INVESTIGATION OF *PUNICA GRANATUM L.* LEAF EXTRACTS ON ANTIMICROBIAL ACTIVITY

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Abstract: Current study was conducted to identify and isolate vital phytochemical constituents of *Punica granatum* leaf extracts and antimicrobial activity of these extracts during March 2021. The experiment was designed under Complete Randomized Design (CRD). The results revealed that leaf extracts of *P. granatum* contained higher contents of bioactive compounds like Total Phenolic Compounds (119.47mg GAE/g), Total Antioxidants (1.71 DPPH inhibition), the enzyme Catalase (3.92 mg/g), Superoxide Dismutase (0.0503 µg/g) and Peroxidase (0.88 µg/g). To assess the antimicrobial activity of *P. granatum* extract of different solvents were applied against micro bacterium *Escherichia coli, Pseudomonas aurigonisa* and *Staphylococcus aureus*. According to the findings of the antibacterial assay, the hexane and chloroform extracts of the *P. granatum* leaves showed inhibitory activity against, *S. aureus* whereas the *E. coli* and *P. aurigonisa* bacteria were resistant against the extract constituents. Hexane leaf extracts zones of inhibition against micro bacterium were 8.6mm, 2mm and 2mm, and chloroform leaf extract had zones of inhibition as 6.3mm, 1.33mm and 1.33 mm against *S. aureus, P. aurigonisa* and *E. coli* respectively. While distilled water leaf extract zones of inhibition against Bactria was 4.6mm, 0.23mm against *S. aureus* and *P. aurigonisa*, but no inhibition against *E. coli* was recorded. The presence of these phytochemicals leads to the conclusion that *P. granatum* leaves have the potential for formation of new drugs and can be used as herbal medicine for treatments of different diseases and is a good natural candidate to produce drugs against micro bacterium.

Key Words: Extract, Antimicrobial Activity, Total Phenolic Compounds, Micro Bacterium, Chloroform Extracts

INTRODUCTION

Synthetic and traditional medicine are both derived from natural products. Phytochemicals are organic substances found in plants. Plants and their derivates are being used as medicines since ancient times. Kamal *et al., (*2014). Medicines obtained from medicinal plants are efficient, inexpensive, and easily available and have no complexities.

The species is mainly subtropics to mild-temperate and naturally adaptable to areas with hot summers and cool winters. *P. granatum* bears heat and blooms in dry areas, requiring constant watering during the dry season to obtain good yields and high quality fruit. Levin *et al.*, (2006).

Current scientific studies confirm the traditional use of pomegranates as a medical remedy. Pomegranate flowers, fruits, leaves and Bark include bioactive phytochemicals which are

antimicrobial and act towards infectious sicknesses which includes cancer, diabetes, excessive blood stress and atherosclerosis. Holland *et al.,* (2009).

Various studied have been revealed that Pomegranate has wonderful result in curing fungal candid, against tumor and malaria fever. Different chemicals such as Tannin and alkaloid are found in bark of the Pomegranate. For the treatment of diarrhea, peel of pomegranate is used by the local healers for the preparation of herbal medicine. Ahmad *et al.,* (2001).

Microbial resistance against different synthetic drugs is reaching at alarming level which is a serious threat for human beings, due to resistance against new synthetic drugs by microbes, treatment is becoming unproductive, prolongs illness, and hospitalizations, and also increases the rate of mortality". WHO (2014).

One of the solutions is to combine antibiotics with other active ingredients to create a cooperative effect. This new generation of phytopharmaceuticals can borrow new phytotherapy and could offer the opportunity to treat diseases treated with synthetic drugs. H. Wagner *et al., (*2009).

Several scientists reported that pomegranate is used in the treatment of lungs and prostate cancer. Khan *et al.,* (2007). Studies further show that aqueous leaves extracts is used to treat stomatitis and diarrhea. Lansky *et al.,* (2004). In ancient times different parts of Pomegranate were used to treat a number of ailments. Jayaprakasha *et al.,* (2006). Pomegranate is also known as hemoglobin boosting and antiulcer agent. Jurenka *et al.,* (2008). In ancient times, Pomegranate was considered a medication having many beneficial outcomes in many ailments. Vidal *et al.,* (2003).

