RESEARCH ARTICLE

Comparative Investigation of *Punica granatum* L. Phytochemical Analysis and *In-vitro* Antimicrobial Activity Screening Using Roots Samples from Urban and Rural Areas Uses Five Different Solvent Extracts

V Naga Ashwini[1], Revathi M[2], J Manjunathan[1*]

[1]*Department of Biotechnology, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies, Chennai, Tamil Nadu, India.*

[2]*Department of Chemistry, School of Basic Sciences, Vels Institute of Science, Technology and Advanced Studies, Chennai, Tamil Nadu, India.*

Received: 20th January, 2024; Revised: 16th April, 2024; Accepted: 01st July, 2024; Available Online: 31st August, 2024

ABSTRACT

Antimicrobial, fungal, and bacterial infections in the human body are the causes of foodborne, waterborne, and airborne illnesses as well as those that arise naturally. Because nosocomial usage increases the presence of both complex and non-complicated microorganisms, urinary tract infections (UTIs) are most common, especially in women. Antibiotics are an amazing tool in the fight against infections; the WHO survey 2022(1) noted that microbial and fungal infections account for 1.5 billion deaths worldwide. The production of natural remedies is becoming a serious concern and is progressing quickly as well. Many phytochemical compounds from flora could be used to treat these infections based on the effect of the respective integrated organism's host defense efficacy. The therapeutic or pathogen-resistant capacity of the phytochemicals under different chemical classes may reverse and control the antibiotic resistance of resistant pathogens. The aim of the study is to find the roots of *Punica granatum* L from the urban and rural regions to find the phytochemical efficacy from extracts like petroleum ether, chloroform, ethanol, carbinol and aqueous. It revealed that a high yield of pharmacological compounds is from a rural sample's carbinol extract. Quantitative analysis showed the presence of high amounts of majorly anti-cancerous agents in high peaks from rural samples. Using the well diffusion method, *in-vitro* antimicrobial activity was determined. Against their negative controls, the inhibition activity of *Escherichia coli* showed a 22 mm inhibition zone from the ethanol extract, *Staphylococcus aureus* showed a 21 mm inhibition zone, and fungal pathogens such as *Candida albicans* and *Candida krusei* showed a 21 mm maximum inhibition zone from the carbinol extracts.

Keywords: *Punica granatum,* Urinary tract infections, Phytochemical, Antimicrobial activity.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.3.22

How to cite this article: Ashwini VN, Revathi M, Manjunathan J. Comparative Investigation of *Punica granatum* L. Phytochemical Analysis and *In-vitro* Antimicrobial Activity Screening Using Roots Samples from Urban and Rural Areas Uses Five Different Solvent Extracts. International Journal of Pharmaceutical Quality Assurance. 2024;15(3):1239-1246.

Source of support: Nil.

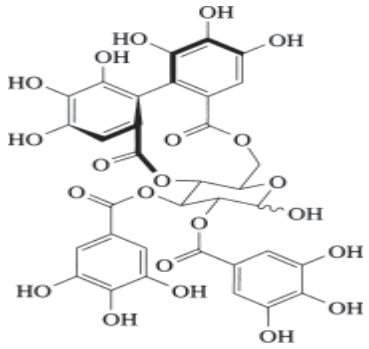
Conflict of interest: None

INTRODUCTION

Mother Nature has gifted us with several medicinal plants in this Universe, most of which are very much available feasibly and at a lesser cost, a must say a thing is India a place where most of the plants have medicinal formulas that can heal or repair disorders and diseases easily.[1] Most of the leaves, fruits, greens, pulses, cereals, etc. which is consumed in day to day lives have exemplary effects on the proper functioning and magical maintenance of the metabolic pathways. The supportive enzymes and co-enzymes that are present in the parts of legumes and arils of a few fruits can actually play the role of supplementary factors. Majorly highlighted corner of a healthy tree is strong and meticulously deepened and has healthy roots. The fertility of the soil and aeration factors of its quality, as well as the water toxicity levels, play a vital role in healthy tree growth and its production.[2] Every living organism is dependent on other fellow living organisms for its food and survival, which everyone is aware of in its ecosystem. Amongst Man is on the Top end of consuming most of the universal and natural productions. Hence the maintenance and balancing of his health and resistance against most commonly occurring infections can be avoided.

