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Research Article

IN-VITRO ANTIBACTERIAL ACTIVITY OF POMEGRANATE AND *DARU* (WILD POMEGRANATE) AGAINST DENTAL PLAQUE BACTERIA

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ABSTRACT

Punica granatum is used widely in tropical and subtropical countries as source of antimicrobial agent against a variety of dental bacteria. A major cause of the dental disease is believed to be commensal bacteria which exist in dental plaque**.** In the present investigation we compared wild pomegranate (daru) and cultivated pomegranate seeds, white membrane and peel extracts were comapred for their *in vitro* antibacterial potentiality. The antibacterial activity of methanolic fruit extract was evaluated against isolated bacteria by agar well diffusion method. Maximum antibacterial activity was shown by methanolic extract of the *daru* peel. The MIC is recorded as the lowest concentration of drug which showed clear fluid without turbidity. MIC of *Punica granatum* peel ranged from 0.2 to 3.2 mg ml^{-1}. The present findings suggest that the methanolic extract of peel of *daru* can be used as a promising novel antibacterial agent in near future.

Keywords: Antibacterial activity, Methanolic seed, Peel and white membrane extracts, Antibiotic resistance, *Punica granatum, Daru* (wild pomegranate)

INTRODUCTION

The oral micro biota functions as a part of the host defence by acting as a barrier, e.g., by competition for essential nutrients and creation of unfavourable conditions to exogenous organisms that may be pathogenic to the host, but under certain conditions some can cause oral infections like caries or periodontal disease [1]. The human oral cavity is habitat for about 500 cultivable and non-cultivable bacterial species [2]. As many as 400 distinct bacterial species may be found in plaque. Dental plaque formation begins with the initial colonization of the pellicle by *S. mutans, S. gordonii, S. oralis and S. mitis*. Once the tooth surface is colonized, subsequent attachment of other bacteria like *Staphylococcus sp., Lactobacillus sp.,* result due to various specific coaggregation reactions. The advent of allopathic medicine turned attention of scientists from plant sources to synthetic preparations as the basis for modern drugs. However, side-effects of many modern drugs along with the development of drug-resistant organisms have brought back into focus the studies of natural sources. Micro-organisms have developed resistance to many antibiotics and this created immense clinical trouble in the treatment of infectious diseases [3, 4]. This resistance has increased due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. Recently interest developed in usages of traditional medicinal plants and their products throughout the world [5-11]. Two hundred and fifty years ago there were few or no synthetic medicines. The 250,000-300,000 species of higher plants were the main source of drug for the world population. Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Attention has been focused on phytochemicals as potential sources of functional substances such as antimicrobial substances [12, 13].

Pomegranate extract kills micro-organisms, the yellowish build-up isolated from the dental plaque of healthy adults. The traditional knowledge reveals that the bark and rind of fruits is used in tanning and as vermifuge especially in cough and cold. Extracts of all parts of the fruit appear to have therapeutic properties [14]. Medically beneficial compounds can be derived from the seed, peel, leaf, flower, bark, and roots of the pomegranate. The broad scope and power of the pomegranate has been expanded recently with the discovery that the peel of the pomegranate contains antimicrobial activity that may be effective in the treatment of bacteria especially methicillin-resistant *Staphylococcus aureus* (MRSA). The use of pomegranate fruit dates back to Biblical times and reports of its therapeutic qualities have echoed throughout the millennia. The pomegranate belongs to the family Punicaceae. The genus *Punica* consists of two species, *Punica granatum* L. and *P. protopunica* Balf. *Punica granatum* is native extending from Iran to the Himalaya in northern India, and has been cultivated and naturalized over the entire Mediterranean region since ancient times [15]. The Indian Himalaya holds a good diversity of wild pomegranate. The evergreen pomegranate cultivars have been reported to be originated in India [16]. Wild pomegranate is found growing in the hills of Himachal Pradesh and Uttrakhand. This local wild variety called *daru* is generally found growing under forest conditions on slopes receiving scanty rainfall. Its roots are very good soil binder for the slopes. Under domesticated conditions pomegranate is grown throughout India. The traditional utilization of these fruits lies in drying the seeds of these cracked fruits to yield a value added bi-product known as anardana used in Ayurvedic and Unani medicines. Thus the present investigation wishes to compare wild pomegranate (*daru*) and cultivated pomegranate fruit parts extract for their *in vitro* antibacterial potentiality.

