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Punica granatum L. (Pomegranate) leaves extract: The study of antioxidant and antibacterial activity

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Abstract
Pomegranate (*Punica granatum*) is a wonder fruit because of its huge pharmacological properties. Pomegranate and its extracts exhibit potent anticarcinogenic properties and the waste part of this wonder fruit, pomegranate has miraculous effects for human health. The potential therapeutic properties are wide-ranging and include treatment and prevention for cancer, cardiovascular disease, diabetes, dental conditions, male infertility, arthritis, and protection from ultraviolet (UV) radiation. The objective of the study was to evaluate the Pomegranate leaves extract by *in vitro* antioxidant methods like DPPH· radical, superoxide radical inhibition, phosphomolybdenum reduction, and ferric reducing power assays. The results showed that the Pomegranate leaves extract has significant antioxidant activity. The DPPH· radical scavenging activity of leaves extract was 79.13±0.37% at 120 µg/mL concentration and its IC_{50} was 39.16 µg/mL concentration. The superoxide radical scavenging activity of leaves extract was 71.48±0.43% at 120 µg/mL concentration and its IC_{50} was 40.31 µg/mL concentration. The phosphomolybdenum (Mo^{6+}) reduction of leaves extract was 53.36±0.39% at 120 µg/mL concentration and its RC_{50} was 104.89 µg/mL concentration. The Fe^{3+} reduction of leaves extract was 81.47±0.13% at 120 µg/mL concentration and its RC_{50} was 21.28 µg/mL concentration. The antibacterial activity showed the highest zone of inhibition of 19 mm against *Micrococcus luteus.*

Keywords: *Punica granatum*, antioxidant, DPPH, antibacterial

Introduction
Pomegranates (*Punica granatum* L.) considered as the "Tree of Life" has prominent medicinal properties [1]. Pomegranate is native in Asian countries including Iran to Northern India. It has been cultivated and naturalized over the whole Mediterranean region since ancient times [2]. Pomegranate has been the subject of current attractiveness as a medicinal agent with a wide variety of therapeutic applications. The pomegranates are used as natural remedies to chemical treatment due to their capability against a wide range of diseases. More or less every part of the pomegranate, including the fruit juice, peel, arils, flowers, and bark has been tested for antimicrobial activities. There are wide ranges of phytochemical properties that have demonstrated antimicrobial activities in pomegranate. Ellagic acid and hydrolyzable tannins, such as punicalagin, have the most activities [3]. The pomegranate peel does hold immense potential as it contains double the antioxidants than the fruit pulp. Compared to the pulp, the inedible pomegranate peel contains thrice the total amount of polyphenols [4] including condensed tannins [5], catechins, gallocatechin, and prodelphinidins [6]. The peels are effective against heart disease and are a rich source of vitamin C. Clinical research shows that pomegranates, when part of a healthy diet, might help prevent heart disease, heart attacks, and strokes. The use of pomegranate juice, peel, and oil has been showing anticancer activities, and interfere with tumour cell proliferation, cell cycle, invasion, and angiogenesis, and may be associated with the anti-inflammatory effects of pomegranate. The phytochemistry and pharmacological actions of pomegranate indicate a wide variety of clinical usage for cancer prevention and treatment, also other diseases where chronic inflammation is reliable to play a main etiologic role [7].

The plant, which may attain 5 or 7 meters (16 or 23 feet) in height, has elliptic to lance-shaped, bright-green leaves about 7.5 cm (3 inches) long. The handsome axillary orange-red flowers are borne toward the ends of the branchlets. The calyx (comprising the sepals) is tubular and persistent and has five to seven lobes; the petals are lance-shaped, inserted between the calyx lobes. The ovary is embedded in the calyx tube and contains several compartments in two series, one above the other [8]. The fruit is the size of a large orange, obscurely six-sided, with a smooth leathery skin that ranges from brownish-yellow to red; within, it is divided into several

chambers containing many thin transparent arils of reddish, juicy pulp, each surrounding an angular elongated seed [9].

