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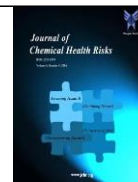

Antimicrobial Properties of *Punica granatum* Extracts: Phenolic Concentration and Antimicrobial potential.

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KEYWORDS

Punica granatum, Phenolic compounds, antimicrobial activity.

ABSTRACT

The antimicrobial attributes of pomegranate are ascribed to its phenolic compounds that works as natural defense mechanisms particularly hydrolyzable tannins such as gallic acid and ellagic acid were assessed for antimicrobial properties. This study aimed to assess ellagic acid and total phenol levels, extraction method yields, and inhibitory effects of *Punica granatum* extracts from various plant parts against *E.coli, Staphylococcus aureus, Candida albicans, Trichophyton rubrum*. Samples of dried peels, seeds, and leaves were used in triplicates, and extracts were obtained via Soxhlet extraction and percolation using water, ethyl alcohol and ethyl acetate solvents. Ellagic acid content was determined using high-performance liquid chromatography (HPLC), while total phenols expressed in gallic acid were assessed spectrophotometrically. Antimicrobial activity was assesed through disc diffusion following Clinical and Laboratory Standards Institute guidelines. The highest of phenol concentrations were detected in leaves followed by pomegranate peel and least were in seed extract.

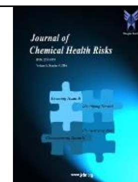
INTRODUCTION

With the resistance of microorganisms to conventional drugs, there has been progress in research on the antimicrobial potential of natural plant extracts and oils, isolated or combined with each other, or with traditionally used anti-infective medications (Ge et al., 2021). Pathogenic bacteria and fungi that develop biofilms can be up to 1000 times more resistant to antibiotics than their microbial counterparts (Rios & Recio, 2005). Therefore, the discovery of agents with antibiofilm activities that are capable of disrupting pre-formed biofilms, thus favoring the action of antimicrobials and avoiding the selection of more resistant organisms, is necessary (Verardo et al., 2014). Among drug-resistant microorganisms, *E.coli, Staphylococcus aureus, Candida albicans, Trichophyton rubrum* have led scientists to pay attention to herbs in traditional medicine because they are responsible for infections in immunocompromised patients with invasive diseases (Barde and Baig, 2023).

Knowledge about the application of plant parts for medicinal purposes in primary human health is the result of practical experiences, observations, and ancient socio-religious rituals passed down through generations (Alshammari et al., 2017). The choice of plants used for human health initially occurs based on ethnobotanical findings. However, the ideal selection must be based on extensive and challenging studies on phytochemical evolution (Reddy et al., 2007).

Pomegranate is the fruit of a plant named Punica granatum. It is native to the Himalayas, from northern India to Iran. It belongs to the kingdom Plantae, phylum Tracheopyta, class Magnoliopsida, order Myrtales, family Lythraceae, and is widespread in Central Asia, Caucasus, and southwestern Mediterranean. The fruit is popularly consumed as fresh, natural, and industrial juice, sweets, and jellies.

The phytochemical properties of pomegranate have been evaluated in studies worldwide using extracts from fresh and dehydrated parts of the entire plant, owing to its antioxidant potential in vivo (Aguilera-Carbo et al., 2008), antibacterial, antifungal, anti-inflammatory (Endo et al., 2010), antimalarial (Faria & Calhau, 2011), antitumor, and antimutagenic (Chinsembu, 2016) activities. These therapeutic properties are attributed to phenolic compounds, which occur from the roots to the leaves of the plant to protect them from environmental stress (Gul et al., 2012).



Ellagic acid and gallic acid are polyphenols, described in the category of ellagitannins and gallotannins, respectively, produced from their hydrolysis. Like other tannins, they are produced by the plant's defense system against microbial and pest attack, presenting recognized antioxidant and microcidal properties among other biological activities (Bakkiyaraj et al., 2013).