MATERIALS AND METHODS

Collection

Ripened pomegranate fruits were collected from local places and, *daru* fruits from different neighbouring forests of Dehradun. For the preparation of extract; the seeds, peel and white membrane were separated, dried under shade and stored into fine powder using electric blender.

Crude solvent extraction

Crude solvent extract of the plants was prepared by taking 50 g of dried powder sample and extracted by Soxhlet distillation apparatus using methanol. The solvent was removed under reduced pressure in a rotary evaporator until the residue become completely dry.

Isolated bacterial strains

A total of 150 dental plaque samples were collected from Uttaranchal Dental College and Hospital Dehradun and different dental clinics of Dehradun. The samples were collected ascetically in sterile 50 ml Oakridge tubes and inoculated in nutrient broth for 24 hrs at 37^0C. Inoculated samples were streaked on nutrient agar and other selective media. The isolates obtained were identified on the basis of colony morphology and biochemical reactions. All the isolates were identified as four bacterial species viz., *Lactobacillus, Proteus, Staphylococcus and Streptococcus* species.

Assay for antibacterial activity using agar well diffusion method

The inoculums were adjusted according to 0.5 McFarland standard which was prepared by adding 0.05ml of Barium chloride ($BaCl_2$) (1.17% $BaCl_2.2H_2O$) to 9.95ml of H_2SO_4 (1%) with constant stirring.

The inoculums of test strains was adjusted to 1.5 x 10^8 CFU/ml equal to that of the 0.5 McFarland standard by adding sterile distilled water. 20 ml of Muller Hinton agar melted and cooled at 45^0C was poured into sterile petri plates and allowed to solidify completely. A lawn of test pathogen was prepared by evenly spreading 100µl inoculums (1.5 x 10^8 CFU/ml) with the help of a sterilized spreader onto the entire surface of agar plate. The antibacterial activity of solvent extracts was done by agar well diffusion method [17]. After the medium was solidified wells of 6mm were made in the plates with the help of a cork borer. 200µl of the extracts (500mg/ml) was introduced into the wells separately and the plates were incubated overnight at 37°C. The experiment was performed under strict aseptic conditions. Bacterial growth was determined by measuring the diameter of the zone of inhibition (DIZ) around the well.

Determination of MIC

MIC of methanolic extracts of pomegranate peel was determined by Mueller Hinton Broth using Broth dilution method [18]. The bacterial suspension was used as a positive control and broth was used as negative control. Two-fold serial dilutions of the extracts in the broth were made from the stock concentration of the extract to obtain (3.2, 1.6, 0.8, 0.4, 0.2, 0.1) mg/ml. Tubes of each dilution were inoculated with the test bacterial suspension adjusted to the 0.5 McFarland standard and tubes were incubated at 37^oC for 24 h.

RESULTS AND DISCUSSION

Isolation, Characterization of isolates

Subsequent to the isolation procedures four types of bacteria were identified. The isolates obtained were identified on the basis of colony morphology and biochemical reactions. Total recovered isolates were 320 out of which 50% were *Streptococcus sp.,* 28.12% *Lactobacillus sp*., 15.62% *Staphylococcus sp.* and 6.25% were *Proteus sp.*

***In vitro* antibacterial activity of plant extracts on the recovered isolates**

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from natural sources including plants [19]. Antibacterial activity of the extracts was recorded when the zone of inhibition was greater than 6mm [20]. The antibacterial activity of methanolic extract of the local variety called *daru* and Pomegranate was assayed by well diffusion method and data on the diameter of inhibition zones produced by using *daru* extract (Table-1), was compared with the data obtained with Pomegranate extract (Table-2).

Table 1: Inhibitory activity of Methanolic *Daru* Extract (Maximum Diameter of Inhibition Zone in mm) (DIZ)

Test organism	Peel	WM	Seed
Streptococcus sp.	28	28	25
Lactobacillus sp.	26	26	24
Staphylococcus sp.	28	27	25
Proteus sp.	24	23	22

*WM- (white membrane).