Punica granatum. L fruit is a "boon on earth". They have high antioxidant, anti-inflammatory and antimicrobial activities. The rich phytodynamics and phytokinetics constituents that are present in all the parts of the fruit, from

**Author for Correspondence: jmanjunathan@gmail.com*



(106)

Figure 1: Ellagitannins

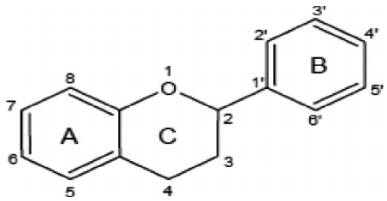


Figure 2: Flavanoids

Figure 3: *P. granatum* plant

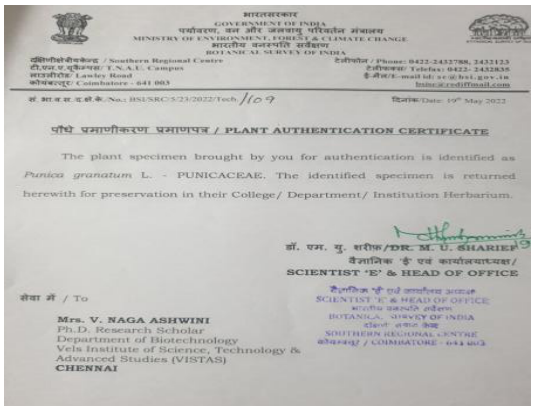
the mesocarp to the arils, has proved to have the best medicinal history to cure many ailments and disorders. There is a belief that the root extracts, when taken in excess, may be poisonous. Limited research on the roots of *P. granatum. L* for the benefit of treating many female reproductive organ-related ailments, disorders and infections lead to this study. Pomegranate is used as medicine in treating many immortalities and it has become a symbol of life, femaleness, fertility knowledge etc.[3]

Biological Taxonomy of *P. granatum* [4]

Kingdom: Plantae
Phylum: Tracheophyta
Class : Magnoliopsida
Order : Myrtales
Family : Lythraceae
Genus : *Punica*
Species: *granatum*

Pomegranate is considered as flavanoid-rich plant, the roots of pomegranate have shown a wide variety of elagitannins like punicalin, punicalgin and numerous piperidine alkaloids.[5] Figure 1 and 2 shows the strcutures of chemical compounds. Lack of proper sanitation and self-hygiene can make them

Figure 4: Root of *P. granatum*



भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
टी.एन.ए.यू. कैम्पस: T.N.A.U. Campus
लॉचरी रोड: Lawley Road
कोयम्बटूर/ Coimbatore - 641 003

टेलीफोन / Phone: 0422-2432788, 2432123
टेलीफाक्स: Telefax: 0422- 2432835
ई-मेल/E-mail id: sc@bsi.gov.in
bsisrc@rediffmail.com

सं. भा.व.स.द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech./1109 दिनांक/Date: 19th May 2022

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Punica granatum* L. - PUNICACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

डॉ. एम. यू. शरीफ/DR. M. U. SHARIEF
वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष/
SCIENTIST 'E' & HEAD OF OFFICE

सेवा में / To
Mrs. V. NAGA ASHWINI
Ph.D. Research Scholar
Department of Biotechnology
Vels Institute of Science, Technology & Advanced Studies (VISTAS)
CHENNAI

वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष
SCIENTIST 'E' & HEAD OF OFFICE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA
दक्षिणी क्षेत्रीय केन्द्र
SOUTHERN REGIONAL CENTRE
कोयम्बटूर / COIMBATORE - 641 003

Figure 5: BSI of the samples collected

prone to many microbial pathogen infections. Challenging factors of organisms are always intricate; however, the hidden solution in flora and fauna also comes hand in hand. Phytodynamics and phytokinetics of Natural medicine have its own importance and is always a boom for the newer generation. The phytochemicals and chemical groups of *P. granatum* as bioactive compounds have their therapeutical factors that can fight against the defense mechanism of the host efficacy of serious and infectious pathogens.[6] A recent update from WHO dated June 2023 says research priorities must be increased for antimicrobial resistance (AMR) pathogens, due to which in a year there are 1.5 billion deaths recorded worldwide by increasing the risk of disease spread, bacteria, fungi, and viruses which change over time and no longer respond to present antimicrobial medicines.