Taxonomy

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Myrtales
Family:	Punicaceae
Genus:	*Punica*
Species:	*granatum*
Binomial name:	*Punica granatum*

Fig 1: *Punica granatum* (Pomegranate)

Materials and Methods

Collection of plant material and preparation of the extract

Pomegranate leaves were collected from Kundrathur, Chennai, Tamilnadu, India. The leaves were washed in distilled water and shade dried for 10 days. The dried leaves were powdered and soaked in methanol for 72 h. The supernatant was filtered by filter paper and condensed by a rotary evaporator at 50 ºC, which yields greenish gummy extract.

Phytochemical analysis

The phytochemical analysis of methanol leaves extract of *Punica granatum* was carried out for different classes of phytoconstituents using specific reagents [10, 11].

Estimation of total phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds with slight modifications[12]. One hundred µL (1mg/mL) of Pomegranate leaves extract was mixed with 900 µL of methanol and 1 mL of Folin Ciocalteau reagent (1:10 diluted with distilled water). Then, 1 mL of 20% (w/v) Na_2CO_3 solution was added and shaken well. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

Estimation of total flavonoids

The total flavonoid content of Pomegranate leaves extract was determined using aluminium chloride reagent method with slight modifications [13]. Five hundred µL (1mg/mL) of leaves extract was mixed with 500 µL of methanol and 0.5 mL of 5% (w/v) sodium nitrite solution. Then, 0.5 mL 10% (w/v) aluminium chloride solution was added followed by 50 µL of 1 M NaOH solution was added and shaken well. Absorbance was measured at 510 nm and the result was expressed as quercetin equivalent (µg/mg of extract), which is a common reference compound.

In vitro antioxidant activity

DPPH˙ radical scavenging activity

The radical scavenging activity of Pomegranate leaves extract was measured based on stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity [14]. One mL of various concentrations (20-120 µg/mL) of leaves extract was mixed with 1 mL of 0.1 mM DPPH solution in methanol and the mixture was then allowed to stand for 30 min incubation in dark. Ascorbic acid was used as the reference standard. One mL methanol mixed with 1 mL DPPH solution was used as the control. The decrease in absorbance was measured at 517 nm. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\%\ \text{of DPPH}^{\cdot}\ \text{radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Superoxide radical scavenging activity

The superoxide radical scavenging activity was carried out by the riboflavin-light-NBT system[15]. The reaction mixture contained various concentrations of (20-120 µg/mL) of Pomegranate leaves extract, 1.5 mM of riboflavin (200 µL), 12 mM of EDTA (100 µL), and 50 mM of NBT (50 µL) and added in that sequence. The reagents should be prepared in 50 mM of phosphate buffer (pH 7.6) solution. The reaction was started by illuminating the reaction mixture for 5 min and the absorbance was measured at 590 nm. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\%\ \text{of superoxide radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Phosphomolybdenum reduction activity

The radicals reduction capacity of Pomegranate leaves extract was assessed by the phosphomolybdenum reduction assay method [16]. The leaves extract with different concentrations (20-120 µg/mL) was mixed with 1 mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM), and sulphuric acid (600 mM). The reaction mixture was incubated in a water bath at 95ºC for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\%\ \text{of } Mo^{6+}\ \text{reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

Ferric (Fe^{3+}) reducing power activity

The reducing power of Pomegranate leaves extract was determined by the potassium ferricyanide method with a little modification [17]. One mL of leaves extract of different concentrations (20 - 120 µg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v) solution. The mixture was then incubated at 50ºC in a water bath for 20 min. Five hundred µL of trichloroacetic acid (10% w/v) was added to each mixture. Then 100 µL of freshly prepared $FeCl_3$ (0.1%,

w/v) solution was added, shaken well and the absorbance was measured at 700 nm. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\% \text{ of } Fe^{3+} \text{ reduction} = \frac{\text{Sample - Control}}{\text{Sample}} \times 100$$

Antibacterial activity

Microorganisms

The Gram-positive microorganisms such as *Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus* as well as Gram-negative microorganisms such as *Escherichia coli*, *Proteus vulgaris*, and *Shigella flexneri* were used for antibacterial activity.

