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Evaluation of the Antimicrobial Potential of *Punica granatum* and *Origanum vulgare*

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Abstract
Aim: To evaluate the antimicrobial potential of *Punica granatum* and *Origanum vulgare* against some bacterial strains. **Methods:** The aqueous extracts were screened for their antimicrobial potential and physiochemical parameters such as extract concentration and temperature were optimized. The MIC of the most sensitive microorganism was worked out. **Results:** The 10% aqueous extract of *Punica granatum* (pomegranate) and *Origanum vulgare* (Oregano) were screened for their antimicrobial activity against bacteria like *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhimurium, Staphylococcus epidermidis*, where the average inhibition zone of *Punica granatum* ranged from 11-19mm with *Salmonella typhimurium* being the most sensitive (19mm). *Origanum vulgare* did not show effectiveness against the test organisms at 10% concentration. Optimization studies showed 20% extract concentration and an extraction temperature of 60°C to be the optimal conditions which would support the best antimicrobial activity of the plant extracts. The MIC of *Punica granatum* against *Staphylococcus epidermidis* was found to be 50mg/ml. **Conclusion:** These findings suggest that these plants can be used for development of antimicrobial compounds in time to come.

Keywords
Antibiotic resistance; Antimicrobial potential; Agar Diffusion Assay; Microorganisms; Medicinal plants; Minimum Inhibitory Concentration (MIC)

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INTRODUCTION
The plants and its products are being used for years as traditional and allopathic medicine. Since ancient times, several herbs and spices were used in food, not only as a flavouring agent and a food preservative but also as a folk medicine [1]. Researchers have found many plants species containing medicinal properties due to the presence of various secondary metabolites such as glycosides, saponins, flavonoids, steroids, tannins, alkaloids and terpenes etc, which are traditionally used by our ancestors to cure diseases [2]. With the advancement in Science and Technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs. However, only one third of the infectious diseases are known have been treated from these synthetic products. This is because of the increasing antibiotic resistance among different microbial species as a result of overuse and misuse of antibiotics. Antibiotic resistance has increased significantly in the recent years and has augmented therapeutic problem among humans. One of the methods to reduce the resistance to antibiotics is by using alternative compounds from plants. Plants are known to produce an array of compounds to protect themselves against a variety of pathogens. Different parts of the medicinally important plants have been

used by the people for treatments of numerous human diseases for thousands of years around the globe. The different herbal plant extracts were traditionally used for their anticancer, antioxidant, antiulcer, analgesic and antidiabetic properties. They are also known to have antiparasitic, antifungal, antibacterial, antimalarial, analgesic and anti-inflammatory activity.

Keeping the antimicrobial activity in mind, two plants, *i.e*., *Origanum vulgare* (Oregano) dried leaves and *Punica granatum* (Pomegranate) dried peels were studied for their antibacterial potential. By studying some of the published literature showing the medicinal importance of above plants, the present protocol has been outlined regarding the antimicrobial activity on these selected plants using aqueous extracts. In view of this, that the present research was set up to evaluate the antibacterial activity of herbal plants (*Punica granatum* and *Origanum vulgare*) extracts against some bacteria.

MATERIAL AND METHODS

Plant material and its extract preparation: Plant materials *i.e*. dried leaves of *Origanum vulgare* and dried peel of *Punica granatum* used in the present research were obtained from the local markets of Amritsar. The plant material was surface sterilized by first washing with autoclaved distilled water and soaking them in 1% mercuric chloride ($HgCl_2$) for 5 min followed by rinsing them 3 to 4 times using sterilized distilled water and then drying at 40˚C for 3-4 days. The dried plant material was grinding separately into powder form using an electric grinder. The aqueous extracts of each plant were prepared by weighing (1g) of powdered plant material and suspending it in autoclaved distilled water (10 ml) under sterile conditions. The suspension was thoroughly mixed and kept in a hot water bath for extraction at 40˚C for 20 min. The extracted material was filtered through muslin cloth and the filtrate was subjected to antimicrobial screening by Agar Well Diffusion Assay (ADA)[3].

Test microorganisms: The reference bacterial strains *viz*., *Salmonella typhimurium, Klebsiella pneumoniae* as Gram negative bacteria and *Staphylococcus epidermidis, Staphylococcus aureus* as Gram positive bacteria were obtained from Department of Microbiology, Guru Nanak Dev University, Amritsar. The bacterial cultures were grown on nutrient agar medium. Each culture was further maintained by subculturing the test strains regularly on the same medium and stored at 4ºC before use in experiments.

Inoculum preparation: The inoculum was prepared by using a loopful of culture and inoculating it into 5mL of nutrient broth followed by 4 h incubation at 37˚C before using it for screening.