The centuries-old use of pomegranate and its bioactive potential make it the target of studies to identify its chemical constituents and how specifically they act on microorganisms. Anibal et al. (2013) evaluated the extracts from pomegranate arils, seeds, pericarps, peels, and whole fruits. They found great inhibitory activity of pericarp and bark extracts on the disarray of cellular structures. However, resistance to aril and seed extracts was observed. It was found that more than 30 active compounds in the extract, with emphasis on the tannins punicalagin and galagildilactone as the main compounds involved in antifungal activity; however, they did not disregard the synergistic effect of the compounds found. There are more than 8000 polyphenols based on molecular weight and polarity, within ecological diversity and in all parts of plants, which could be explored and evaluated due, for the most part, to their beneficial effects on human health. Therefore, the objectives of this study were to evaluate the levels of ellagic acid and total phenols, yield of extraction methods, and inhibitory potential of extracts from parts of Punica granatum on selected microbes.

MATERIALS AND METHODS

Origin of plant materials, chemicals, solvents, and equipment.

Pomegranate plants from the various farm in Nanded (Maharashtra, India) were collected in January and June, 2021. An exsiccate of the vegetable drug (dehydrated peel) was deposited in the Herbarium of the Department of Botany, Yeshwant Mahavidyalaya, Nanded . The peels, seeds, and leaves were separated and completely dried in an oven at 45 °C for 72 h, crushed, pulverized using a sieve with a 1.70 mm opening and 12 mesh mesh, and separated in triplicates for extraction and storage.

Ellagic acid and gallic acid standards were purchased from Thermo Fisher Scientific India Pvt. Ltd. Folin-Denis reagent was prepared using Phosphotungstic-Phosphomolybdic reagent and sodium carbonate High-performance liquid chromatography (HPLC) grade methanol was purchased from Thermo Fisher Scientific India Pvt. Ltd. and purified water was obtained using the Milli-Q Direct Q-3 filter system (Millipore, Bedford, USA). The analytical reagents Acetic acid, sodium hydroxide, and hydrochloric acid were purchased from Thermo Fisher Scientific India Pvt. Ltd..

Extraction

Extracts from the bark, leaves, and seeds were produced using the following methods. Extraction (95ºC) in a closed Soxhlet system, in which three samples of 10 g of dried powder from each part of the plant were used and 70% ethanol (v/v) , 90% ethyl acetate as the solvent as well as the extraction (More and Baig, 2013.).

Determination of ellagic acid and total phenols

The products from the extraction of leaves, bark, and seeds were prepared to determine the content of hydrolyzable tannins. The ellagic acid content was determined using high-performance liquid chromatography (HPLC) (Shimadzu, Shimpack ODS C 18 reverse phase column; 100 mm × 2.6 mm). HPLC grade methanol was used as the mobile phase in 2% aqueous acetic acid solution with gradient elution (0-7 min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95% v/v methanol, 7.5-8.5 min 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol) at a flow rate of 1.0 mL/min. The separation was achieved at 25ºC. The injection volume was 5 μL and the wavelength was 254 nm. An ellagic acid standard curve was prepared at concentrations between 6.25 and 100 μg/mL from a stock solution at a concentration of 200 μg/mL in methanol.

Total phenols were quantified in the extracts of pomegranate peel, leaves, and seeds using the spectrophotometric method, and the results were expressed as gallic acid. Aliquots of 0.5 ml were taken in clean test tube and 2.5 ml of Folin-Denis reagent and 5.0 ml of 29% sodium carbonate were added, were kept protected from light and readings were taken after 30 minutes in a spectrophotometer. UV-Vis at 760 nm, all of which were analyzed in triplicate. The gallic acid standard curve was prepared from a stock solution (24.97 mg/100 ml of distilled water) in aliquots of 0.8; 0.9; 1.0; 1.1 and 1.2 mL, added with 2.5 mL of Folin-Denis reagent and 5.0 mL of 29% sodium carbonate, read on a UV-Vis spectrophotometer at 760 nm.