Many diseases have been cured by the use of plants extracts and still provide a great source of potential source of new chemotherapeutic providers. It further increases the production of vitamin E and decreases bad cholesterol, it also helps in the fight against free radicals and oxidation. Kadam *et al*., (2012).

In Consumers patterns and request it has turned out to be basic for the cultivators to created items which have high nourishing just as medical advantages. In this specific circumstance, because of its fantastic nutritive esteem, high tastefulness and accessibility in wealth is a perfect produce for handling. It is ordinarily devoured crisp as treat natural product that is enjoyably sweet and reviving in flavor. Braga *et al.,* (2005).

The juice of Pomegranate has been used for several years as a wonderful and effective fruit which provides beneficial nutrition. Guo C., *et al.,* (2008). *P. granatum* peel is used to treat genital herpes and mastitis, folliculitis, allergic dermatitis, acne, antioxidant, diarrhea, scalds, and tympanitis,. Singh, R. P., *et al., (*2002).

MATERIALS AND METHODS

Current study "Investigation of *Punica granatum L*. leaf extracts on antimicrobial activity" was conducted at Biosciences Lab of University of Wah during March 2021 on the basis of following parameters and treatments. Experiment was conducted in complete randomized design (CRD) factor factorial with 3 replications.

Sample preparation:

The fresh and tender leaves of Pomegranate were collected from University of Wah orchard and dried out at room temperature for twenty to thirty days and afterward smothered into

pulverized fine powder by using electric grinder processor. The coarse fine powder was got dried and was then screened out to get fine powder using the fine plastic screen. Three different extraction solvents i.e. hexane, chloroform and distilled water were utilized for the extraction process. The leaf powder was added to every one of flasks for making a 20% fixation. Each of the three extracts were filtrated with Whatman paper, were kept at 4˚C. Data was recorded on the basis of following parameters.

Proximate Chemical Analysis of *Punica granatum* Leaves:

1. The pH of leaves extract:

pH of three extracts (Hexane, Chloroform & Distilled Water) were calculated, according to 1:5 soil water interruptions with a pH cadence Jackson (1962).

2. Ash Content:

Three gram of *P. granatum* leaves samples were taken in a crucible before shifting sample into the dish to record the fresh weight. Then it was ignited in the muffle furnace at 600°C for 8 hrs. Finally ash formed, the weight of ash was recorded, and ash contents of samples were learned through the technique delineated by Anndy *et al., (*2003).

3. Organic Matter:

Organic Matter (OM) of *P. granatum* leaves were calculated by using the following formula. Anndy *et al., (*2003).

Organic Matter (%) = 100 – % Ash.

4. Moisture Content & Dry Weight of *Punica granatum* Leaves Extract:

Moisture content & dry matter of *Punica granatum* leaves was surveyed consenting prominent unvarying temperature broiler procedure by the method of ISTA (2006). Sample of fresh leaves collected and pulverized. 5g of pulverized leaves of *P. granatum* were got dehydrated in broiler at 130 to 133°C temperature for one hour. Share of moisture content was deliberated using following technique:

% Moisture content = (M2-M3) / (M2-M1) × 100

M1= Container weight/g

M2= Container & pulverized fresh leaves of *P. granatum* weight/g

M3= Container & pulverized Dehydrated leaves of *P. granatum* weight/g

Phytochemical Analysis:

Solvent extracts of *P. granatum* leaves (Hexane, Chloroform & Distilled Water) were investigated for phytochemical screening as determined by the method of Tiwari *et al.*, (2011). Following phytochemicals were screened during the study.

1. Investigation of Alkaloids (Mayer's Test):

Solvents extract of *P. granatum* leaves were dissolved in diluted HCL (Hydrochloric Acid) and filtered by using Whatman filter paper. Filtrated solvents extract were treated with potassium Mercuric Iodide (Mayer's reagent) with concentration of (2ml dilute HCL + 2ml of *P. granatum* leaves extract + 2ml potassium Iodide added). Creation of yellow colored precipitate shows the presence of Alkaloids. Tiwari *et al.,* (2011).

