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Comprehensive Antibacterial and Antioxidant Assessment of Essential Oil and Extracts of Punica granatum for Pharmacological Prospects

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Abstract

Objective: To evaluate the phytochemicals estimation, antibacterial and antioxidant activity of peel extracts and seed oil of *Punica granatum* against acne pathogens.

Methods: Extracts of alcoholic solvent (ethanolic, methanolic) of *P. granatum* peels and its seed oil were phytochemically evaluated for total phenolic, flavonoid and other essential phytochemicals by suitable specific test procedures as well as by FTIR (Fourier Transform Infra red) spectroscopy. Additionally, Estimation of antioxidant activity was also carried out by DPPH, Nitric oxide scavenging activity of plant extracts. Further, antibacterial screening of these extracts was also estimated against pathogen causing acne vulgaris by broth micro dilution methods recommended by CLSI.

Results: The peel extracts of *P. granatum* and seed essential oil showed high phenolic and flavonoid content, free radical, nitric oxide scavenging activity as well as antibacterial activity against the gram-positive anaerobic bacteria (*Propionibacterium acnes*) and aerobic bacteria (*Staphylococcus epidermidis*). The MIC range 0.093, 0.933, 1.027 µg/ml of SO, PE, PM respectively, against *P. acnes* whereas 1.512, 1.861, range of SO, PE, PM against *S. epidermidis.* The IC_{50} ranges 0.061, 0.636, 1.012 of SO, PE, PM. The FTIR analysis proved the presence of alkenes, alcohols, ethers, carboxylic acid, esters, nitro compounds, alkanes, H-bonded H-X group, hydrogen bonded alcohols and phenols.

Conclusions: *P. granatum* seed oil had the great potential value against both Gram-positive anaerobic bacteria *(P. acnes*) and aerobic bacteria (*S. epidermidis*).

***Keyword**: DPPH; Punica granatum; FTIR; CLSI; Propionibacterium acnes; Staphylococcus epidermidis*

Introduction

The evolution of multidrug-resistant (MDR) property of pathogenic bacteria is increasing due to prolonged treatment of commercially available drugs in recent scenario. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [1].

Hence, there is an immense need to search herbal based solutions. Natural products with their diverse biological and pharmacological activities represent a treasure for scientists in terms of treating health disorders and infections. Among constituents of plants, polyphe-

nols have received a great deal of attention, due to their diverse biological functions. The antimicrobial activity of the polyphenols, tannins and flavonoids, is well documented [2,3]. As a result, plant based formulations are being used in reducing side effects of conventional antibiotics treatment [4]. In last few years, with an increasing interest in aromatherapy, emphasis has been mainly on the anti-inflammatory and antioxidant properties of plant essential oils [5,6].

In this group, acne is common multi-factorial chronic disease of the pilosebaceous unit which involves androgen induced sebaceous hyperplasia, follicular keratinisation, hormonal imbalance, immune hypersensitivity and bacterial colonization i.e. *Staphylococcus epidermidis, Propionibacterium acnes* [7]. Thus, Present study focused on antibacterial and anti-inflammation activity of the essential oils, obtained from the seeds and extract obtained from the peel of *P. granatum* fruits against these pathogens *P. granatum* rich in tannins which have remarkable antimicrobial activity [3,8]. Pomegranate peel is a by-product whose production has increased because of an exponential rise in the production of pomegranate juice and 'ready-to-eat' arils. Further, estimation of its anti-inflammatory, antioxidant activity (free radical scavenging activity) carried out by DPPH, including Nitric oxide scavenging assay with all relevant phyto-chemical analysis were done. The functional groups of essential oils were observed using FTIR technique which makes current data more authentic. Moreover, antibacterial screening of these extracts was also estimated against pathogen causing acne vulgaris (aerobic bacteria *Staphylococcus epidermidis* and anaerobic bacteria *Propionibacterium acnes*) using CLSI recommended broth micro dilution technique.

Materials and Methods

Collection of plant part: The pomegranate fruits *(Punica granatum* L.) belongs to the Punicaceae family, ripen fruits waste were collected from local vendors of Prayagraj, India.