Antifungal activity of extracts

The disk diffusion method was used to evaluate the antifungal potential of the extracts obtained from different parts of pomegranate. It followed the standards of the Clinical and Laboratory Standards institute (CLSI

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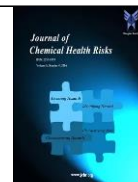

2022.)24. For this test, a strain of *Candida albicans* (SC 5314), extracted from a patient with candidemia, was used. After activating the microorganism for 24 h at 35 °C under aerobic conditions, the inoculum was prepared with a turbidity equivalent to 0.5 on the MacFarland scale for subsequent seeding with a cotton swab over the entire surface of Mueller Hinton agar (MH, Difco) contained in a Petri dish. .

Sterile filter paper discs were then soaked in the solution of each sample for 5 min. The excess solution was removed, the disc was positioned on the inoculated agar, and the plate was incubated aerobically for 20 h at 35 °C. The formation of inhibition halos was observed with the naked eye using a millimeter ruler and photographed. It was interpreted as "extract sensitive" when the zone of inhibition formed from the edge of the disc had a diameter of 10 mm or more. As an "intermediate reaction" in an inhibition zone ≤9 mm and > 0, and non-reactive if = 0. This interpretation was adapted from the NCCLS (2003)24.

All stages of the methodological processes were carried out in the following methods described earlier (Baig, 2022).

Antibacterial and Antifungal Properties of a Plant Extract

The antibacterial activity of plant extracts (devoid of alcohol or ethyl acetate and converted to aqueous) was determined using the well-diffusion method and expressed as a mm-diameter zone of inhibition for *E.coli, Staphylococcus aureus, Candida albicans, Trichophyton rubrum* as test organisms.

The bioassay was performed using 1ml of inoculum ($1X10^6$ colony forming units) produced overnight from a Nutrient Broth or Sabouraud agar culture of the test bacteria or fungus. 1ml of the resulting spore/cell suspension was placed into each Petri plate, followed by the addition of the medium used to seed each prepared plate. Allowing the medium to harden was permitted. Using a sterilised cork borer, wells 5 mm in diameter were drilled into the solidified infected medium and uniformly distributed around the plate area. 0.5 ml of extract was added to each well. After that, plates were incubated aerobically for 24 hours at 37 **ºC** for bacteria and 72 hours at **25ºC** for fungi.

Similarly, wells with a standard antibiotic concentration (Streptomycin) were utilised to compare the plant extract's antibacterial activities. Antibiotics (Streptomycin) were obtained in their purest form from Hi-media in Mumbai. Each antibiotic was dissolved in 1000 mL of sterile distilled water at a concentration of 100 mg. 0.5 ml of this was used to fill the wells. Amphotericin B was acquired from Hi-media, Mumbai, and 1 gm was dissolved separately in sterile distilled water, followed by 0.5 ml for filling the wells. This antibiotic solution was used as a control (Mahadevan and Sridhar, 1982).

HPLC analyses

The standard phenolic compounds were purchased from reputed manufacturers. The samples were prepared using 5 mg of each standard compound was dissolved in 10 ml of HPLC grade Methanol resulting in a sample concentration of 600 µgm/ml. This was sonicated and then passed through Whatman Nylon Membrane Filter (0.45µm & 47mm diameter) before injecting it in the column. Analysis of all the standard samples was performed using 515 HPLC pumps and 2489 UV/VIS detectors of Waters company, USA, having reverse phase water guard using Symmetry C18 (5µm, 4.6X250mm) column and Hamilton microliter syringe using an injection volume of 20 µl. The data analysis was done using Empower 2 software.

RESULTS

Experiments were designed to assess the antibacterial and antifungal properties of the leaves, peel and seeds of *P. granatum* L. against selected pathogenic bacteria and fungal dermatophytes. The extraction of the leaves was done in distilled water, ethyl alcohol, and ethyl acetate as described. The appropriate dilutions were made to assess the activity of the extract by the agar well method. The extracts were also subjected for characteriziation of phenilcs in the extract. The results are given in.