2. Investigation of Saponins (Froth Test):

20ml Distilled Water was added to the solvents of *P. granatum* leaves extract and then vortex for 15 mints. Establishment of 1cm foam layer indicates the Saponins presence. Tiwari *et al.,* (2011)

3. Investigation of Phenols (Ferric Chloride Test):

Solvents extracts of *P. granatum* leaves were treated with ferric chloride solution 3 to 4 drops. Bluish black color formation shows the presence of phenols. Tiwari *et al.,* (2011)

4. Investigation of Flavonoids (Alkaline Reagent Test):

Several drops of NaOH (Sodium Hydroxide) solution were treated with solvents extract of *P. granatum* leaves. Strong yellow color formation, which becomes pallid when diluted acid was added, specifies the flavonoids presence. Tiwari *et al.,* (2011)

5. Investigation of Protein & Amino Acid (Xanthoproteic Test):

The solvent extract of *P. granatum leaves* were treated with few drops of concentrated HNO_3 (Nitric Acid).Yellowish color formation point out the presence of proteins in the leaves extracts. Tiwari *et al.,* (2011)

6. Investigation of Diterpenes (Copper Acetate):

Solvent Extract of *P. granatum leaves* have been disbanded in DW and then copper acetate solution of 3 to 4 drops were added for the investigation of Diterpenes. The creation of emerald green color indicates the presence of Diterpenes. Tiwari *et al.,* (2011)

Total Phenolic Content (TPC): The Total Phenolic Content of *P. granatum* leaves extract was determined by the method of Folin - Ciocalteu. 2ml *P. granatum* leaves extract + 5ml extraction mixture (Methanol: Acetone: HCL (90:8:2)) were made up. Supernatant collected from the mixture. 200µl F.C (Folin-Ciocalteu) reagent added into 100µl supernatant, vortex it for 2 to 3 mints and then added 800µl Na_2CO_3 into the mixture. Stand it for a further 60 min in the dark, and absorbance was measured at 650 nm. The Total Phenolic Content was calculated from the calibration curve, and the results were represented as mg of Gallic acid equivalent per gram dry weight. Kaur *et al., (*2002).

Activity of Antioxidant (AOA): The Antioxidant Activity of solvents extracts of *P. granatum* leaves was calculated by trial of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), as portrayed above with a few changes. 50µ L of each extracts of *P. granatum* + 5 mL .004% DPPH solution mixed together and then vortex. Incubated it in dark room temperature for 30 mints. The mixture absorbance was deliberated at 517 nm. For positive control Ascorbic Acid was used. Villano et al. (2007)

Enzyme Activity of *Punica granatum* leaves:

1. Assay of Peroxidase Activity (POX):

To decide the estimation of Peroxidase action (POX), the Guaicol oxidation strategy was utilized. The 3 ml reaction mixture contains an extract of 10mM potassium phosphate (pH 7.0), 8mm Guaicol and 100µL enzyme extract. The reaction was initiated by adding 1% H_2O_2. The absorption was calibrated at (470nm) in 30 S. The activity unit of peroxidase was articulated change in absorption per minute and per mg soluble protein unit of enzyme (extinction coefficient 6.39). Anndy *et al. (*2003).

2. Assay of Catalase Activity (CAT):

Activity of Catalase was estimated as described by UV spectrophotometric method. To initiate the reaction; 1ml of reaction mixture which contain potassium phosphate buffer (pH 7.0), 250µl of enzyme extract and 60 mm H2O2 was taken. The activity was calibrated at wavelength of 240 nm for 3min in which H2O2 consumption was determined through molar extinction coefficient, 39.4mM-1cm-1. Anndy *et al. (*2003)

3. Assay of Super Oxide Dismutase (SOD):

Super oxide dismutase activity was examined through spectrophotometric ally by the method of for the preparation of incubation medium; then reaction mixture of 3ml, 50mM potassium phosphate buffer (pH 7.8), 5.3mM riboflavin, 45µM methionine, and 84µM NBT and 20µM potassium cyanide was taken. The test tubes place inside aluminum foil box and 15W fluorescent lamps to regulate the temperature 25 ° C. The remaining NBT was measured at 600 nm following contact to light. Chen *et al.*, (2009).

