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Structural and Antimicrobial Analysis of Methanolic Extract of *Punica granatum* PEEL

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ABSTRACT

Introduction: The ancient fruit *Punica granatum*, native to Iran is gaining significant attention from contemporary researchers due to its manifold therapeutic and pharmacological properties. It is well known for antibacterial, antifungal, anti-inflammatory, immunomodulatory and antioxidant activity since time immemorial. Besides, recent studies are revealing its potent anti-cancerous properties with evident anti-metastatic, anti-proliferative and anti-invasive effects on numerous cancer cell lines. Anti-acne property and using Punica as a functional food supplement is an active research area.

Objective: The study mainly focuses on identifying the rich diversity of phytochemicals and bioactive compounds present in the methanolic extract of pomegranate fruit peel.

Methods: Pomegranate peel powder was soxhlet extraction with 75% methanol to obtain peel extract. Preliminary phytochemical analysis was performed for quantification of phytochemicals. The antimicrobial assay was carried out against 3-gram positive and 3-gram negative bacteria using well diffusion method, with varying concentrations (25%, 50%, 75%, 100%) of Punica peel extract. FTIR, GC-MS analysis and DPPH assay were performed to identify the functional group, chemical composition and to quantify the antioxidant scavenging activity.

Results: Presence of tannins, saponins, flavonoids, alkaloids, terpenoids, phenols, steroids, and cardiac glycosides were confirmed by various phytochemical tests. The peel extract exhibited a significant zone of inhibition against 3-gram positive (*Bacillus subtilis*, *Listeria monocytogenes*, *Streptococcus aureus*) and 3-gram negative (*Escherichia coli*, *Klebsiella aerogenes*, *Klebsiella oxytoca*) bacteria. GC-MS revealed the presence of compounds like Ethyl Acetate, Pentanoic Acid, Succinamic Acid and FTIR confirmed functional groups like O-H, C=O, S=O.

Conclusion: This culminates the promising pharmacological and medicinal characteristics of *Punica granatum*. Its high antioxidant activity favours efficient utilization for cancer studies.

Key Words: *Punica granatum*, Phytochemicals, GC-MS analysis, FTIR analysis, DPPH assay

INTRODUCTION

Punica granatum commonly referred to as the Holy Quran is the oldest known fruit species belonging to the order of Myrtales and Lythraceae family.¹ The Roman name for carthage from which the genus name Punica was derived and it is where the regal pomegranates have been grown. In northern India, it is the native plant of the region from Iran to the Himalayas. Since classical time, it has been naturalized and cultivated. It was celebrated since time immemorial in the Jewish Torah, old testament of bible and the Babylonian Talmud as a sacral fruit granting the potentials of good luck fertility and abundance. According to Saudi Arabian traditional

medicine pomegranate is considered as a health-giving fruit and nearly the whole plant is used in their day-to-day life.² These strong traditional beliefs about Punica granatum was the driving force of this research.

The pomegranate tree customarily grows at an altitude of 1000 to 2000 meters to the height of 12 to 16 feet tall.² It has large variegated red-white flowers and the fruit eventually developed from the tubular calyx. A fully matured pomegranate fruit can have an extensive size up to 5 inches with leathery skin and dark red colouration.³ As the tree ages, the bark turns grey. The leaves of pomegranate are lance-shaped and glistening. Different parts of the pome-

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granate tree were used in traditional medicine for the treatment of a large array of diseases. It is used as a medication for erectile dysfunction, skin inflammation, and wounds due to the rich antioxidant property. For the treatment of tapeworm infection, diarrhoea and amoebic dysentery, the dried rind of the fruit can be used.^{4,5} The decoction of the fruit rind is used as douche after the childbirth and in case of leucorrhea and mouth sour by a local herbalist. The extract of dried peel is vastly used in the treatment of colitis, stomach aches, and gut spasms.⁶

The edible portion of the fruit is fairly the 50% (40% mace and 10% seeds), and therefore the rest is that of the non-edible peel. Peel is a rich source of phenolics, minerals and sophisticated polysaccharides, whereas arils, except water (85%), contain sugars, pectin, organic acids, phenolics, and flavonoids—principally anthocyanins. Seeds contain proteins, crude fibres, vitamins, minerals, pectin, sugars, polyphenols, isoflavones, and therefore the oil that's derived from them (12–20%) is characterized by a high content of unsaturated fatty acids like linolenic and linoleic acids, also as alternative lipids like punicic acid, oleic acid, saturated fatty acid, and hexadecanoic acid.⁷ Contemporary researchers believe that pomegranate can be consumed intensively for a balanced diet.