Table 2: Inhibitory activity of Methanolic Pomegranate Extract (Maximum Diameter of Inhibition Zone in mm, DIZ)

Test organism	Peel	WM	Seed
Streptococcus sp.	27	26	25
Lactobacillus sp.	25	24	24
Staphylococcus sp.	25	25	24
Proteus sp.	23	23	20

In both *daru* and pomegranate, the maximum inhibitory effect was recorded by peel extract followed by white membrane; however, the seed extract had very less inhibitory effect. For *Streptococcus sp.* which is Gram-positive both, peel and white membrane extract of *daru* showed highest DIZ 28 mm, followed by seed with 25 mm. In case of *Lactobacillus sp.* the DIZ values for seed extract of both *daru* and pomegranate were same, 24mm. *Lactobacillus sp.* showed maximum sensitivity towards peel extract of *daru*, 26 mm and 25 mm in pomegranate. White membrane extract of pomegranate showed lesser zone, 24 than 26 mm of *daru*. Also in *Staphylococcus sp.* high antibacterial effect was exhibited by both peel and white membrane *daru* extracts 28 and 27 mm. Pomegranate peel and white membrane showed 25 mm zone, followed by *daru* seeds and pomegranate seed extract 25 and 24 mm. In Gram-negative *Proteus sp.* the least activity was observed for all peel, white membrane and seed extract with DIZ values of 24, 23 and 22 mm, in *daru* and 23, 23 and 20 mm, in pomegranate. As shown in the table *Streptococcus sp.* showed the highest DIZ value followed by *Staphylococcus sp.* The MIC is recorded as the highest dilution (least concentration) of extract, which shows clear fluid without turbidity after 24 h of the incubation at 37°C [21]. As methanolic extract of *daru* peel showed the maximum activity, so MIC of *daru* peel was determined. MIC of *daru* peel ranges from 0.2 to 3.2 mg ml^{-1}.

From the results of antibacterial activity of the extracts against the isolates, it has been observed that *Streptococcus sp.* and *Staphylococcus sp.* are most susceptible to extracts of both varieties and *Proteus sp.* are the least susceptible. The evaluation of the antibacterial activity of both extracts was found higher against Gram-positive bacteria than Gram-negative bacteria. These results support Tian *et al.,* [22], who stated that the structural difference of bacteria plays an important role in their susceptibility. The structural differences include cytoplasmic membrane and cell wall components which are different in gram-positive and gram-negative bacteria. Gram negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering, and without outer membrane, the cell wall of gram-positive bacteria can be permeated more easily. Individuals heavily colonized by cariogenic bacteria are considered to be at high risk for dental caries. Hence eradication of these microorganisms is important for dental treatment. Prevention of dental diseases is easier than a cure. At the present time, considerable importance is given to functional foods, which, in principle, apart from their basic nutritional functions, provide physiological benefits and play an important role in disease prevention or slow the progress of chronic diseases [23]. There has been a virtual explosion of interest in the pomegranate as a medicinal and nutritional product because of its multifunctionality and its great benefit in the human diet as it contains several groups of substances that are useful in disease risk reduction. As a result, the field of pomegranate research has experienced tremendous growth [24]. The antimicrobial activity of some of the common pomegranate cultivars has been widely studied and several *in vitro* assays demonstrated its bactericidal activity against many highly pathogenic and sometimes antibiotic-resistant organisms [25]. Braga *et al.,* [26], showed that pomegranate extracts inhibit or delay *Staphylococcus aureus* growth and subsequent enterotoxin production. At a low extract concentration bacterial growth was delayed, and at a higher concentration such growth was eliminated.

After extensive research on the medicinal properties of pomegranate it is easy to understand why Longtin [27], referred the pomegranate

as "nature's power fruit". It is rich in antioxidants, has antibacterial properties, and has been found useful in treating dental health. This research will hopefully lend insight into new and improved treatment and prevention methods and/ or drugs for a variety of ailments. The extract of *daru* (wild pomegranate) fruit can be used as a promising novel antibacterial agent in the coming years. It should be studied further to elucidate and determine structural identification of the active principle.

CONCLUSION

The experimental findings and discussion clearly conclude that peel extracts of *Punica granatum* and *daru,* have important antibacterial activities. The antibacterial activity of wild pomegranate (*daru*) fruit is reported for the first time. Further studies are required to determine the nature of compound(s) responsible for the antibacterial effects. The findings in the present study may be considered an effective approach in the discovery of new antibacterial agents from *daru.*

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