MATERIALS AND METHODS

Collection of Samples

P. granatum roots were collected from urban regions (Erode district, Tamil Nadu) and rural regions (Kothagiri, Ooty Hills).

The plant parts are labelled as PGE and PGK. The collected samples were endorsed by BSI (Botanical Survey of India) Agri University, Coimbatore. Figure 3 to 5 shows the root samples of *P. granatum* and the BSI of the samples.

Experimental Procedure

Preparation of plant extract

The roots were transferred to the lab within 24 hours, washed under tap water and dried in room temperature for 4 hours. Then fresh samples were coarsely powdered and 20 g of the sample is mixed with petroleum ether, chloroform, ethanol,

methanol solvents in different conical flasks, stirred at (900 rpm) for 10 minutes and filtered in 25 mesh sieves. After the filtration process, the extract was transferred to a rotary device to remove excess of solvent.

Extract preparation

About 20 g of the powdered root samples of *P. granatum* were boiled with ethanol, carbinaol, distilled water, petroleum ether, and chloroform as solvents. The solvent extracts were cooled for 30 minutes and then filtered in different conical flasks with filter paper. All the extracts, once prepared, were transferred into amber-colored reagent bottles, labeled and stored at room temperatures (20–22º C, 68–72ºF) for not letting the light to pass through.

Phytochemical Analysis

Qualitative analysis

The chosen solvents have the root samples soaked in them for 72 hours. The extent of extraction is analysed by the respective protocols qualitatively. Each qualitative analysis was performed three times to confirm the phytochemicals.

Quantitative analysis

Presence from various previous studies, it was believed that Pomegranate fruit has rich sources of tannins, flavonoids, anthocyanin, polyphenols, and natural sugar content, which can promote several health characteristics and benefits. The below-mentioned qualitative assay has been performed to check the presence of secondary metabolites in the roots, where it is always believed that the root is only a bank of primary metabolites.[7,8]

Dragendorff's Test

A few milligrams of the extract were heated for two minutes in a test tube containing 2% H_2SO_4 before being filtered in the same test tube. After that, a small amount of Dragendorff's reagent bismuth carbonate (5.2 g) and sodium iodide (4 g)—was heated to a boil in 50 mL of glacial acetic acid for a short while. After 12 hours, the precipitated crystals of sodium acetate were filtered through a sintered glass funnel. Added to it was a reddish-brown filtrate made from 40 mL of clear solution combined with 60 mL of ethyl acetate and 1-mL of water, all stored in an amber-colored container. The orange-red precipitates indicate the presence of alkaloids.

Hagger's test

Alkaloids are present when a vivid yellow precipitate forms after 2 mL of the plant extract and a few drops of Mayer's reagent (saturated picric acid solution).

- *Mayer's test*

About 2 mL of Con.HCl (Hydrochloric acid) was added to 2 mL of extract, and then a few drops of Mayer's reagent were added. Precipitate with a white or green color indicates the presence of alkaloids.

Terpenoids Detection

Salkowski test: About 1-mL of chloroform was added to the 500 µL of extract and thoroughly stirred. The test is positive when a reddish-brown film forms on the test tube's sidewalls after a few cautious drops of concentrated sulfuric acid are applied.[8]

Phenolic Substance Detection

Ferric chloride test: About 1-mL of distilled water was used to dissolve the 500 µL of extract. A small amount of neutral 5% ferric chloride solution was added to this. The presence of phenolic compounds is indicated by a dark green or violet color.[9,10]

Detection of Flavanoids

Flavonoid detection using the alkaline reagent test. After adding a few drops of NaOH solution and thoroughly shaking the 500 µL of extract. A vivid yellow hue emerges[11].