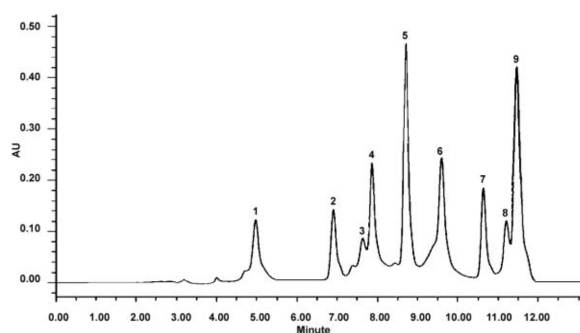

Figure1. HPLC chromatogram of phenolics in *Punica granatum* L.

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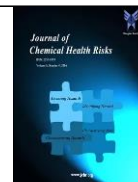

Concentration of ellagic acid and total phenols

Ellagic acid and total phenols (expressed as gallic acid) were found in higher concentrations in the leaves and peel of pomegranate samples and at very low concentrations in the seeds using Soxhlet extraction and percolation methods with 70% hydroalcoholic solvent. When comparing the concentration of ellagic acid in pomegranate peels harvested at different times of the year (January and June 2021) from the same plant.

Table 1 Retention times of phenolic peaks

	Peak Name	RT	Area
1	Ellagic acid	31.889	12075841
2	Catechol	15.601	23238735
3	Gallic acid	5.388	31071773
4	Resorcinol	12.353	14270535
5	Tannic acid	32.743	766953
6	Vanillin	28.998	67301243
7	Acetyl Salicylic acid	39.188	3486930
8	Benzoic acid	40.507	4155117
9	Ascorbic acid	2.794	766998

The mobile phase consisting of Acetonitrile and 0.1% Phosphoric acid in water for 45 minutes run time, the HPLC analysis of 9 phenolic compounds, namely Ellagic acid (31.88 min), Catechol (15.60 min), Gallic acid (5.38 min), Resorcinol (12.35 min), Tannic acid (32.74 min), Vanillin (28.99 min), Acetyle Salicylic Acid (39.18 min), Benzoic acid (40.50 min) and Ascorbic acid (2.79) was done. The HPLC profile of these phenolic compounds when combined together exhibited the same sequence of elution, with the combined Chromatogram of nine phenolic compounds giving the appearance of being a superimposition of individual chromatography profiles with similar peak elution times.

Antimicrobial potential of extracts

The disk diffusion test was carried out with extracts obtained from the peel and again from other parts of the plant (leaves and seeds). The diameter of the microorganism growth inhibition zone varied between 6 and 33mm around the disc.

The aqueous leaf extract assay for the zone of inhibition of *Punica granatum L.* varied with the dilutions. The maximum zone of inhibition was recorded in 1 dilution against *E. coli* exhibiting 11 mm and *T. rubrum* with 17 mm, while the minimum was recorded in both bacteria and *C. albicans* with 8 mm in 0.25 dilution. In the ethanolic leaf extract of *P. granatum L.* maximum zone of inhibition was recorded in 1 dilution against E. *coli* exhibiting 13 mm and *T. rubrum* with 22 mm, while the minimum was recorded in *S.* aureus i.e. 11 mm in 0.25 dilution. The ethyl acetate leaf extract of *P. granatum L.* showed a maximum zone of inhibition in 1 dilution against E.coli exhibiting 19 mm and 27 mm with *T.* rubrum while a minimum of 14 mm was recorded in *S. aureus* with a 0.25 dilution.

The aqueous bark extract assay for the zone of inhibition of *P. granatum L.* showed a maximum zone of inhibition in 1 dilution against *E.coli* exhibiting 17 mm and *T.*rubrum with 23 mm, while a minimum was recorded in *S. aureus* and C.albicans with 10 mm in 0.25 dilution. The ethanolic bark extract of *P. granatum L.* exhibited the maximum zone of inhibition in 1 dilution against *E. coli* exhibiting 21 mm and *T. rubrum,* with 29 mm, while the minimum was recorded in *S. aureus,* i.e. 14 mm in 0.25 dilution. The ethyl acetate bark extract of *P. granatum L.* showed the maximum zone of inhibition was recorded in 1 dilution against *E. coli* exhibiting 25 mm and 32 mm with *T. rubrum,* while the minimum was recorded in *S. aureus* with 19 mm in 0.25 dilution.

