The dried fractions were then redissolved in methanol to yield the solution containing 100 mg of extract per milliliter solution.

Test microorganisms and growth media

The following enteric pathogenic bacteria were used for antimicrobial activities studies included *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 19430 and *Shigella dysenteriae.* The bacterial strains were grown in Mueller–Hinton agar (MHA) plates at 37 °C. The stock culture was maintained at 4 °C.

Antimicrobial disk diffusion assay

Antibacterial activities of peel of *Punica granatum* extract was investigated by the disk diffusion method [3-4]. The MHA plates, containing an inoculum size of 10^6 colony-forming units (CFU)/ml of bacteria were spread on the solid plates with an L-shaped glass rod. Then disks (6.0-mm dia) impregnated with 25 µl of each extracts at concentration of 100 mg/ml were placed on the inoculated plates. Similarly, each plate carried a blank

disk by adding solvent control alone in the center to serve as a control, and antibiotic disks (6.0-mm dia) of Gentamicin (10µg/ml) and kannamycin (30µg/ml) were also used as positive controls. All of the plates were incubated at 37 °C for 18 to 24 h. The zones of growth inhibition around the disks were measured after 18 to 24 h of in incubation at 37 °C. The sensitivity of the microorganism species to the plant extracts was determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values <8 mm were considered as not active against microorganisms. All of the experiments were performed in triplicate. The results are reported as the average of 3 experiments.

Determination of minimum inhibitory concentration (MIC)

MIC was determined by both agar and broth dilution methods [5]. Two-fold serial dilutions (0 to 100.0 mg/ml) of the extract were prepared in Mueller-Hinton broth. For broth dilution tests, 0.1 ml of standardized suspension of bacteria (10^6 CFU/ml) was added to each tube (containing fractions of extracts at a final concentration of 0 to 100.0 mg/ml) and incubated at 37 °C for 24 h. MICs were taken as the average of the lowest concentration showing no growth of the organism and the highest concentration showing visible growth by macroscopic evaluation [6]. Each assay was performed in triplicate.

Toxicity testing against the brine shrimp

Hatching shrimp

Brine shrimp eggs, *Artemia salina*, were hatched in artificial seawater prepared by dissolving 38 g of salt in 1 l of distilled water. After a 24-h incubation period at room temperature (22-29°C), the larvae were attracted to one side of the vessel with a light source and collected with a pipette. The larvae were separated from the eggs by aliquoting them three times in small beakers containing seawater.

Brine shrimp assay

The bioactivity of the extract was monitored by the brine shrimp lethality test [7]. Samples were dissolved in DMSO and diluted with artificial seawater. Two milliliters of seawater was placed in all the bijoux bottles. A two-fold dilution was carried out to obtain the concentration from 10 to 0.1525 mg/ml. The last bottle was filled with sea salt water and DMSO only, serving as a drug-free control. Suspensions of larvae, (100 µl) containing about 10-15 larvae were added into each bottle and incubated for 24 h. The bottles were then examined and the number of dead larvae in each bottle was counted. The total number of shrimp in each bottle was counted and recorded. The mean percentage mortality was plotted against the logarithm of concentrations, and the concentration that could kill 50% of the larvae (LC_{50}) was determined from the graph.

Data analysis

The mean results of brine shrimp mortality against the logarithms of concentrations were plotted using the Microsoft Excel computer program, which also presents regression equations. The regression equations were used to calculate the LC_{50} value. Extracts giving LC_{50} values greater than 1 mg/ml are considered to be nontoxic [8-9].

RESULTS

Antimicrobial activity

The results of antimicrobial activity of the extract against tested microbes are given in the Table 1. The extract exhibited a favorable activity against the bacteria tested. The zone of clearance produced by the commercial antibiotic disk was lower than those produced by the extract disk. The agar dilution method recorded the MIC value of 12.50 mg/ml.