Many years back Hippocrates declared that “death sits in the bowl” and a bad digestive system is the root cause for evil. And Metchnikoff proclaimed that probiotics can help in improving gut and intestine by diminishing the negative effects of putrefying bacteria. Pomegranate is a potent prebiotic; prebiotics is defined as the food for probiotics in other words it enhances the activity of probiotic bacteria.⁷ Ellagitannins is the most important constituent of pomegranate polyphenols. Ellagitannins are hydrolyzed into ellagic acid in the gut which is subsequently metabolized by colon microbiota into urolithin A and B, and it serves the function of prebiotics. Recent in-vitro studies have shown that by-products of the pomegranate can enhance the growth of *Lactobacillus species* by acting as a prebiotic.⁸ Cancer is the leading cause of medical death in the world and for years it is shaking public health. The chemotherapeutics available today tend to produce more ill effects, so many researchers started focusing on natural remedies. Pomegranate peel contains infinite health-giving phytochemicals and enormous antioxidant activity. The pomegranate peel extract contains 10 times more antioxidants than the pulp extract.⁹ The peel extract can neutralize superoxide and hydroxide anion radicals.¹⁰ Pomegranate peel has anticancer activity and exhibits anti-proliferative properties against various cancer cell lines like colon, breast, prostate and oral. Pomegranate extract also exhibits promising anti-tumour activity against various vital organs. Potential anticancer effect of pomegranate extract is due to a significant amount of hydrolysable tannins and polyphenols.¹¹ The main purpose of the study is to study the antimicrobial,

antioxidant activity and to analyse the bioactive compounds of pomegranate peel.

MATERIALS AND METHODS

Identification and handling of sample

Collection of samples: The sample of *Punica granatum* collected from the local market in Sathyamangalam is brought to the laboratory. The fruits were washed and the leathery exocarp was peeled off then chopped into small pieces. Later the chopped peels were dried at room temperature for a week. Then the dried peels were powered by pulverizer and stored in an air-tight storage container for future use.

Peel extract preparation: The pulverized peel powered was weighed for 10g and packed in a filter paper. Then the desired compounds were extracted using Soxhlet's extraction at 65°C for 12 cycles using methanol-water mixture in the ratio of 3:1 of 300ml. Later the extract was concentrated to 10ml using the steam distillation process.¹² This concentrated Punica peel extract was used for further analysis.

Preliminary phytochemical screening

The following procedures were employed for the qualitative estimation of phytochemicals of concentrated Punica peel extract:

Test for proteins: Biuret's test- The peel extract of 1ml was added with 2% NaOH and 0.3% CuSO₄. The appearance of a pinkish colour confirms protein presence.¹³

Test for tannins: Ferric chloride test- A few ferric chloride drops were added to the peel extract of 1ml and mixed well. Formation of bluish-black colour symbolises the existence of tannins.¹³

Test for saponins: Foam test- The peel extract of 1ml was mixed vigorously with 5ml distilled water. Persistence of foam after 15 minutes confirms the presence of saponins.¹⁴

Test for flavonoids: Alkaline reagent test- Peel extract of 1ml was added with 1ml of 2N NaOH and mixed well. Existence of flavonoids was affirmed by the colour change from reddish-brown to yellow colour.¹⁵

Test for Alkaloids: Mayer's test- To the peel extract of 1ml add a few drops of Mayer's reagent and mix well. Development of white precipitate symbolizes alkaloids presence.¹⁵

Test for Glycosides: Borntrager's test- 1ml of peel extract was added to 3ml of chloroform and a few drops of 10% ammonium solution. The pinkish-red colour of the ammonia layer points out the presence of glycosides.¹⁵

Test for Terpenoids: To 1ml of peel extract, add 1ml of chloroform. After a couple of minutes add a few drops of concentrated sulphuric acid. The crimson brown colouration at the interface confirms the presence of terpenoids.¹³

Test for phenols: Ferric chloride test: Peel extract of 1ml is added with 2ml of distilled water and mixed. Then a few drops of 10% ferric chloride was added, the appearance of bluish-black colour confirms the presence of phenols.¹³

Test for steroids: 2ml of chloroform and few drops of concentrated sulphuric acid was added with 1ml of peel extract the development of violet-brown ring confirms the presence of steroids.¹³