Procurement and maintenance of bacteria: The test organisms *Propionibacterium acnes* (MTCC1951) and *Staphylococcus epidermidis* (MTCC435) were procured from microbial type culture collection, Chandigarh, India and media were procured from Hi-Media. The culture of *P. acnes* was maintained on anaerobic blood agar medium supplemented with fresh sheep blood. The proper anaerobic environment was maintained by Anaxomate advance instrument followed by the incubation period of 48 hours at 37°C in CO_2 incubator (Figure 1). The culture of aerobic bacteria was maintained on Nutrient Agar by maintaining in BOD incubator for 24 hours at 35 ± 2°C. (Figure 1).

Preparation of plant extracts

All 3 solvents were prepared separately for extracting phenolics from peels: methanol, ethanol, and deionized water (ddH_2O). Peels (1g) were grinded with a Mortar and Pistle. The grinded peels were transferred to a centrifuge tube containing 10 mL of each solvent. The mixture was kept overnight in the dark. After centrifugation (5000 rpm for 10 min), filtration (Whatman No. 1); the extracts were subjected to pour in rotary evaporator to make thick slurry under vacuum at operating temperature below 45°C. Extracts were tested for the presence of tri terpenoids, steroids, alkaloids, tannins, and vitamin C along with the quantitative phytochemical screening of total phenols and total flavonoids and antioxidants [9].

Steroids and triterpenoids test: -The extract was mixed with few drops of acetic anhydride, boiled and cooled followed by addition of conc. sulphuric acid and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids and triterpenoids respectively (Liebermann Burchard test).

Test for alkaloids: The extracts were treated with few drops of Hager's reagent (saturated picric acid solution), lead to the formation of yellow precipitate (Hager's Test).

Test for tannins: The extracts treated with gelatin solution would give white precipitate indicating the presence of tannins (Gelatin Test).

Test for vitamin C: Test extracts were treated with dinitrophenyl hydrazine dissolved in concentrated sulphuric acid, lead to the formation of yellow precipitate (DNPH Test) [10].

Phenolic content: 20 µl of each extract solution and standard (tannic acid) were mixed with 1 ml of ddH_2O and 100 µl of Folin-Ciocalteu reagent, followed by addition of 300 µl of 20% Na_2CO_3 solution after 1 minute. The resulted mixture now incubated in shaking incubator (temp 40°C, 30 min). The phenolic content was determined as milligram tannic acid equivalents (TAE)/g of dry weight powder (DW) [11].

Flavonoids content: 4 ml of ddH_2O was mixed with 1 ml of each olive extracts. Subsequently, a 5% sodium nitrite solution (0.3 mL) and 10% aluminum chloride solution (0.3 mL) were added and incubated at room temp for 5 - 10 minutes. Then 2 mL of 1 M NaOH was added to the mixture, the volume makes upto 10 ml with ddH_2O and subjected to vortex thoroughly. The pink colour developed and show absorbance at 510 nm. Total flavonoids content was determined as mg catechin equivalents per gram of dry weight powder [11].

Antioxidant assay or DPPH assay

DPPH: It is a method to measure the antioxidant/ free radical scavenging activity of extract. This activity of leaves extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH solutions (100 µg/mL) in methanol were made and method was performed in 96 well microtitre plates in triplicates. All the extracts were tested at 20, 17.5, 15, 12.5, 10, 7.5, 5, 2.5 mg/mL concentration and serially diluted to 2.5 mg/ml concentrations using Ascorbic acid as reference standard. After incubation period of 30 minutes in dark at 25°C, decrease in absorbance was measured at A = 517 nm against blank. The Percentage effect of scavenging ability of each plant extract on DPPH was calculated using the equation:

Percentage effect (E%) =

Where Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample extract or standard [12].