Antibacterial Activity of *Punica granatum* Leaves Extract:

1. Agar Diffusion Method:

Media Used: 10 gram Sodium Chloride, Peptone extract 10 gram and 5 gram Yeast, 20g Agar material dissolved in distilled water 1000 ml. To begin with, bacteria's stock cultures. It is revitalized by inoculation in broth media and grew for 18 hours at temperature of 37 ºC. The above media's agar plates have been organized and wells have been made on media in the plates. Every plate was injected with 18 h old crops (100 µl) and evenly spread on the plate. The wells were filled with different solvents distill samples after 15 minutes. With solvent, Gentamycin is used for filling of control sample well. All plates were incubated for 24 hours at temperature of 37 ºC and the inhibition zone diameter was noted.

2. Growth of Bacteria's:

Antimicrobial susceptibility testing was done utilizing the well-dispersion method as per the National Clinical Laboratory Committee's financial standard. The implantations of the constituent were analyzed on the plates of Mueller Hinton II to watch the activity of bacteria. 5 mm measurement wells were connected to the medium utilizing a sterile borer before mottling the plates with microscopic organisms. The agar plate surface was mottled over the unblemished aseptic agar surface pivoting the plate to confirm even inoculant scattering with a last swab around the edge. The plates are left to dry the additional dampness for 3 to 5 minutes. After immunization of the plates with bacterium, 50 µl aliquots of each test distillate were circulated to each well. The wells were likewise set 2 inches separated in a triangle arrangement. On each plate, the proportional distillate was utilized, with an aggregate of three plates utilized for every bacterium determination separate. Instead of the concentrate, unadulterated solvents were utilized for each bacterial strain. The plates are fixed with parafilm, checked and set in a hatchery set at 37 C. Each plate was broke down for concealment zones following 24 hours of hatching. A ruler was utilized to assess in millimeters the concealment zones. Each investigation was led in parallel and the outcomes incorporated a normal of no less than three autonomous tests.

Statistical analysis

Data was analyzed by application of standard error and analysis of variance (ANOVA) using Least significant difference (LSD) at $p<0.05$ by using latest version of Statistix 8.1.

Results & Discussion

The results and discussions of experiment comprises of following Parts:

Table 1 shows the value of moisture content and dry matter of *P. granatum leaves*. As per results of the experiment, the leaves of Pomegranate consisted of 89.40 % moisture and 19.82% of dry matter. In this experiment moisture content is higher than dry matter of leaves. These results are also in agreeing with Bose *et al* (1999). Moisture content is maximum in all parts of plants.

1.1 Organic Matter & Ash Content of *P. granatum* Leaves:

(Table 1) demonstrate the values of organic matter and ash content of *P. granatum* leaves. Organic matter of pomegranate leaves was calculated 91.88% and Ash percentage of *P. granatum* leaves obtained by experiment was calculated 5.51%. This study discloses that organic matter is maximum in *P. granatum* leaves against ash content. It also reveals that *P. granatum* leaves use to increase the soil fertility as a natural source.

Table 1: Proximate Analysis of *P. granatum* Leaves extract.

Proximate Analysis of *P. granatum* Leaves%	
Moisture Content	89.40%
Dry Matter	19.82%
Organic Matter	91.88%
Ash	5.51%

2. Phytochemical Analysis:

(Table 2) demonstrates the screening of outlined phytochemical constituents of *P. granatum* extracts under examination on subjective premise. Presence and absence of phytochemicals of *P. granatum* leaves extract as discussed under in details. Along these lines, the phytochemical investigation revealed the Hexane, Chloroform and Distilled Water extracts have synthetic intensifies that have been found to have antibacterial exercises.

2.1 Alkaloid:

For the detection of Alkaloid in the solvents extract of *P. granatum* leaves "Wagner test" perform during experiment. Study reveals that Alkaloid are absent in all three solvent extract of *P. granatum* leaves.

2.2 Flavonoids (Alkaline Reagent Test):

By Alkaline Reagent test that perform for the detection of Flavonoids in the solvent extracts of *P. granatum* leaves, all three solvents extracts Hexane, Chloroform and Distilled Water of *P. granatum* leaves show the presence of flavonoid. Flavonoids are hydroxylated polyphenolic compounds that are famous to be created by plants in reaction to microbial contagions, which have been extensively studied and ascertained to be antimicrobic against a variety of in vitro microorganisms. Trease *et al.*, (1999).