Test for lipids: 1ml of peel extract was introduced with 0.1N alcoholic potassium hydroxide and a few drops of phenolphthalein. Heat the combination for 1 hour in the water bath. Soapy appearance indicates the presence of lipids.¹³

Test for cardiac-glycosides: Keller-killani test: To 1ml of peel extract add 0.5ml of glacial acetic acid, 2 drops 5% ferric chloride and concentrated sulphuric acid. The occurrence of cardiac-glycosides was observed by the colour disappearance at the junction of two layers and bluish-green in the upper layer.¹³

Antimicrobial analysis

Antimicrobial activity of *Punica granatum* was tested against 3-gram positive (*Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*) and 3-gram negative bacteria (*Escherichia coli*, *Klebsiella aerogenes*, *Klebsiella oxytoca*) by adopting agar well diffusion method.¹⁶ The microbial strains were maintained in their respective specific broth at 5°C in the refrigerator. Culture media were prepared for each organism with specific media and 0.8% agar and autoclaved at 121°C, 15 psi for 30 minutes. Autoclaved sterile media were poured into clean Petri dishes and left for solidification. After solidification 100µL volumes of microbial culture were inoculated into agar plated and spread using a clean swab inside the sterile laminar hood.¹⁷ Four wells of 3.5mm in a quadrant form and one in the centre were punched in an agar plate using a good puncher. Rind extract was diluted to 25%, 50%, 75%, 100% using sterile distilled water. This diluted extract was injected into four quadrant wells and sterile distilled water as a negative control was injected into the centre well. Then the plates were kept at $\pm 37^\circ\text{C}$ in an incubator. After 24 hours, the zone of inhibition was measured in millimetres using a measuring scale.^{18,19}

GC-MS analysis

The peel of the studied *P. granatum* was dried in the sunlight. The dried peel was pulverized to a powder and using the methanol as a solvent the Soxhlet extraction was carried out. The crude extract obtained was concentrated using the water-bath evaporator at 60°C for 25 to 35 minutes then it is used for GC-MS analysis. The gas chromatography (GC) and mass spectrometry (MS) are used to identify the chemical composition of the rind extract.²⁰ The chemical composition examination was carried out using the Perkin Elmer

Clarus 680 model which is furnished with Clarus 600 electron ionization using Turbo Mass ver 5.4.2 software. A fused silica column is packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m $\times$ 0.25 mm ID $\times$ 250µm df) and the constituents present in the extract were isolated using carrier gas which is Helium with a 1ml/min consistent flow rate.²¹ The temperature of the injector during the running time of chromatographic was set at 260°C. The temperature of the oven was set at 60 °C for 2 min when the 1µL of the concentrated test sample was infused into the instrument and followed by 300°C for 10°C min⁻¹ rate and 300°C, where it was held for 6 min. The detector mass spectrum conditions were: temperature of transfer line 230°C; mode of ionization in electron impact at 70eV; the temperature of particle source 230°C, the output scan time is 0.2 sec and scan 0.1 sec. the fragments from 40 to 600 Da. The spectrum database of known components was compared with the spectrum range of components in the NIST (National Institute of Science and Technology) library (2008).²²

FTIR Analysis

The functional group analysis and the type of chemical bonds present in the methanolic peel extract of *Punica granatum* were analyzed using FTIR (Fourier-transform infrared spectroscopy) analysis. The characteristics of the chemical bond present in the sample are determined by the range of wavelength it absorbs from the given source.²³ The powder of dried *Punica granatum* peel is taken in the ratio of 1:9 with the KBr used for FTIR analysis. The mixture of KBr and peel sample were pressed with a system press tip flap.²⁴ The detection was carried out using the scan range of 400 to 4000cm⁻¹ in the infrared region using the resolution of 4cm⁻¹.²⁵ The resultant graph shows the stretching range at 1613 cm⁻¹ related to C=C Stretching of α , β -unsaturated ketone (4H-PYRAN-4-ONE, 2,3-DIHYDRO-3,5-DIHYDROXY-6-METHYL-) presence. The bands at 2935.69 cm⁻¹ and 1445.68 cm⁻¹ shows C-H Stretching of Alkanes (2-PROPENE-1,1-DIOL, DIACETATE). In the 1728.84cm⁻¹ stretch to C=O Stretching of Aldehydes (4,4'-BISCYCLOHEXANONE, 2,2',6,6'-TETRAMETHYL-). The bands identified in the range of 875.17cm⁻¹ and 3280.77cm⁻¹ is C-O stretching and O-H Stretching is Alcohols and primary and secondary alcohols (1,3-DIOXANE, 2,4-DIMETHYL-). The FTIR result shows the presence of alkanes, aldehydes alcohols and so forth.