Antimicrobial screening: Ten percent aqueous extract of dried peel of *Punica granatum* and dried leaves of *Origanum vulgare* were tested for their antimicrobial activity against two Gram negative and two-Gram positive bacterial strains. For this 0.1mL of the activated test organisms (prepared above) were inoculated on sterile medium plates by spread plate method. The diameter of wells (8mm) was made on sterile plates with the help of sterile stainless-steel borer. 0.1 mL of plant extracts were poured into wells and water was used as control. Plates were incubated in upright position at 37˚C for 24 hours. Sensitivity was measured by the diameter of resultant zone of inhibition. Any organism with a zone of inhibition < 12 mm was considered to be resistant. The experiment was performed in duplicate and repeated twice [3].

Optimization of physiochemical parameters using one factor-at-a-time (classical) approach: Two physiochemical parameters *i.e.,* extract concentration and temperature, were optimized for obtaining the best antimicrobial activity using Agar Diffusion Assay (ADA).

Effect of concentration: After screening the *Punica granatum* and *Origanum vulgare* extracts against the test organisms, the effect of concentration of aqueous plant extracts on on the microbial strains was determined by using different concentrations (5%-20%) for their anti- microbial activity using Agar Diffusion Assay (ADA).

Effect of Temperature - In order to ascertain optimum extraction temperature, the plant material at a concentration, optimized above, was extracted in hot water bath at different temperatures *i.e*.(30˚C-100˚C) for 20min. The extracts were then filtered and subjected to antimicrobial screening by Agar Diffusion Assay method [3].

Minimum Inhibitory Concentration (MIC)

It was performed by using Broth microdilution method against the most sensitive organism *Staphylococcus epidermidis*. The two-fold serial dilution of the aqueous extract of *Punica granatum* was made using sterile nutrient broth, with concentrations ranging from 20%-0.156%. To these concentrations, 50µl of the 4h activated culture was added. The plates were incubated at 37˚C for 24h. The minimum concentration where no visible growth was seen was taken as MIC. The well containing only culture was taken as positive control.

RESULTS

Preliminary screening of the aqueous extract for antimicrobial activity

Aqueous extract (10%) of dried peel of *Punica granatum* (Pomegranate) and dried leaves of *Origanum vulgare* (Oregano) were tested for their antimicrobial activity against four bacterial strains by Agar Diffusion Assay (ADA). *Punica granatum* exhibited a broad-spectrum antimicrobial activity as it showed inhibitory action against all four tested strains with its average zone of inhibition ranging from 16-21 mm (Table 1). *Staphylococcus aureus* showed maximum sensitivity closely followed by *Staphylococcus epidermidis> Salmonella typhimurium> Klebsiella pneumoniae,* while no zone of inhibition was observed in the case of *Origanum vulgare* at this particular concentration.

Table1. Screening of *Punica granatum* and *Origanum vulgare* 10% aqueous extracts for their antimicrobial activity against bacterial strains.

Bacterial Strain	Zone of Inhibition (mm)	
	Punica granatum	***Origanum vulgare***
Salmonella typhimurium	18	-
Klebsiella pneumoniae	16	-
Staphylococcus epidermidis	20	-
Staphylococcus aureus	21	-

Based upon the above results, various physicochemical parameters affecting the antimicrobial potential such as concentration and temperature were screened against tested organisms.

Effect of concentration: An increase in the anti-microbial activity of *Punica granatum* and *Origanum vulgare* was observed with increase in the concentration of the extract.

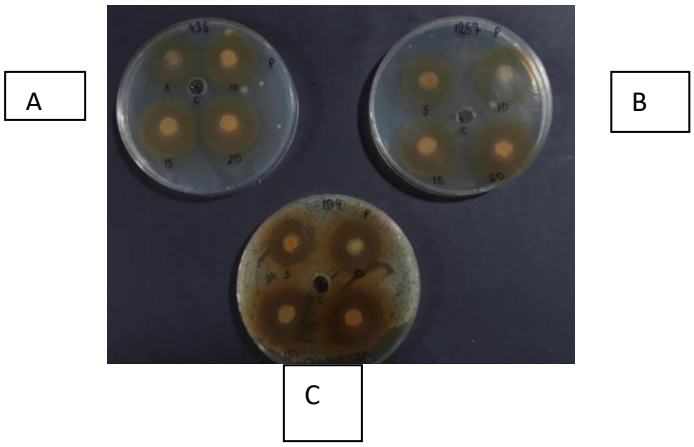
In case of *Punica granatum* (Figure 1), diameter of zone of inhibition increased with increase in concentration such that maximum activity was observed at a concentration of 20% with average inhibition zones ranging 18-20 mm where *Salmonella typhimurium* and *Staphylococcus epidermidis* (21mm) were most sensitive followed by *Klebsiella pneumoniae* and *Staphylococcus aureus* (Table 2).