2.3 Di Terpenes (Copper Acetate Test):

Copper acetate test performed for the detection of Di Terpenes in the solvents Hexane, Chloroform and Distilled Water extract of *P. granatum* leaves. Experiment demonstrated that Di Terpenes are absent in all three extracts of *P. granatum* leaves. Terpenoids were mainly used for their fragrant characters, they were also potential agents against bacteria suppression. H. Tsuchiya *et al.*, (1996).

2.4 Protein (Xanthoproteic Test):

Xanthoproteic test perform for the detection of Protein in solvents extract of *P. granatum* leaves that reveals all three solvents Hexane, Chloroform and Distilled Water extract of *P. granatum* leaves show absence of Protein.

2.5 Saponins (Froth's Test):

Froth's test was performed in experiment for the detection of Saponins in the solvents extract of *P. granatum* leaves which show that Saponins were absent in Hexane and Chloroform extract of *P. granatum* leaves but was present in the Distilled water extract of *P. granatum* leaves.

2.6 Phenols (Gelatin Test):

Gelatin test was performed in experiment to check the availability of Phenols in the solvents extract of *P. granatum* leaves that reveals Phenols were present in all three solvents extract of *P. granatum* leaves.

Table. 2 Phytochemicals Screening of *P. granatum* Leaves Extract

Phytochemicals Constituent	Hexane Extract	Chloroform Extract	Distilled Water
Alkaloid	-	-	-
Saponins	-	-	+
Diterpenes	-	-	-
Flavonoids	+	+	+

Protein & Amino Acid	-	-	-
Phenols & Tannins	+	+	+

Presence of Constituent (Positive); Absence of Constituent (Negative)

3. Antibacterial Activity:

The outcome of the study evidenced that only two of the raw dissolvent distills from the leaves of *Punica granatum*, hexane and chloroform, showed bacterial inhibitory activity (Table 3). Bacteria *S. aureus*as susceptible to the two extracts, while *E. coli* bacteria showed high resistance to all three extract of *Punica granatum* leaves. Hexane distill have significantly maximum antimicrobic activity with average zone of inhibition of 8.6 and 2 mm and 2.3 mm compared to chloroform extract with a mean inhibition zone of 6.3 mm, 1.6 mm and 1.33 mm. *S. aureus, P. aurigonisa* & *E. coli*

3.1 Bacteria *Escherichia coli*:

Hexane extract of *P. granatum* leaves inhibit the bacteria maximum 2mm and distilled water extract did not shows any inhibition to bacteria *E. coli* and chloroform extract of leaves inhibit Bactria 1.33mm. *E. coli* bacteria show highest resistance of solvent extracts of *P. granatum* leaves as compare to other two bacteria *S. aureus* & *P. aurigonisa*. Its cell wall structure could be attributed to the resistance of the *E. coli* and *P. aurigonisa* bacteria. *E. coli* microbes have a wall to permeability, consisting of a thin external membrane of lipopolysaccharide, that refrain the penetration of plant distill, *E. coli* bacteria have previously been reported to be more resistant to the extract of *P. granatum* leaves and even have no effect compared to *S. aureus*. Kumar *et al.,* (2012).

3.2 Bacteria *Staphylococcus aureus:*

*S. aureus*as bacteria maximum exhibit by the Hexane extract of *P. granatum* leaves extract followed by chloroform and distilled water extract. All three solvent extract gives significant result against the bacteria *S. aureus*as compare to other both bacteria. Hexane extract of *P. granatum* gives highest inhibition value 8.6 mm and chloroform extract of leaves inhabit 6.3mm but distilled water extract of *P. granatum* leaves gives less inhibition against the bacteria *S. aureus* which is 4.6mm.

3.3 Bacteria *Pseudomonas aurigonisa:*

Bacteria *P. aurigonisa* gives significant value against Hexane extract, distilled water extract and chloroform distill of leaves (*P. granatum*). The values of inhibition of hexane distill of leaves (*P. granatum*) 2.00mm and chloroform extract leaves 1.33mm but distilled water extract 0.23mm inhibition. *S. aureus*has foam- like layer of peptidoglycan that is more reachable to extract osmotic pressure. Stefanello *et al.,* (2008).

Table 3: Antibacterial Activity of *Punica granatum leaves* extract.