The seed extract assay for the zone of inhibition of *P. granatum L.* in 1 dilution against *E.* coli exhibited 19 mm and *T.* rubrum had 27 mm while a minimum was recorded in *S. aureus* and C. *albicans* with 17 mm in 0.25 dilution. The zone of inhibition of the seed extract of *P. granatum L.* in 1 dilution against E. *coli* exhibited 25 mm and *T. rubrum* had 32 mm while a minimum of 18 mm was recorded in *S. aureus,* i.e. 18 mm in 0.25 dilution. The zones of inhibition of seed extract of *P. granatum L.* was recorded in 1 dilution against *E. coli* exhibiting 27 mm and 35 mm with *T.* rubrum while the minimum was recorded in *S. aureus* with 21 mm in 0.25 dilution.

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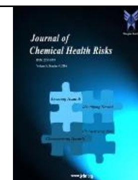

Table 2: Effect of *Punica grantum* L. on growth of test bacteria and fungi.

part	*Dilution*	*Zone of Inhibition (in mm)*											
		Aqueous Extract				**Ethyl Alcohol Extract**				**Ethyl Acetate Extract**			
		E	*S*	*C*	*T*	*E*	*S*	*C*	*T*	*E*	*S*	*C*	*T*
Leaf	1	11	10	13	17	13	12	18	22	19	17	21	27
	0.5	10	9	11	15	12	11	17	19	17	15	20	25
	0.25	9	8	10	13	11	10	15	17	15	14	18	23
Peel	1	17	13	20	23	21	19	23	29	25	23	29	32
	0.5	15	11	18	21	19	17	21	27	23	22	27	29
	0.25	12	10	16	19	18	14	19	24	21	20	25	27
Seed	1	19	17	23	27	25	22	28	32	27	25	30	35
	0.5	18	15	21	25	23	20	26	28	24	23	28	32
	0.25	17	14	19	22	21	18	24	26	22	21	26	30
Control	Stm	17	19	-	-	17	19	-	-	17	19	-	-
Control	Amp. B	-	-	18	16	-	-	18	16	-	-	18	16

E: E. coli *S: S. aureus* *C:. C.albicans* *T:. T.rubrum*

DISCUSSION

The indiscriminate use of antibiotics has contributed to and accelerated the emergence of antibiotic-resistant microorganisms, in association with the unwanted effects of antifungals, which has led to the search for new antimicrobial agents. The results of ethnobotany on the use of plants with an effect on microorganisms, increased the number of studies on identification, isolation and quantification of natural bioactive compounds that have such a function, with emphasis on ellagic acid and total phenols (standard gallic acid) present in pomegranate for their antioxidant and antifungal effects (Fawole et al., 2012).

All parts of the pomegranate tree (from root to leaves) are widely used, and consequently, in scientific research on natural antimicrobials (Viuda-Martos et al, 2010). In the present study, the highest concentrations of ellagic acid and total phenols were found in the leaves and bark of three parts of the plant (leaves, fruit peel, and seeds). However, the concentration in leaves was approximately four times lower than that found in a Soxhlet system and hydroalcoholic solvent by Li et al. (2016). These authors used leaves dehydrated at room temperature, which differed from the present study, where drying took place in an oven at a temperature of approximately 45 °C. According to the literature, the differences between the results of extraction in plants are due to variations in the extraction techniques used, the chemical and genotypic composition of the plant, variety, type of soil, place of origin, harvest time, maturation, and method of storage (Orak et al, 2012).