Nitric oxide scavenging activity

Sodium nitroprusside (SNP) generates nitric oxide (NO) in aqueous solution (pH 7.4) spontaneously, which on interacting with the oxygen produces nitrite ions which diazotize with sulphanilic acid and couple with naphthyl ethylene diamine (Griess reagent), producing pink color, which can be measured at 546 nm 2 mL of Sodium nitroprusside (10 mM) solution mixed with 0.5 mL of plant extract and standard at various concentrations (20 ug/ml - 100 ug/ml). The mixture was incubated at room temperature for 150 minutes.1 mL of Griess reagents added to 0.5 mL of the obtained solution and incubated at room temperature for 30 min. The absorbance was then measured at 546 nm [13,14]. The amount of nitric oxide radical was calculated using the equation:

NO radical scavenging activity = × 100

Where Abs control is the absorbance of NO radical + methanol.

Abs sample is the absorbance of NO radical + sample extract or standard.

Griess reagents =1.0 mL of sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthylethylenediamine chloride (0.1% w/v).

Fourier transform infrared spectrophotometer (FTIR): The functional groups of the essential oil were estimated by Fourier transforms infrared spectra (FTIR). FTIR spectra were recorded using IR beam of IR spectrometer (FTIR spectrum RX-1, Perkin Elmer) with detector at 4000 - 500 cm^{-1} resolution.

Antibacterial assay of plant extracts

The extracts were tested for antibacterial activity using broth micro dilution methods against test pathogens. Freshly prepared Muller Hinton Broth (MHB) medium was used as a base media for the experiment. Stock solutions of all the extracts and standard (50 mg/ml) were prepared by dissolving Dimethyl Sulfoxide (DMSO). Bacterial inocula were prepared as per 0.5 McFarland standards. The experiment was performed in flat bottom sterile 96-well microtitre plates [19,20] (Figure 1). The cultured 96 well plate of anaerobic bacteria was then incubated in CO_2 incubator (Galaxy 170 S New Brunswick, USA) for 48 hours and aerobic bacterium plates were placed in BOD incubator for 24 hours.

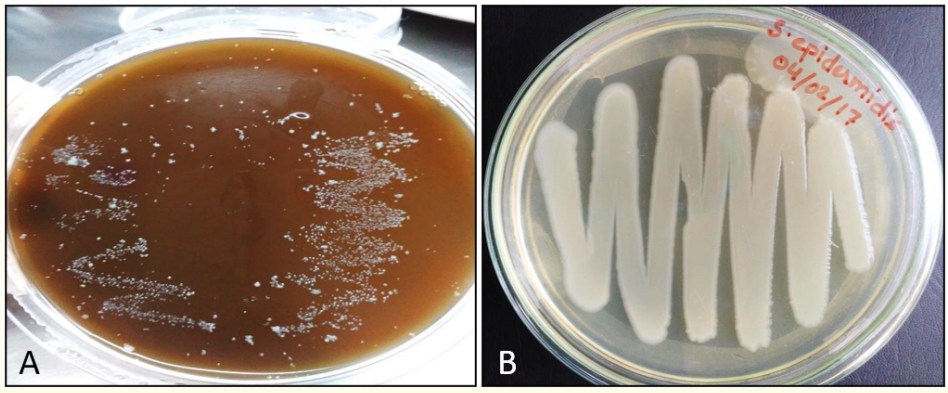


Figure 1: *The pictorial presentation of test pathogen A: Culture of P. acne, an anaerobic bacterium, growth maintained on anaerobic blood agar supplemented with fresh sheep blood. B: Culture of S. epidermidis an aerobic bacterium, growth maintained on Muller Hinton agar.*

Determination of minimum inhibitory concentrations (MICs) and IC_{50}: The MIC is defined as the lowest concentration of antimicrobial that can inhibit the visible growth of microorganisms. For extracts the MIC was determined as the lowest drug concentration showing absence of growth visually or 80% growth inhibition compared with the growth in the drug-free well. IC_{50} defined as the drug concentration that produces 50% of growth inhibition compared to the growth in the drug-free well. Comparative inhibition percent of bacteria inoculums in media treated with extracts were calculated by using formula [15]:

% Inhibition = × 100.

Statistical analysis: All experiments were carried out in triplicate. The data were analyzed using analysis of variance (ANOVA) and significant differences ($p < 0.05$) among means were determined by GraphPad Prism version 5.01 (San Diego, CA).