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was employed to measure the antioxidant activity of *Punica granatum* peel. Stable radical DPPH was administered to measure the antioxidant activity in terms of hydrogen donating or radical scavenging capability.²⁶ The DPPH solution was prepared in 0.1mM concentration by the addition of methanol and the volume of standards and samples were adjusted by adding

100µL of methanol. Samples and standards (3ml) of different concentrations were mixed with 0.1mM of 1ml DPPH. The mixture was mixed vigorously and incubated in a dark chamber at room temperature for 30 minutes. Colour change of the mixture from violet to yellow depicts the presence of antioxidants. The antioxidant activity is quantified spectrometrically at 517nm. Standard used as ascorbic acid and percentage of inhibition formula is used for the numerical representation of DPPH scavenging.^{27,28}

Percentage inhibition of DPPH radical = $[A0-A1/A0] \times 100$

Where A0 was the absorbance of the control and A1 was the absorbance of the sample/standard.

RESULTS AND DISCUSSION

The presence and absence of secondary metabolites of *Punica Granatum's* methanolic peel extract were tested using various methods. The secondary metabolites like Saponins, Tannins, Flavonoids, Terpenoids, Phenols, Glycosides, Steroids, Alkaloids were checked. Methanolic extract of pomegranate peel shows positive for tannins, saponins, flavonoids, alkaloids, terpenoids, phenols, steroids and cardiac glycosides. Proteins, glycosides, and lipids were absent (Table 1). The result of GC-MS compounds presented in the methanolic extract showed the presence of 10 components at different retention times (Table 2, Figure 1 and 2). It has 2 bioactive compounds like succinamic acid which has the property of antibacterial and also used as a food additive and dietary supplement and pentanoic acid -4-oxo which is used for potential biofuels and also used for cigarettes to increase the nicotine delivery.

The peel extract was tested against the bacteria for the sensitivity. By increasing the concentration of extract the zone inhibition was increased, the concentration has direct contact with the bacterial inhibition. Table 3 shows the diameter of the zone of inhibition in millimetres with bacterial exposure towards the peel extract. *Escherichia coli* has a higher inhibition rate compared to other bacterial species. *Listeria monocytogenes* has a lower inhibition rate. The FT-IR analysis of a methanolic extract of *Punica granatum's* peel shows the compounds at particular stretching vibrations and bands at 3280.77, 2935.69, 1728.84, 1613.08, 1445.68, 1324.55, 1225.46, 1017.92, 875.17 cm^{-1} (Table 4). This revealed the presence of primary and secondary alcohols, aldehydes, aromatic group, alkanes, vinyl ether, sulfone and fluoro compounds. Using the peak values of the infrared region, the active compounds can be identified based on the functional group stretching.

Based on the stable DPPH and free radical electron acceptance of a molecule, the antioxidant activity is determined. It can be visualized through the colour change from purple

to yellow. The article Polterait¹⁶ states that the scavenging effect of free radical is may because of the presence of flavonoids, tannins and phenols. Because of the presence, it possesses higher anti-inflammatory, anti-oxidant, and antimicrobial activity (Figure 5).

The phytochemical analysis performed in this study revealed the presence of several valuable phytochemicals like tannins, saponins, flavonoids, terpenoids, alkaloids, phenols, steroids and cardiac glycosides. These phytochemicals have immense applications as pharmaceuticals and in the development of commercial-scale internal and external body products. *Punica granatum* peel extract displays significant antimicrobial activity against a range of both gram-positive and negative bacteria namely *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Klebsiella oxytoca*, *Streptococcus aureus*. Specifically, the antimicrobial activity was more against *Bacillus*, *Escherichia coli* and *Streptococcus*. The GC-MS analysis showed diverse bioactive compounds that *Punica granatum* peel constitutes. Ranging from ethyl-acetate, pentanoic acid, methyl acetoxyacetate, succinamic acid, 2-propene-1,1diol, diacetate. These compounds are of predominant industrial and pharmacological importance. One of the main reasons pomegranate being a fruit of great medicinal value during the ancient period was due to the presence of an array of phytochemicals and bioactive compounds. Presence of many functional groups like alcohols, alkanes, aldehydes, alkanes, sulfone, vinyl ether and fluoro compounds are confirmed by FTIR analysis (Figure 4). From this, we can confirm that *Punica granatum* has significant properties which can be wisely tweaked against bacterial, fungal pathogens and tumour cells in vital organs.