Tannin Detection

Lead acetate test: About 1-mL of a 10% Lead acetate solution was added after 500 µL of extract had been diluted in distilled water. A large, white precipitate12 indicated the presence of tannins.

Carbohydrates Test

Molisch test

Add two drops of alcoholic α-naphthol solution to the 500 µL of extract and thoroughly shake. A small amount of concentrated sulfuric acid was added to the test tube's walls. The formation of a violet ring indicates the presence of carbohydrates.[11,12]

Detection of saponins

Foam Test: To make 5 mL, 500 µL of extract were diluted with distilled water. The suspension was given a good, hard shake. A lingering froth suggested the existence.[12]

Protein detection

- *Xanthoproteic test*

After adding a few drops of concentrated nitric acid to 1-mL of extract (500 µL), a yellow color formed, signifying the presence of proteins.[13]

- *Glycoside detection*

Legal's Test 500 µL of pyridine was used to dissolve the 500 µL of extract. To make it alkaline, 500 µL of sodium nitroprusside solution was added next, and then a few drops of NaOH solution. The presence of glycosides was shown by the formation of blood red color.[11,12]

Quinione Detection

After adding 1-mL of methanol and 500 µL of extract, thoroughly mix them. Next, a small amount of Con. Sulfuric acid was gently applied along the test tube's sides to create a red ring[11,12].

Quantitative Analysis

Five separate petri plates containing five distinct extract samples each have 1-mL of root sample condensed at room temperature (25ºC)[14,15] Following the condensed state, silica gel is employed, the samples are scraped, and then the samples are collected.[16,17] The absorbance readings are then recorded

Table 1: Standard secondary metabolite and its absorbance level

Name of secondary metabolite	*Standards used and absorbance in nm*
Alkaloids	Atropine @ 470
Phenolic compounds	Gallic acid @ 765
Flavanoids	Quercetin @ 510
Tannins	Tannic Acid @ 700
Terpenoids	Linalool @ 538
Saponins	Diosgenin @ 544
Carbohydrates	Glucose @ 495
Proteins	BSA @ 595
Glycoside	Sennoside @495
Quinone	Quinones @ 505

** BSA = Bovine Serum Albumin: All of the subsequent quantitative tests were run every trimester, and the average result was noted. Ethanol and carbinol (methanol) were equally important in quantitative investigations; however, methanol outperformed all other solvents in terms of extraction of secondary metabolites.

against the standards. The standards that are applied to the quantitative metrics are listed in Table 1.

The following formula further calculates the quantitative values

$$= \frac{Absorbance\ of\ the\ test\ value}{absorbance\ of\ the\ standard\ used} X\ 100\ g$$

All five extracts were prepared to check the absorbance values and were derived with the above mentioned formula and finalized that the methanol (carbinol) extract yields have more standard values derived.

Microorganisms and Culture Medium

This study will focus on gram-positive and gram-negative bacteria as well as fungal infections. *Esherichia coli* is a gram-negative bacteria[18,19] and *Staphylococcus aureus* (Gram-positive bacteria). Fungal infections: *Candida Albicans* (CA) and *C. Krusei* (CK) were determined using the Kirby – Bauer disc diffusion method, and the percentage of inhibitory concentration is measured.[20,21] It is discovered that Carbinol methonal extracts have higher inhibition values than other solvents.

Preparation of Culture Medium

The Culture medium was prepared for antibacterial and antifungal activity. The ingredients for culture medium preparation are listed in Table 2.