Results

The Broth micro dilution assay of *P. granatum* extracts reflects strong antagonistic activities against both the test pathogens. The MIC range 0.093, 0.933, 1.027 ug/ml of SO, PE, PM respectively against *P. acnes* whereas 1.512, 1.861, range of SO, PE, PM against *S. epidermidis.* The IC_{50} ranges 0.061, 0.636, 1.012 of SO, PE, PM respectively against *P. acnes* whereas 1.209, 1.436 and NA against *S. epidermidis* compared with standard (Table 1 and figure 2). The *P. granatum* shows effective pattern of inhibition against anaerobic bacteria *P. acnes* than *S. epidermidis* (Table 1). The peel extracts and seed oil of *P. granatum* has revealed the presence of Triterpenoids, Steroids, Alkaloids, Tannins and Vitamin C (Table 2) [9]. Table 3 represents the quantities of total polyphenols and flavonoids of pomegranate peel and seed

oil. The data demonstrated the level of polyphenols and flavonoids varied according to the pomegranate botanical part. Seed oil contains higher amounts of total phenols and flavonoids (58.27, 48.06 mg/g respectively) than PE (52.63, 41.04) and PM (42.03, 37.32 mg/g) (Figure 3). The DPPH assays of all the extracts are shown in table 3. SO (11.5 to 79.5%) shows strong scavenging activity than PE (12.9 to 81.8%) and PM (14.7 to 93.6%) (Figure 4).

Pathogenic microbe	Bactericidal activity (IC_{50} and MIC)						Standard drug (Tetracycline)	
	Punica granatum							
	PM		PE		SO			
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
P. acnes	0.636	0.933	1.012	1.012	0.061	0.093	0.013	0.028
S. epidermidis	1.436	1.861	NA	NA	1.209	1.512	0.106	0.159

Table 1*: Antibacterial activity of Punica granatum extracts of different solvent and seed essential oil along with standard against P. acnes and S. epidermidis.*

NA: No Activity.

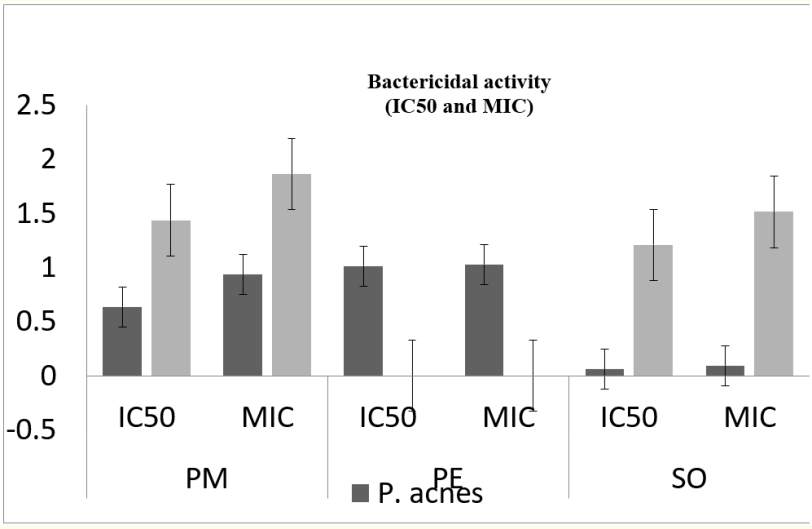


Figure 2:*Antibacterial activity of Punica granatum extracts of different solvent and seed essential oil against P. acnes and S. epidermidis.*

Phytochemical test	Compound detected	PEE	PME	SO
Liebermann Burchard Test	Triterpenoids, Steroids	-	-	+
Hager`s Test	Alkaloids	+	-	+
Gelatin Test	Tannins	+	-	+
DNPH Test	Vitamine C	+	+	++

Table 2: *Phytochemical testing of the peel extract of Punica granatum and its seed oil.*

Extraction solvent	Phenolic content	Total Flavonoid
PGM	42.03 ± 0.33	37.32 ± 0.66
PGE	52.63 ± 0.88	41.04 ± 0.33
PGO	58.27 ± 0.66	48.06 ± 0.88