Other studies corroborate this, in which pomegranate bioactive compounds, such as polyphenols, ellagitannins, and flavonoids, were mainly concentrated in the peel extract (Zhang et al, 2010) which has greater antioxidant power than juice and seed extracts, including metal chelating capacity, which is important in delaying the oxidation of reactive oxygen species catalyzed by metals. The lowest concentrations of these compounds are present in the seeds, which have 12%–20% of their total weight made up of lipids (Viuda-Martos et al., 2010). This was also demonstrated in the present study, where ellagic acid values were the lowest compared to those found in other parts of the plant in both extraction methods. Although it has a low amount of tannins the higher concentration of oil in the seed extract, compared to the juice and peel extracts, increases the antioxidant power of its oil-soluble phytochemicals, such as beta carotene, tocopherols, and flavonoid punicic acid (omega 5), which is an isomer of the omega 327 fatty acid.

Zhang et al. (2010) quantified polyphenols extracted from different parts of the plant and observed higher amounts of polyphenols in the bark (63.65 mg/g) and leaves (61.53 mg/g) and lower amounts in the seeds (2.17 mg /g). These values were approximately five times lower than those of the total phenols quantified in the present study. One of the factors that may have determined this difference was the freezing of the sample at -80 °C in liquid nitrogen before being pulverized and

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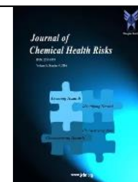

the extraction method in which the authors used water at 65 °C as the extracting vehicle. Drying, freezing, and storage at negative temperatures for a long time (30 days) affect the flavonoid content of plant extracts (Orak et al, 2012).

The antifungal potential, in this study the both the fungal isolates were sensitive to the extract. There was a slight variation in the peel and leaf extracts obtained. These are variations in the concentration of bioactive compounds determined by the environment as well as the extraction method. As verified by Fawole et al. (2012), methanolic and aqueous extracts of pomegranate peels obtained from seven different cultivars in South Africa showed variable antibacterial potency against two Gram-negative bacteria (Escherichia coli ATCC 11775 and Klebsiella pneumonia ATCC 13883) and two Gram-positive bacteria (Bacillus subtilis ATCC 6051 and Staphylococcus aureus ATCC 12600). Only the methanolic extract was found to be effective. According to the authors, potency differences may be linked to intra- and inter-genetic variability and geo-environmental spatial variation, which generate chemical heterogeneity because plants face different abiotic and biotic challenges that alter the expression of secondary metabolites.

The high antimicrobial activity is due to the phenolic and flavonoid content 20 to 110 times higher in the pomegranate peel extract than in the juice (Dey et al, 2015). Furthermore, the presence of compounds such as epigallocatechna-3-gallate, quercetin, ellagic acid, punicalagin and caffeic acid would act, respectively, on the effectiveness against microorganisms and the antioxidant capacity of the peel (Foss et al., 2014). As for seeds, there is a higher content of punicic polyunsaturated fatty acid (omega 5) and sterols, in addition to the antioxidants tocopherols (vitamin E) and flavonoids, the latter in smaller quantities (Orak et al, 2012). The inhibition of enzyme by phenolics has been mode of growth inhibition of microorganisms (Kothari and Baig, 2013).

Pagliarulo et al. (2016) evaluated the antimicrobial activity of juice and 50% alcoholic extract of pomegranate peel and identified the highest amounts of total phenols in the peel extract. Using the disk diffusion method, the authors reported that the bark extracts, regardless of concentration, demonstrated antibacterial activity against *S. aureus* and *E. coli*, forming an inhibition zone of 15 – 30 mm like the control with Ampicillin. These results were considerably higher than in the present study;

CONCLUSIONS

In pomegranate leaves and peels, higher concentrations of total phenolic compounds (expressed as gallic acid) and ellagic acid were found slightly hinger as compared to seeds. Although the leaves presented the highest average yield, the peel presented the highest efficiency. There was no difference in the concentration of total phenols in the extracts obtained from the peel. There is antimicrobial potential of pomegranate peel extract against the selected bacterial and fungal isolates are recommended for further antimicrobial testing.

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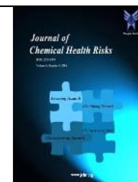

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