Antibacterial Activity of the Plant Extracts

The antibacterial activity of the different solvent extracts of *P. granatum* was studied as per the standard procedures. The host defence mechanism of negative bacterial strains, such as gram-negative *E. coli* and gram-positive *S. aureus*, was assessed. After filling the plates with the agar medium and allowing it to firm, the pathogens were streaked and marked in relation to the control and test solutions. Next, the well was sliced with cork borers.[22,23] The antibacterial assay uses fluconazole and tetracycline as controls. Following the inoculation of the test sample with 250, 500, and 1000 µL, the plates were incubated at 37ºC for the entire night, and the following day, the inhibitory zones were determined.[24]

Table 2: Culture medium of for antibacterial and antifungal activity

Antibacterial activity	*Antifungal activity: (PDA medium potato dextrose agar medium)*
Peptone – 5 g	Potato Infusion – 200 g
Yeast extract – 3 g	Dextrose (Glucose) – 20g
Sodium chloride – 5 g	Agar – 25g
Distilled water – 1000 mL	Distilled water – 1000 mL
Agar – 25 g	-
(PH: 7 ± 0.02)	(PH 6.5 ± 0.02)
Control used is dimethyl sulphoxide	Control used is dimethyl sulphoxide
Standard used is tetracycline	Standard used is fluconazole

Table 3: Petroleum ether extract of *P. granatum* from erode (PGE) and Kothagiri hills (PGK)

Secondary metabolite	*PGE*	*PGK*
Alkaloids:		
Dd	---	---
Ht	---	---
Mt	---	---
Terpenoids	---	---
Phenols	---	---
Flavanoids	---	---
Tannins	+++	+++
Carbohydrates	---	---
Saponins	+++	+++
Proteins	---	---
Glycosides	+++	+++
Quinone	---	---

PGE: *P. granatum.L* Erode (Urban sample) PGK : *P. granatum.L* Kothagiri hills (Rural sample)

Antifungal Activity of the Plant Extracts

The antifungal activity of the different solvent extracts of *P. granatum* was studied as per the standard procedures. The fungi *Candida auris* and *C. krusei* were selected for this study because they each pose different risks to human health and immunity. They can cause candidiasis in people, which can be fatal if left untreated.[25] Following the filling of the plates with potato dextrose agar (PDA) and a 20 minute solidification period, the fungi parasites were streaked and left out for a 24 hour period to allow for fungal development.[26] Next, hygienic cork borers cut the wells. After pathogen streaking and organism culture for 24 hours, control and test fungal samples were placed in the well for the antifungal assay[27]. Fluconazole is the control utilized in this experiment. By calculating the zone's extent, the drug's responsiveness is determined.

RESULT AND DISCUSSION

Table 3 illustrates the presence of fundamental preliminary phytochemical components in petroleum ether extracts of

Table 4: Chloroform extract of *P. granatum* from Erode (PGE) and Kothagiri hills (PGK)

Secondary metabolite	*PGE*	*PGK*
Alkaloids:		
Dd	---	---
Ht	---	---
Mt	---	---
Terpenoids	---	---
Phenols	---	---
Flavanoids	---	---
Tannins	+++	+++
Carbohydrates	---	---
Saponins	+++	+++
Proteins	---	---
Glycosides	+++	+++
Quinone	---	---

Table 5: Ethanolic extract of *P. granatum* from Erode (PGE) and Kothagiri hills (PGK)

Secondary metabolite	*PGE*	*PGK*
Alkaloids:		
DD	+++	+++
HT	+++	+++
MT	+++	+++
Terpenoids	+++	+++
Phenols	+++	+++
Flavanoids	+++	+++
Tannins	+++	+++
Carbohydrates	+++	+++
Saponins	+++	+++
Proteins	---	---
Glycosides	+++	+++
Quinone	+++	+++

P. granatum from Erode (PGE) and Kothakigri hills (PGK). Tannins, saponins and glycosides were observed in PGE – petroleum ether extract and PGK – petroleum ether extracts.

Table 4 illustrates the presence of fundamental preliminary phytochemical components in chloroform extracts of *P. granatum from* erode (PGE) and Kothagiri hills (PGK). Tannins, saponins and glycosides were observed in PGE – Chloroform extract and PGK – chloroform extracts.

Table 5 illustrates the presence of fundamental preliminary phytochemical components in Ethonalic extracts of *P. granatum from* Erode (PGE) and Kothagiri hills (PGK). Tannins, saponins and glycosides were observed in PGE – Ethonalic extract and PGK – Ethonalic extracts. alkaloids, terpenoids, phenolic compounds, flavonoids, tannins, carbohydrates, saponins, glycosides and quinones were present in both PGE and PGK.