Table 3: *Phenolic content, flavonoid content of Punica granatum extracts along with standard.*

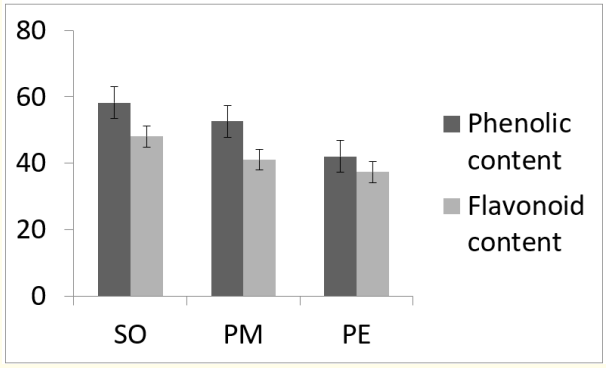
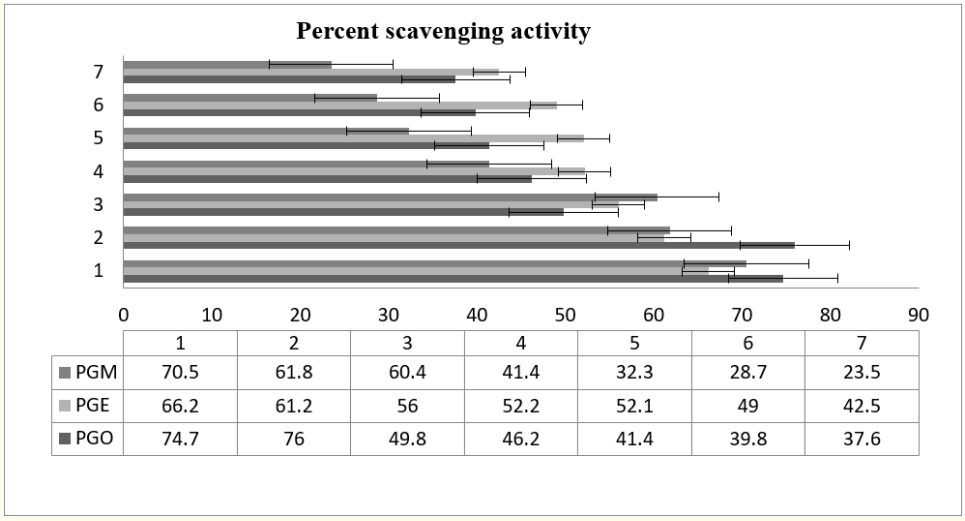


Figure 3:*Phenolic content, flavonoid content of Punica granatum extracts along with standard.*



	1	2	3	4	5	6	7
PGM	70.5	61.8	60.4	41.4	32.3	28.7	23.5
PGE	66.2	61.2	56	52.2	52.1	49	42.5
PGO	74.7	76	49.8	46.2	41.4	39.8	37.6

Figure 4

Considering nitric oxide assay, *P. granatum* exhibits significant nitric oxide scavenging activity (Table 4 and figure 4). SO, shows 62.31% Nitric oxide scavenging activity at 100 µg/mL concentration (consider as high NO activity comparing to standard). Whereas PM showed

74.06% of NO scavenging activity i.e. comparatively low activity [15]. FTIR analysis of seed oil of *P. granatum* proved the presence of Alkenes (predominantly C-H stretch), Aliphatic fluoro compounds, Alcohols, Ethers, Carboxylic acids (O-H stretch), Esters (C-C(O)-C stretch (all others), Nitro Compounds, Alkanes (C-H in-plane bend, C-H bend disubstituted - 1,1 and C=C stretch conjugated), H-bonded H-X group, Hydrogen bonded Alcohols and Phenols which shows major peaks at 754, 875, 920, 1145, 1200, 1316, 1338, 1408, 1600, 2750, 2866, 2929, 3300, 3334, 3465, 3534 (Figure 5) [17].