Nutrient broth agar medium

Nutrient broth agar medium was prepared according to the standard methods (peptone-5 g, yeast-3 g, NaCl-5 g, distilled water- 1000 mL, agar-20 g). The reagents were calculated for medium preparation depending on the organisms to be used. Then, all the reagents weighed and suspended in 150 mL of distilled water in a conical flask, stirred, boiled to dissolve, and then autoclaved at 15 lbs and 121ºC for 15 min. The hot medium was poured in sterile petri plates which were kept in the aseptic Laminar airflow chamber and allowed to solidify for 15 min.

Agar well diffusion method

Antibacterial activity of Pomegranate leaves extract was carried out using the agar well diffusion method. The solidified nutrient agar in the petri plates was inoculated by dispensing the inoculum using sterilized cotton swabs which are previously immersed in the inoculum containing test tube and spread evenly onto the solidified agar medium. Five wells were created in each plate with the help of a sterile well-borer of 8 mm diameter. The leaves extract was then poured into each well containing 250, 375, and 500 µg/mL concentrations. All the plates with leaves extract loaded wells were incubated at 37ºC for 24 h and the antibacterial activity was assessed by measuring the diameter of the inhibition zone formed around the well[18]. Tetracycline (25 µg) was used as a positive control.

Results and Discussion

Qualitative phytochemical analysis

The phytochemical analysis of methanol Pomegranate leaves extract showed the presence of alkaloids, terpenoids, steroids phenolic compounds, flavonoids, tannins, glycosides, carbohydrate, and saponins.

Table 1: Phytochemical analysis of Pomegranate leaves extract

S. No	Phytochemicals	Test	Inference	Result
1	Alkaloids	Hager's test: To the extract, the saturated aqueous solution of picric acid was added and shaken well.	Yellow precipitate	+
2	Terpenoids	Salkowski test: To the extract, chloroform was added and mixed well. Then, a few drops of Conc.H_2SO_4 were added along the sides of the test tube.	A red ring appears.	+
3	Steroids	Libermann-Burchard's test: To the extract, 1 mL of acetic anhydride was added and shaken well. To this, few drops of Conc.H_2SO_4 were added along the sides of the test tube.	Various shades of dark colour appear	+
4	Phenols	Ferric chloride test: To the extract, a few drops of 5% $FeCl_3$ solution were added and shaken well.	The dark violet colour appears	+
5	Flavonoids	Alkaline Reagent test: To the extract, a few drops of 2% NaOH solution were added and shaken well.	Yellow colour appears	+
6	Tannins	Lead acetate test: To the extract, a few drops of 5% Pb $(CH_3COO)_2$ solution were added and shaken well.	White colour appears	+
7	Glycosides	Legal's test: To the extract, few drops of pyridine and few drops of alkaline sodium nitroprusside solution was added and shaken well.	Blood red color appears.	+
8	Carbohydrate	Molisch test: To the extract, two drops of alcoholic α-naphthol solution was added and shaken well. To this, a few drops of Conc.H_2SO_4 were added.	Violet ring appears	+
9	Saponins	Foam test: To the extract, 3 mL of distilled water was added and shaken vigorously.	Foam appears	+

Total Phenol and Flavonoid content

Phenolic compounds are secondary metabolites that are found naturally in all plant species, and plant-based food products. These compounds are thought to be an integral part of human and animal diets and represent the most important group of natural antioxidants [19]. The most common phenolic compounds in plants are phenolic acids, tocopherols, and flavonoids. It has been reported that phenolic compounds and flavonoids, act as antioxidants to exert antiallergic, anti-inflammatory, antidiabetic, antimicrobial, antipathogenic, antiviral, antithrombotic, and vasodilatory effects [20]. After proton donation, these compounds are oxidized to resonance-stabilized radicals that can further act as prooxidants at high concentrations, high pH, and in the presence of metal ions. Flavonoids and phenolic acids present in food such as quercetin, myricetin, caffeic acid, gallic acid, chlorogenic acid, coumaric acid, ferulic acid, and ellagic acid which are antioxidant and prooxidant behavior, have been proven to exhibit dual character [21]. The total phenolic content of Pomegranate leaves extract was 220.83±3.06 µg/mg of GAE and flavonoid content of Pomegranate leaves extract was 35.38±2.59 µg/mg of QE.