Table 2. Effect of different concentrations of *Punica granatum* extracts on test microorganisms

	Zone of Inhibition (mm)			
Microorganism	**5%**	**10%**	**15%**	**20%**
Salmonella typhimurium	17	19	20	21
Klebsiella pneumoniae	15	18	20	20
Staphylococcus epidermidis	-	19	21	21
Staphylococcus aureus	15	19	20	20

(-) means no zone of inhibition

Figure 1: Effect of different concentrations of *Punica granatum* on tested organisms (A): *S.epidermidis* (B) *S. typhimurium*(C) *K. pneumoniae*



In case of *Origanum vulgare*, no zone was observed upto a concentration of 15% in *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Staphylococcus epidermidis,* while *Staphylococcus aureus* showed sensitivity at a concentration of 15% *i.e*. (13 mm). Concentration of 20% was sensitive against *Salmonella typhimurium* and *Staphylococcus aureus,* with larger zone of inhibition (18 mm) in case of *S. aureus* (Table 3). Therefore, in case of both plants, 20% extract concentration was chosen to be the optimal.

Table 3 Effect of different concentrations of *Origanum vulgare* extracts on test microorganisms

	Zone of Inhibition (mm)			
Concentration	**5%**	**10%**	**15%**	**20%**
Salmonella typhimurium	-	-	-	16
Klebsiellapneumoniae	-	-	-	-
Staphylococcus epidermidis	-	-	-	-
Staphylococcus aureus	-	-	13	18

(-) means no zone of inhibition

Effect of temperature: In this study a temperature range of 40°C-100°C was used for extracting the plant material. *Punica granatum* has showed maximum activity at the temperature of 60°C showing maximum activity against *S. epidermidis* (24 mm) followed by *K. pneumoniae* (23mm), *S. typhimurium* (22mm), *S. aureus* (21mm). At 80°C, the activity marginally decreased, falling in the range of 19- 21 mm at 80°C while the extract was not active at 100°C (Table 4).

Table 4 Effect of extraction temperature of plant extract of *Punica granatum* on its antimicrobial activity

	Zone of Inhibition (mm)			
Microorganism	**40°C**	**60°C**	**80°C**	**100°C**
Salmonella typhimurium	20	22	21	-
Klebsiella pneumoniae	20	23	21	-
Staphylococcus epidermidis	22	24	19	-
Staphylococcus aureus	19	21	19	-

In case of *Origanum vulgare,* zone of inhibition was observed in *S. typhimurium and S. aureus* at a temperature of 40°C (15mm and 18mm), 60°C (18mm and 19mm) and 80°C (none and 16mm), respectively with best extraction at 60°C. *S. aureus* has showed maximum inhibition zone *i.e*. 19 mm. With an increase in extraction temperature, the antimicrobial activity decreased further. The effect of different extraction temperature on *S. typhimurium* showed increase in their activity as zones observed at 40°C was 16 mm and at 60°C it was 18mm. Extracts at 100°C showed no antimicrobial activity against any tested organism as it has lost antimicrobial activity due to increased temperature (Table 5).Therefore, in case of both plants, 60°C extract temperature was chosen to be the optimal.

Table 5. Effect of extraction temperature on the anti-microbial activity of *Origanum vulgare* on test microorganisms

	Zone of Inhibition (mm)			
Microorganism	**40°C**	**60°C**	**80°C**	**100°C**
Salmonella typhimurium	15	18	-	-
Klebsiella pneumoniae	-	-	-	-
Staphylococcus epidermidis	-	-	-	-
Staphylococcus aureus	18	19	16	-

Minimum Inhibitory Concentration (MIC)

The MIC of *Punica granatum* against *Staphylococcus epidermidis* was found to be 50mg/ml.

DISCUSSION

Natural products of microbial or plant origin have played an invaluable role in drug discovery. Now a day, antibiotic resistance is an emerging global

problem, caused by misuse and overuse of antibiotics, and has serious negative effects in designing the treatment. Natural resources especially actinomycetes, fungi and plants have been a rich repository for producing various antimicrobial agents. Recent years have witnessed a renewed interest towards exploring plants [4]. In the present study, dried peel of *Punica granatum* and dried leaves of *Origanum vulgare* were subjected to antimicrobial activity screening against some test strains. *Punica granatum* showed a promising potential against all the four pathogens in the range 16-21mm. These results are in consonance with the previous studies on the same plant against various pathogens [5, 6]. The 10% extract of *Origanum vulgare* did not show any antimicrobial potential against any test pathogen may be due to non-extractability and low abundance of the bioactive compound at the used extraction concentration and temperature. Further, 20% concentration and 60°C extraction temperature were found to be most suitable for the antimicrobial activity. It may be due to abundant amount of the bioactive components at this concentration which showed the antimicrobial activity and better extractability of the antimicrobial compounds at this temperature. These results are in agreement with previous studies on other medicinal plants [3, 7]. The MIC of *Punica granatum* against *Staphylococcus epidermidis* was 50mg/ml, which was comparable or even better the values obtained in another study against various medicinal plants [8].

CONCLUSIONS

These finding highlight the importance of the study and make these plants a suitable candidate for development of potential antimicrobial compounds in the time to come.

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