Extracts	Percentage scavenging (Mean ± SD) of Triplicates				
	Concentrations (ug/ml)				
	20	40	60	80	100
PE	48.23	51.63	57.38	61.94	68.41
PM	52.61	58.64	65.06	71.43	74.06
SO	36.38	40.06	46.91	52.23	62.31
AA	21.59	30.86	38.06	50.04	54.14

Table 4: *Percentage scavenging of Punica granatum extracts along with standard.*

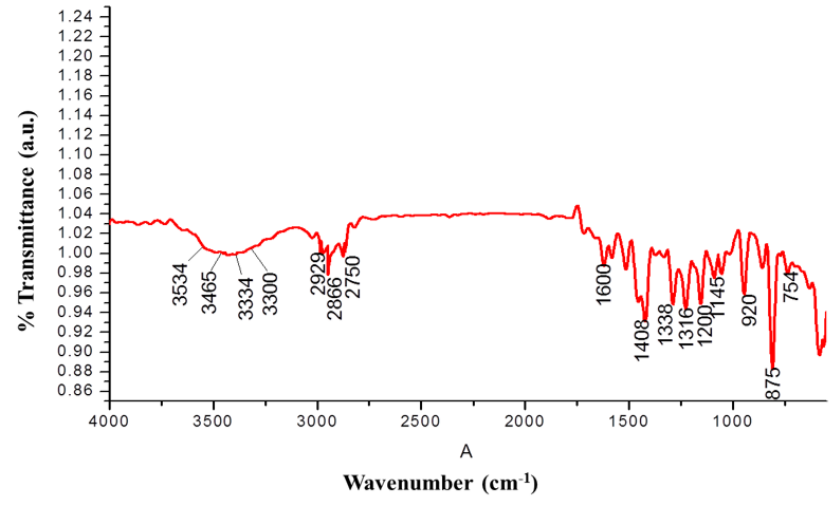


Figure 5: *FTIR spectrum peaks of Punica granatum seed oil.*

Discussions

In the present study, preliminary qualitative and quantitative analyses of the *P. granatum* L. peel extracts (methanol, ethanol) and seed oils were executed to analyze antibacterial [18] and antioxidant [19] properties against *P. acnes* and *S. epidermidis* (causative microbe of Acne vulgaris). The obtained data clearly portray significant antibacterial, antioxidant and anti-inflammatory properties of *P. granatum.* These properties of *P. granatum* may attribute to the presence of phytochemicals such as polyphenols, flavonoids triterpenoids, steroids, alkaloids, tannins, vitamin C etc [10]. The presence of these compounds in *P. granatum* extract may give credibility to its indigenous usage for treatment of oxidative stress induced disorders [21]. It has also been shown to increase the amount of short chain fatty acid of our

body microbiome. Short chain fatty acids are thought to have an anti-inflammatory effect on the sebaceous glands so the pomegranate extract may act as a prebiotic in the body and subsequently increasing the gastrointestinal microbial diversity and by producing short chain fatty acids that may have systemic beneficial effects especially on the acne or any other skin diseases [20]. As far as antioxidant property is concern, phenolic compounds, including flavonoids, anthocyanins and tannins, are accredited as a main group of antioxidant phytochemical and has free radical scavenging activities [11,21,23]. Correspondingly, estimation of the total phenolic and flavonoid contents of plant extracts [10,11,15] reveals seed oil having comparatively high phenolic and flavonoid content compare to ethanolic and methanolic extracts [28]. Whereas, *P. granatum* seed oil consist triglyceride composition and fatty acid profiles and greater amounts of conjugated linolenic acids exhibit characteristic biological activities. Pomegranate seeds contain about 6.3 - 12.2% oil on a dry matter basis [24].

FT-IR analysis was carried out to analyze the role of the plant extract functional groups, each peak in the FTIR spectra corresponds to the functional groups and vibration modes responsible for infrared absorption. FTIR analysis of SO also evidenced the presence of phytochemicals (Phenolics, alkanes, alkenes, Nitro compounds etc.) that are accountable for antibacterial and anti-inflammatory and antioxidant effects plant extracts [18]. The intensive bands with maxima at 2924 and 2852 cm^{-1} arise, respectively, from the asymmetric and symmetric stretching vibrations of CH_2 methylene and terminal methyl groups of the fatty acid chains in triacylglycerols. The range of 1000 - 700 cm^{-1} probably correspond with punicic acid, which is only present in *P. granatum* seed oil. The peak range of 1600 - 1500/1420 - 1300 cm^{-1} represent carbonyl compound [16].