Table 2: Quantitative estimation of Pomegranate leaves extract

S. No	Phytochemical	Amount (µg/mg)
1	Phenols	220.83±3.06 GAE
2	Flavonoids	35.38±2.59 QE

DPPH˙ radical scavenging activity

The DPPH˙ radical scavenging (1,1-diphenyl-2-picrylhydrazyl) capacity of Pomegranate leaves extract was evaluated by reducing the purple coloured stable DPPH (1,1-diphenyl-2- picrylhydrazyl) radical into the yellow coloured non-radical form of 1,1-diphenyl-2-picrylhydrazine and the reducing capacity increases with increasing concentration of the extract [22]. The DPPH˙ radical scavenging activity of Pomegranate leaves extract was 79.13±0.37% at 120 µg/mL concentration and the IC_{50} was 39.16 µg/mL concentration

(Table 3; Fig. 2). The results revealed that the Pomegranate leaves extract showed good radical scavenging activity and it was compared with the standard ascorbic acid (IC_{50} = 11.98 µg/mL concentration).

Table 3: DPPH˙ radical and superoxide radical scavenging activities of Pomegranate leaves extract

S. No	Concentration (µg/mL)	% of inhibition	
		DPPH˙ radical	Superoxide ($O_2^{\cdot-}$) radical
1	20	16.90±0.46	42.97±0.12
2	40	51.07±0.41	49.61±0.18
3	60	57.55±0.17	58.47±0.20
4	80	61.51±0.28	61.57±0.27
5	100	66.54±0.25	63.42±0.15
6	120	79.13±0.37	71.48±0.43

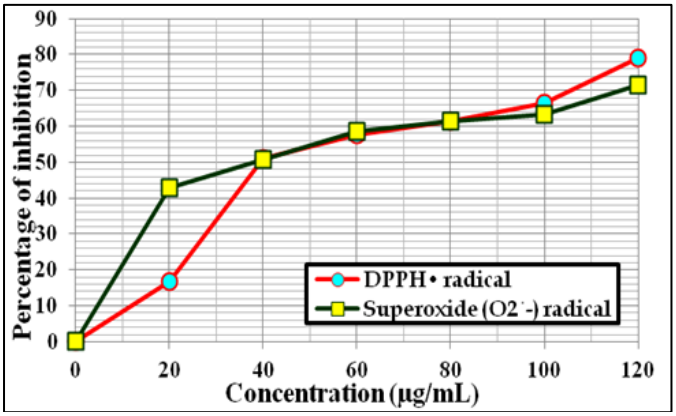

Fig 2: DPPH˙ radical and superoxide radical scavenging activities of Pomegranate leaves extract Superoxide radical scavenging activity

Superoxide is considered to be poorly reactive, and cell damage has been attributed to the generation of HO• radical via the Haber-Weiss reaction and produces other kinds of free radicals and oxidizing agents [23]. Superoxide radicals are the major initial form of ROS produced by mitochondria. Superoxide radicals are produced by mitochondria and are converted into hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase and if the metal ion catalyzes the H_2O_2 the most dangerous HO• radicals coming out. Superoxide or hydrogen peroxide increases the rate of DNA replication and cell proliferation. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which was measured at 590 nm. Antioxidants can inhibit the blue NBT formation and the decrease in absorbance indicates the consumption of superoxide anion by the Pomegranate leaves extract. The maximum superoxide radical scavenging activity of the Pomegranate leaves extract was 71.48 ± 0.43% at 120 µg/mL concentration (Table 3; Fig. 2) and the IC_{50} was 40.31 µg/mL concentration. It was compared with the standard of ascorbic acid (IC_{50} = 9.65 µg/mL concentration).