CONCLUSION

The study about the *Punica granatum* using methanol extract shows that it has antimicrobial properties and antioxidant properties which was evident from the above research. The bioactive compounds present in the peel extract was confirmed by GC-MS and their structural prediction was carried out for functional groups using FT-IR. It is proven from the DPPH assay, that the *Punica granatum* bioactive compound will have an excellent antioxidant effect that favours for the utilization of cancer studies. These culminating properties of *Punica granatum* methanolic peel extract show the pharmacological properties.

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Table 1: The qualitative results of the *Punica Granatum*'s test are as follows,

Phytochemical constituents	Presence in Methanol
Proteins	-
Tannins	+
Saponins	+
Flavonoids	+
Alkaloids	+
Glycosides	-
Terpenoids	+
Phenols	+
Steroids	+
Lipids	-
Cardiac glycosides	+

(+ = Presence, - = Absence).

Table 2: The GC-MS analysis for major phytocomponents in *Punica granatum* extract

SL No.	COMPOUND	MOLECULAR FORMULA	STRUCTURE	MOLECULAR WEIGHT	RETENTION TIME (Min)
1	ETHYL ACETATE	$C_4H_8O_2$		88	7.530
2	N-METHYL-N'-NITRO-GUANIDINE	$C_2H_6O_2N_4$		118	15.734
3	PENTANOIC ACID, 4-OXO-	$C_5H_8O_3$		116	15.954
4	4H-PYRAN-4-ONE, 2,3-DI-HYDRO-3,5-DIHYDROXY-6-METHYL-	$C_6H_8O_4$		144	16.074

5	METHYL ACETOXYAC- ETATE	$C_5H_8O_4$		132	16.339
6	SUCCINAMIC ACID	$C_4H_7O_3N$		117	16.474
7	1,3-DIOXANE, 2,4-DIME- THYL-	$C_6H_{12}O_2$		116	16.584
8	2-PROPENE-1,1-DIOL, DIAC- ETATE	$C_7H_{10}O_4$		158	16.784
9	1,2:5,6-DIANHYDROGALAC- TITOL	$C_6H_{10}O_4$		146	16.889
10	4,4'-BISCYCLOHEXANONE, 2,2',6,6'-TETRAMETHYL-	$C_{16}H_{26}O_2$		250	18.935

Table 3: Antimicrobial activity

S. No.	Test Organism	Negative Control	25µl	50µl	75µl	100µl
1	<i>Bacillus subtilis</i>	C	11	18	26	33
2	<i>Listeria monocytogenes</i>	C	08	12	17	22
3.	<i>Streptococcus aureus</i>	C	10	16	17	24
4.	<i>Klebsiella aerogenes</i>	C	05	10	14	21
5.	<i>Escherichia coli</i>	C	07	19	25	27
6.	<i>Klebsiella oxytoca</i>	C	05	11	16	23

Table 4: The functional group present in the FT-IR analysis are:

S.NO	Wave number (cm ⁻¹)	Functional groups	Compound class	Appearance
1.	3280.77	O-H Stretching	Alcohols	Strong, broad
2.	2935.69	C-H Stretching	Alkanes	Medium
3.	1728.84	C=O Stretching	Aldehydes	Strong
4.	1613.08	C=C Stretching	α, β-unsaturated ketone	Strong
5.	1445.68	C-H Bending	Alkanes	Medium
6.	1324.55	S=O Stretching	Sulfone	Strong
7.	1225.46	C-O Stretching	Vinyl ether	Strong
8.	1017.92	C-F stretching	Fluoro compound	Strong
9.	875.17	C-O stretching	Primary and secondary alcohol	Strong

Figure 1: GC-MS chromatogram graphic representation of *Punica granatum* peel extract.

Figure 2: The MS spectrum of ethyl acetate.

Figure 3: Antimicrobial activity chart.

Figure 4: The graphical representation of FTIR analysis of the *Punica granatum*.

Figure 5: The DPPH Assay plot.

GRAPHICAL ABSTRACT