Table 6 illustrates the presence of fundamental preliminary phytochemical components in Carbinolic extracts of P. granatum from Erode (PGE) and Kothagiri hills (PGK). In both PGE Carbinolic extract and PGK – Carbinolic extracts alkaloids, terpenoids, phenolic compounds, flavonoids, tannins, carbohydrates, saponins, proteins, glycosides and quinones were present.

Table 7 illustrates the presence of fundamental preliminary phytochemical components in *Aqueous* extracts of *P. granatum from* Erode (PGE) and Kothagiri hills (PGK). In both PGE – *Aqueous* extract and PGK – *Aqueous* extracts few alkaloids (DD & HT) , terpenoids, phenolic compounds, flavonoids, tannins, carbohydrates, saponins, glycosides and quinones were present.

Table 6: Carbinol extract of *P. granatum* from Erode (PGE) and Kothagiri hills (PGK)

Secondary metabolite	*PGE*	*PGK*
Alkaloids:		
DD	+++	+++
HT	+++	+++
MT	+++	+++
Terpenoids	+++	+++
Phenols	+++	+++
Flavanoids	+++	+++
Tannins	+++	+++
Carbohydrates	+++	+++
Saponins	+++	+++
Proteins	+++	+++
Glycosides	+++	+++
Quinone	+++	+++

Table 7: Aqueous extract of *P. granatum* from erode (PGE) and Kothagiri hills (PGK)

Secondary metabolite	*PGE*	*PGK*
Alkaloids:		
DD	+++	+++
HT	+++	+++
MT	-----	----
Terpenoids	+++	+++
Phenols	+++	+++
Flavanoids	+++	+++
Tannins	+++	+++
Carbohydrates	+++	+++
Saponins	+++	+++
Proteins	---	---
Glycosides	+++	+++
Quinone	+++	+++

Antibacterial and Antifungal activity study

Five plant species were investigated to evaluate their antibacterial activity against food poisoning bacteria, including two strains of gram-positive bacteria (*B.* cereus & *S. aureus*), three strains of gram-negative bacteria (*E. coli*), fungal pathogen *C. aureis*, *C. krusei* using disc diffusion method. Evaluation of antibacterial activity and antifungal of these

plant extracts were recorded in Tables 8 to 11 and illustrated in Figures 6 (A, B, C, D) & 7 (E, F, G, H).

Antifungal Activity Studies

When secondary metabolites were discovered, the analysis confirmed the presence of polysaccharides, nucleic acids, proteins, and esters in the roots. The extraction process was carried out in accordance with the protocols specified, and the presence of the secondary metabolites was examined.[28] Not only do the metabolite components need to be present, but their quantities also matter. In the standard graphs, the values were indicated in relation to the corresponding standards, and all quantitative measures were estimated. *P. granatum* roots have a significant potential for antimicrobial action; when the antimicrobial analysis was conducted on the roots,[27] the results

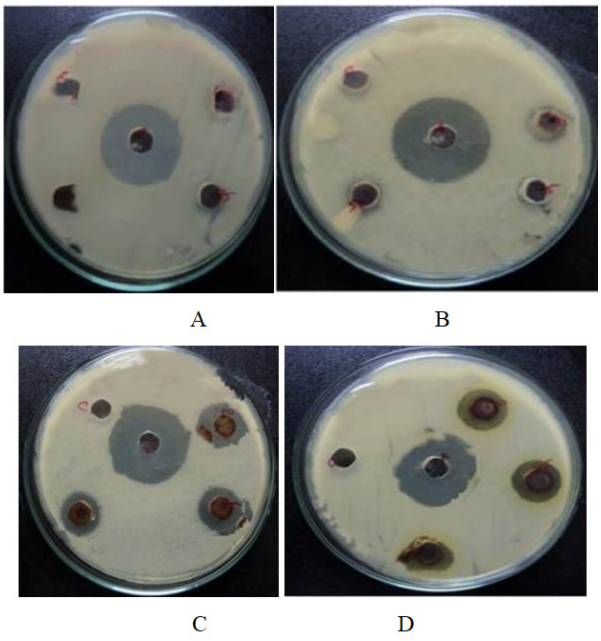


Figure 6: Antibacterial activity of plant extracts (A: Petroleum Ether extract; B: Chloroform extract; C: Ethanolic extract; D: Carbinolic (Methanol) extract)