Phosphomolybdenum reduction activity

The reduction capacity of Pomegranate leaves extract was measured by the phosphomolybdenum reduction assay method. In this method, Pomegranate leaves extract reduces the Mo^{6+} complex into Mo^{5+} complex and the formation of green to blue phosphate/Mo complex at acidic pH, which was measured at 695 nm [24]. The phosphomolybdenum reduction of Pomegranate leaves extract was 53.36 ± 0.39% at 120 µg/mL concentration and the RC_{50} was 104.89 µg/mL concentration (Table 4; Fig. 3). It was compared with the standard ascorbic acid (RC_{50} = 6.34 µg/mL concentration).

Table 4: Phosphomolybdenum reduction and Fe^{3+} reducing power activities of Pomegranate leaves extract

S. No	Concentration (µg/mL)	% of reduction	
		Mo^{6+} reduction	Fe^{3+} reduction
1	20	9.09±0.23	47.00±0.46
2	40	30.23±0.16	54.45±0.18
3	60	38.35±0.19	68.62±0.30
4	80	43.03±0.41	81.14±0.35
5	100	47.67±0.36	81.30±0.22
6	120	53.36±0.39	81.47±0.13

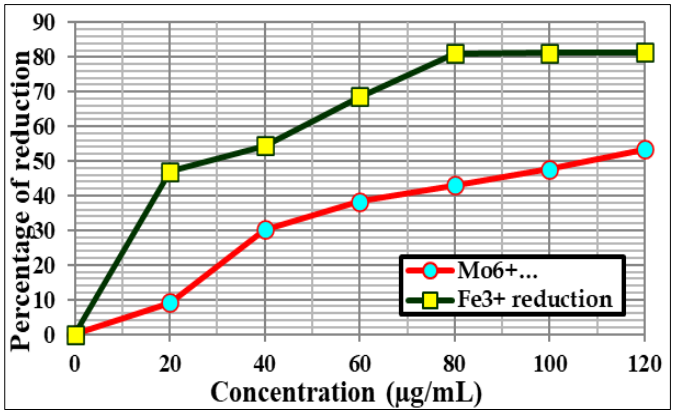

Fig 3: Phosphomolybdenum reduction and Fe^{3+} reducing power activities of Pomegranate leaves extract

Ferric (Fe^{3+}) reducing power assay

The reducing power of Pomegranate leaves extract was measured and the reduction ability from Fe^{3+} to Fe^{2+} increases with increasing concentration of the extract (Table 4; Fig. 3) due to the formation of the ferro-ferric complex [25]. Ferric ion reducing power activity determines the electron-donating ability of an antioxidant present in the extract. The flavonoids and phenolic compounds present in the Pomegranate leaves extract to have the ability to donate electrons, which reflects strong antioxidant activity. The Fe^{3+} reduction of Pomegranate leaves extract was 81.47 ± 0.13% at 120 µg/mL concentration and the RC_{50} was 21.28 µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50}= 7.72 µg/mL concentration).

Antibacterial activity

The antibacterial activity of Pomegranate leaves extract was studied against Gram-positive microorganisms such as *Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus* as well as Gram-negative microorganisms such as *Escherichia coli*, *Proteus vulgaris Shigella flexneri*. The Pomegranate leaves extract showed the highest zone of inhibition of 19 mm against *Micrococcus luteus* at 500 µg/mL concentration (Table 5; Fig 4). An important quality for an antimicrobial drug is selective toxicity, meaning that it selectively kills or inhibits the growth of microbial targets while causing minimal or no harm to the host. The antibiotics of secondary metabolites present in the Pomegranate leaves extract may interfere with specific steps in homeostatic cell wall biosynthesis and a cell wall synthesis inhibitor can result in changes to cell shape and size, induce cellular stress responses, and induces cell lysis [26]. For example, β-lactams derivatives block the cross-linking of peptidoglycan (PG) units by inhibiting the peptide bond formation reaction catalyzed by transpeptidases [27].