Zone of Inhibition (mm)

P. granatum leaves Extracts	E. coli	Staphylococcus Aureus	Pseudomonas Aurigonisa	Means
Distilled water	0.00 e	4.66 c	0.23 e	1.633 c
Hexane Extracts	2.00 d	8.66 a	2.00 d	4.22 a
Chloroform	1.33 d	6.33 b	1.33 d	3.00 b
Mean	1.11 b	6.55 a	1.18 b	

LSD for extracts= 0.535
LSD for Bacteria= 0.535
LSD for extracts and bacteria= 0.928

4. Enzyme Activity:

4.1 Super-Oxide Dismutase(SOD) (µg/mg):

Activity of super-oxide dismutase (SOD) were significantly varied in different solvent concentration of *P. granatum* leaves extract. The SOD activity was higher in chloroform extract 0.050 as compared to Hexane 0.042 and distilled water extract 0.026 of leaves (*P. granatum).* Activity of SOD was lower investigated in distilled water extracts of leaves 0.026 (*P. granatum).* SOD plays a considerable part in regulating some oxidative stresses like in atherosclerosis and other diseases. SOD is found to one of the primary antioxidant defensive component of human body.

4.2 Peroxidase (POX) (µg/mg):

Peroxidase (POX) activity was significantly different in different solvent extract of *P. granatum* leaves. (Table 4) showed the enzymatic activity of leaves (*P. granatum)* extract. POX activity higher in Hexane extract of leaves 0.88 *(P. granatum)* but lower in distilled water extract 0.64. Variation in POX activity among various leaves (*P. granatum)* extract is due to distill chemical composition.

4.3 Catalase (CAT) (µg/mg):

The activity of Catalase (CAT) enzyme was significantly different in different *P. granatum leaves* extracts. CAT enzyme activity is higher in Hexane distill of Pomegranate leaves extracts 3.92 but lower in Distilled water extract of *P. granatum leaves* extract 2.30. This is outstanding to biochemical medication of plant & their extraction of solvent.

Table 4: Enzyme Activity of *Punica granatum leaves* extract.

Solvent for Extraction	CAT µg/mg	POX µg/mg	SOD µg/mg
DW Extract	2.30 c	0.64 c	0.026 c
Hexane Extract	3.57 b	0.88 a	0.042 b
Chloroform Extract	3.92 a	0.78 b	0.050 a
Mean	3.26	0.77	0.039
LSD(0.05)	0.3317	0.082	0.0059

5. pH of Different Solvent of Leaves (*Punica granatum*) Extract:

Table 5. Demonstrate the pH of different solvent extract of *P. granatum leaves* that are significantly different. pH of Distilled Water is 5.10 and Hexane extract 4.53 and Chloroform extract of *Punica granatum leaves* extract was 4.16 Found significant between Hexane, Chloroform and distilled water. Maximum acidity value of leaves extract was found in Hexane and Chloroform as compare to distilled water extract.

5.1 Total Phenolic Content & Antioxidant Action of leaves (*Punica granatum)*:

Table 5 shows the result of Total phenolic content and Antioxidant Activity of different solvent extract of leaves. All result were significant. The value of TPC in distilled water extract of leaves is 119.47, Hexane extract 107.20 while Chloroform extract 97.93 with LSD value 4.90. Total phenolic content value is maximum calculated in the distilled water extract of leaves 119.47 while chloroform extract of leaves show minimum value 97.93. Antioxidant value of *Punica granatum* leaves in distilled water extract is calculated 0.67, hexane extract 1.71 and Chloroform extract of *Punica granatum* leaves 1.26. Maximum value of antioxidant of *Punica granatum* leaves calculated in Hexane extract of *P. granatum* leaves. All the value of antioxidant of *P. granatum* leaves are significantly different among different solvent extract of leaves.

Table 5: Total phenolic Content, Antioxidant Activity & pH of *Punica granatum leaves* extract.

Solvent for Extract	pH	TPC mg/g	AOA
DW Extract	5.10 a	119.47 a	0.67 c
Hexane Extract	4.53 b	107.20 b	1.71a
Chloroform Extract	4.16 b	97.93 c	1.26 b
Mean	4.59	108.2	1.21
LSD (0.05)	0.376	4.90	0.131

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