DPPH is considered as the lipophilic radical, it accepts electron from the antioxidant compound and converts its color from purple to light brown detected at 517 nm [31] owing to the antioxidant activity. The plant extracts react (depicted with colour change) with DPPH radicals and converts the radical to α diphenyl-β-picryl hydrazine [17]. The degree of dis-coloration indicates the potential of the plant extract to scavenge free radicals due to its ability to donate hydrogen. Our data depicts the adequate radical scavenging activity on DPPH radicals subsequently from essential seed oil to ethanolic and methanolic extracts in descendings. The concentration dependent readings of *P. granatum* extracts were compared with the ascorbic acid, the findings corresponded with the findings of Rajan., *et al*. [11] and Oyedemi., *et al*. [32] indicates statistically significant correlation of high antioxidant activity of *P. granatum* (SO > PE > PM) (Table 3) with the high contents of phenolics compounds.

Furthermore, regarding pathogenesis of acne, another factor of concern is Nitric oxide, which is considered as a key factor that incites inflammation [25,26]. It is a pro-inflammatory mediator that persuades inflammation due to disproportionate production in anomalous situations [27]. Nitrite (oxides of nitrogen) was repressed by plant extract through direct competition with oxygen and other oxides of nitrogen in the reaction medium [28]. The nitric oxide scavenging property of these extracts was found to be significant, therefore, it may help to reduces inflammatory responses incites by oxides of nitrogen.

Determining the antibacterial effect of *P. granatum*, the results of the minimal inhibitory concentration (MIC) and the half maximal inhibitory concentration (IC_{50}) evaluated by the 96 well Plate microdilution method suggest that both extracts of pomegranate peel and oil exert different degrees of antibacterial activity (Table 1). The most powerful inhibitory effect of seed oil was observed against *P. acnes* with MIC of 0.097 mg/mL followed by methanolic extract (0.93 mg/ml) and ethanolic extracts (1.027 mg/ml). Whereas, the strains of S. epidermidis were comparatively lesser sensitive to these extracts (1.512, 1.861, range of SO, PE, PM). The IC_{50} ranges 0.061, 0.636, 1.012 of SO, PE, PM. The antibacterial activity of *P. granatum* peel extract is owing to its phytochemical composition, precisely, to the nature of its major phenolic compounds. It can also be accredited to one or more molecules, present in in the extract [36]. These molecules are conferred to substantial amounts of polyphenols present in peel extracts such as ellagic tannins, ellagic acid, and gallic acid [33]. Polyphenols played an important role in protein precipitation and enzyme inhibition of microorganisms [23]. It is reported that ellagic acid content has a significant influence on the antimicrobial activity [34]. Ahmad and Beg [2] reported that other phytochemical components found in alcoholic extract of pomegranate are alkaloid, flavonoid, glycoside, phenol, and tannin. Li., *et al*. [37] reported that phenolic compounds in pomegranate juice are punicalagin isomers, ellagic acid derivatives that are also found to possess bactericidal effects.

Conclusion

The methanolic extract of *P. granatum* peels showed the highest antibacterial efficiency against the tested bacterial strains. Accordingly, the impressive antimicrobial efficiency of the extracts of *P. granatum* peels against tested Acne pathogens confirmed the potential utilization of *P. granatum* extracts as safe dermal formulation, evading the harmful complications of synthetic treatments. Furthermore, the extracts also revealed a high antiradical activity against DPPH radical, from essential seed oil to ethanolic and methanolic extracts are in descending, encouraging utilization of these extracts in fabrication of safe and natural antioxidants, thereby avoiding the possible toxic effects of the synthetic antioxidants. The overall analytical data suggest that this extract could be a potential source of natural drug that could be of great importance for the treatment of acne prone skin.

Conflict of Interest Statement

I declare that I have no conflict of interest.

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