Table 5: Antibacterial activity of Pomegranate leaves extract

Microorganisms	Zone of inhibition (mm)			
	250 µg/mL	375 µg/mL	500 µg/mL	Standard (Tetracycline)
B. subtilis	13	15	16	12
M. luteus	16	17	19	12
S. aureus	13	15	17	11
E. coli	14	16	18	13
P. vulgaris	14	15	16	14
S. flexneri	15	16	17	14

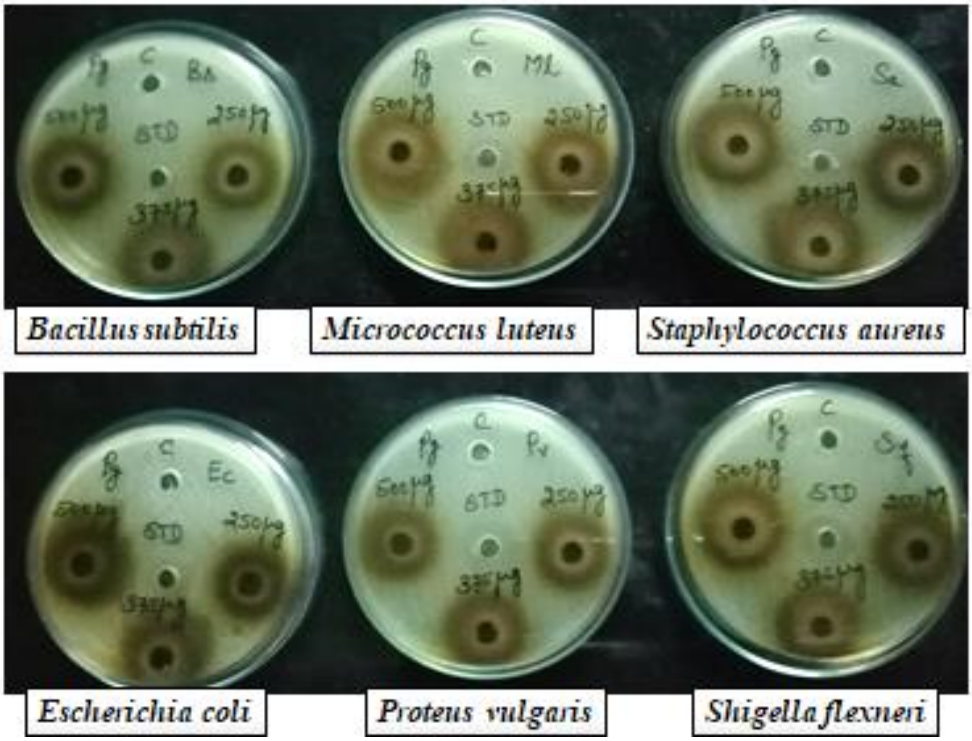

Fig 4: Antibacterial activity of Pomegranate leaves extract

Conclusion

The body cells face threats every day, as many viruses and infections attack them and free radicals also can damage cells and DNA. Some cells can heal from the damage, while others cannot and such free radicals can contribute to the aging process. Free radicals also play a part in diseases, like cancer, diabetes, and heart disease. Antioxidants are stopped or limit the damage caused by the free radicals and balance them. Antioxidants can protect and reverse some of the damage and also boost immunity. Drug-resistant bacterial infections are becoming more prevalent and are a major health issue facing today. The complex effects of bactericidal antibiotics provide a large playing field for the development of novel antibacterial compounds, as well as adjuvant molecules and synthetic biology constructs that could enhance the potency of current antibiotics. It will be very important to find out new antibiotics and antioxidants from natural sources for clinical treatments and approaches so that effectively fight the growing threat from resistant pathogens and boost up immunity to scavenge radicals. The experimental data showed that the Pomegranate leaves extract has good antioxidant and antibacterial activities.

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