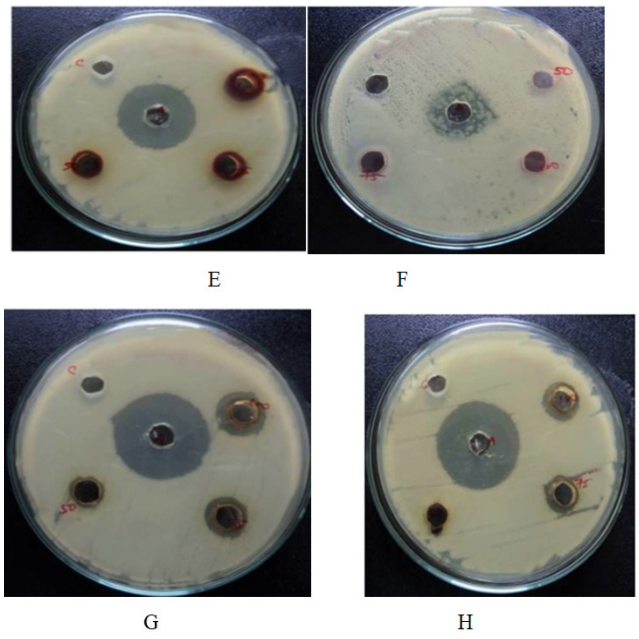


Figure 7: Antifungal activity of various extract (E: Petroleum Ether extract, F: Chloroform extract, G: Ethanolic extract; H : Carbinolic (Methanol) extract)

Table 8: Zone of inhibition of pathogen *E. coli* (Gram-negative bacterium)

S. No	*Concentration (µl)*	*Standard*	*Petroleum ether*	*Chloroform*	*Ethanol*	*Methanol*
1.	250	19	0	10	13	14
2.	500	20	0	11	16	19
3.	1000	22	13	12	18	22

Table 9: Zone of inhibition of pathogen (Gram-positive bacterium)

S No	*Concentration (µL)*	*Standard*	*Petroleum ether*	*Chloroform*	*Ethanol*	*Methanol*
1.	250	14	0	8	14	14
2.	500	14	0	10	16	16
3.	1000	16	0	11	18	20

Table 10: Zone of inhibition of fungal pathogen ***C. aureis***

S. No	*Concentration (µL)*	*Standard*	*Petroleum ether*	*Chloroform*	*Ethanol*	*Methanol*
1.	250	14	6	8	12	16
2.	500	14	6	8	12	18
3.	1000	16	8	10	14	22

Table 11: Zone of inhibition of fungal pathogen *C. krusei*

S.No	*Concentration (µL)*	*Standard*	*Petroleum ether*	*Chloroform*	*Ethanol*	*Methanol*
1.	250	12	0	6	10	14
2.	500	12	4	8	10	16
3.	1000	14	6	8	12	20

*All tests were performed thrice for the interference.

were comparable to those obtained from the other components of the pomegranate plant, including the fruits, arils, leaves, juice extract, and secondary metabolites.[29-32]

It is demonstrated in these striking comparative experiments that pathogens are much more reactive in the methanol solvent than in the other, less polar solvents. Additionally, it is observed that the most or extremely contagious diseases have also demonstrated reactivity to the pharmacological components found in *P. granatum* roots extracted in methanol solvent.

CONCLUSION

The current investigation showed that the various solvent extract from *P. granatum* reduced the inhibition of bacteria and fungi. Furthermore, a variety of bioactive chemicals were found in the extracts. In-depth research is needed to gain a better understanding of the crucial therapeutic role of *P. granatum* through in vivo studies, as well as the *in-silico* molecular docking processes of the bioactive chemicals in the metabolic pathway of obesity and hypertension in diabetes mellitus.

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