Toxicity testing against brine shrimp

The result of the toxicity evaluation against the brine shrimp of the extract is shown in Table 2. The extract showed no significant toxicity against brine shrimp as the LC_{50} value

Table 1. Antimicrobial activity (Zone of inhibition) of *P. granatum* peel extract

Microorganism	Zone of inhibition (mm)[a]				
	Crude extract 100 (mg/ml)	Gentamicin (10µg/ml)	kannamycin (30µg/ml)	Methanol	MIC (mg/ml)
Escherichia coli	26	10	14	0	12.5
Salmonella typhi	25	13	23	0	12.5
Shigella dysenteriae	26	14	13	0	12.5

MIC, minimum inhibitory concentration.
[a] Agar dilution method, mean value n = 3.
[b] The values (average of triplicate) are diameter of zone of inhibition
[c] MIC of *P. granatum* peel extract

Table 2. The toxicity effects of the *P. granatum* peel methanol extract using brine shrimp lethality assay after 24 h

Concentration (mg/mL)	Log_{10} concentration	Number survived	Number dead	Percent mortality (%)
Control	-	15.00	0.00	0.00
10.00	1.000	0.00	15.00	100.00
5.00	0.699	1.00	14.00	93.33
2.50	0.398	6.00	9.00	60.00
1.25	0.097	8.00	7.00	46.66
0.63	-0.204	9.00	6.00	40.00
0.31	-0.505	13.00	2.00	13.33
0.61	-0.903	13.00	2.00	13.33

The LC_{50} was obtained by linear regression equations.
LC_{50} value greater than 1.00 mg/ml was considered to be nontoxic.
LC_{50} value of extract of *P. granatum* peel was 1.42 mg/ml.

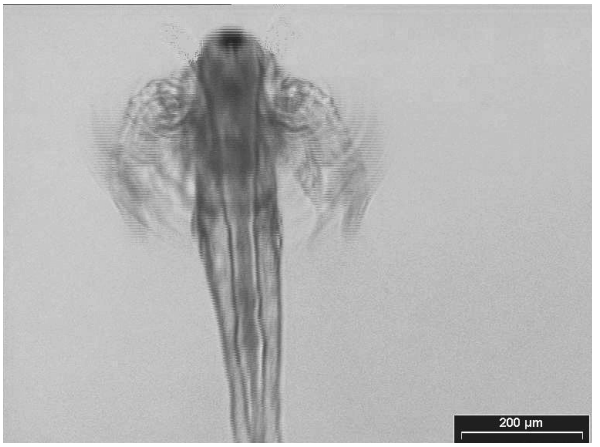

Figure 1. Light microscope micrograph of the P. granatum peel extract treated Artemia salina.

was 1.42 mg/ml. Figure 1 showed the light microscope micrograph of dead *A. salina.*

DISCUSSION

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain [10]. Therefore actions must be taken to reduce this problem, for example to regulate the use of antibiotic, concerted research efforts should be focused to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs [11]. The miracle of plants as an alternative approach to treat diseases is now being investigated, as modern medicine is declining in its function.

In this study, we particularly concentrated on enteric pathogen because of the increasing occurrence of drug resistance and wide spread of incidence of diarrhea and dysentery to human. Hence, antimicrobial substances found in certain plants could solve the problem of these enteric pathogens.

From this study, it appears that the extract of the peel of *P. granatum* exhibited a favorable antimicrobial activity against enteric pathogens tested. The results on brine shrimp assay indicate that the extract has a LC_{50} value greater than 1 mg/ml, which is the recommended cutoff point for detecting cytotoxic activity [9]. This suggests that this plant may not be toxic. The results of the current study concur with the use of this plant by native people for food.

Therefore, infection of enteric pathogenic bacteria could be treated by the extract of peel of *P. granatum*, as the MIC for these bacteria was found to be 12.00 mg/ml. In addition, it may be used as an antibacterial agent in known dosages especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities are inaccessible.

Further purification of the active compounds and *in vivo* evaluation of antibacterial activity of the extract from peel of *P. granatum* are therefore suggested for further studies on the basis of the present preliminary study.

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