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RESEARCH ARTICLE
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FOURIER-TRANSFORM INFRARED SPECTROSCOPY, ANTIOXIDANT, ANTI-INFLAMMATORY AND PHYTOCHEMICAL SCREENING OF DIFFERENT EXTRACTS OF PUNICA GRANATUM PEELS

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ABSTRACT

Different types of drugs have been used over the centuries to cure various diseases due to their potential biological activities. However, these drugs have severe side effects on the human body over time. Medicinal plants are known for their great ability to fight against different types of diseases. As a result, it is thought that using these medicinal plants for healing represents an enormous potential source of therapeutic support for maintaining excellent health. *Punica granatum* is an important medicinal plant with many beneficial constituents that act as anti-inflammatory and antioxidant agents. Four different extracts of *Punica granatum* peels such as aqueous, methanol, ethyl acetate, and n-hexane were prepared and tested to determine phytochemical screening antioxidant activity and anti-inflammatory activity. Antioxidant activity was performed by Iron chelating activity and $FeCl_3$ reducing power activity at different time intervals. Methanol extract showed the lowest IC_{50} value (0.2) at different time intervals which shows its higher antioxidant, n-hexane extract showed the highest IC_{50} value (39.4) at 10 mins time intervals as compared to other extracts. The phytochemical composition of the pomegranate extracts was thoroughly examined using FTIR. This analysis verified the presence of a wide array of bioactive compounds in the pomegranate peels, including carbohydrates, alkaloids, tannins, flavonoids, terpenoids, proteins, glycosides, coumarins, and total phenols. *In vitro,* the anti-inflammatory activity of all four extracts was carried out. Methanol extract has shown the highest %age inhibition value of 348% for its higher concentration of 100µg/ml. These results showed that pomegranate peels proved to be better antioxidant and anti-inflammatory agents and can be used for medicinal purposes.

Keywords: Pomegranate; Antioxidants; Anti-inflammatory; FTIR; Drugs; Phytochemical screening

1: INTRODUCTION

For thousands of years, medicinal plants have been fundamental in treating various ailments. This traditional knowledge has sustained its relevance as plants offer affordable, accessible, and safe therapeutic options[1]. Many cultures have utilized specific plants not only for nutrition but also for their healing properties, given that plants contain unique bioactive compounds that promote health[2]. The Greek physician Dioscorides, regarded as "the father of pharmacognosy," documented the medicinal uses of hundreds of plants in 77 AD, a foundation that established the therapeutic use of

plants[3]. In recent years, synthetic drugs' side effects have renewed interest in natural remedies, especially with the growing concern over antibiotic-resistant pathogens. This has spurred extensive research into plants as alternative therapies for health issues, including infections, inflammation, and chronic diseases[4].

One prominent medicinal plant is *Punica granatum*, or pomegranate, belonging to the *Lythraceae* family. This small tree or shrub can grow up to 10 meters and produces a unique, round fruit with tough skin and ruby-red arils (seeds) encased in jelly-like flesh[5]. Native to regions from the Middle East to the Indian subcontinent, pomegranate's distinct taste and health benefits have made it a cultural and medical icon[6]. Historically, the fruit has symbolized fertility, abundance, and vitality, featured in religious and cultural rituals[7]. Pomegranate's rich history extends to traditional medicine, where it is used to treat ailments such as diarrhea, intestinal parasites, and infections, and is valued as a heart and throat tonic[8].

The therapeutic properties of pomegranate are attributed to its bioactive compounds found in both edible and non-edible parts, including the peel, seeds, leaves, and roots[9]. Its phytochemical profile includes phenolic acids, flavonoids, tannins, anthocyanins, and ellagitannins, which exhibit significant antibacterial, antioxidant, anti-inflammatory, and anticancer properties[10]. The peels, often discarded, are particularly potent, containing more beneficial compounds than the edible parts. These bioactive compounds act synergistically to produce various health benefits. For instance, punicalagin, a type of ellagitannin found in high concentrations in the peel, is known for its antioxidant and anti-inflammatory effects[11].

The research underscores the potent antibacterial properties of pomegranate peel extracts against both Gram-positive and Gram-negative bacteria[12]. The peel contains polyphenols and tannins that disrupt microbial cell membranes, inhibit enzyme activity, and suppress microbial growth. Studies have demonstrated that methanolic extracts of pomegranate peel inhibit pathogens like *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae[12]*. These properties make it a promising alternative for tackling antibiotic-resistant bacteria. Additionally, pomegranate peel extracts show anti-protozoal and antifungal effects, further solidifying its broad-spectrum antimicrobial potential[13].

Pomegranate's strong antioxidant content protects against inflammation and oxidative stress, among other health benefits[14]. Because polyphenols like ellagic acid, gallic acid, and punicalagins neutralize free radicals, they lower the chance of developing chronic illnesses like diabetes, cancer, and cardiovascular disease[14]. Frequent pomegranate juice drinking has been associated with decreased blood pressure, heart disease, and oxidized LDL cholesterol levels, which are indicators of atherosclerosis. By preventing the growth and spread of breast and prostate cancer cells, the peels' antioxidant qualities also hold promise for the prevention and treatment of cancer[15]. Its polyphenolic compounds have the ability to cause cancer cells to undergo apoptosis and may also prevent angiogenesis, the process by which tumors create new blood vessels[16]. Additionally, pomegranates have strong anti-inflammatory properties that are crucial for controlling chronic inflammation associated with diseases like arthritis, heart disease, and neurological problems. Its antioxidants, including flavonoids, tannins, and punic acid, suppress pro-inflammatory enzymes and lessen pain, swelling, and redness[17]. Pomegranate peel extract's potential to lessen the symptoms of inflammation-driven diarrhea is even demonstrated by studies conducted on rats, confirming its therapeutic usefulness[18].

The present study aims to study the qualitative and quantitative (total phenolic contents) examination of pomegranate peels by four different solvents. Furthermore, antioxidative and anti-inflammatory activities were determined to explore its medicinal impact.

2: MATERIALS AND METHODS

2.1: Collection and Preparation of Plant Material

Fresh Punica granatum fruits were sourced from a Lahore market, washed, rinsed in distilled water, and air-dried for five minutes. The peel was then removed, dried, crushed into powder, and stored in refrigeration for further analysis.

2.2: Extract preparation

25g of weighed sample was dissolved separately in 150ml methanol, ethyl acetate, n-hexane, and distilled water. The dissolved sample was macerated for 1 hour. Then the sample was placed on a shaker for 72 hours at the speed of 140 rpm at room temperature. The sample extract was then filtered through filter paper (Whatman No. 4) and residue left behind was re-extracted. The obtained sample extracts were dried using a desktop constant temperature drying oven. The final residue was used to study their activities[19].

2.3: Phytochemical Screening of Pomegranate Extracts

The following tests were employed to qualitatively analyze extracts from *Punica granatum*:

2.3.1: Test for Carbohydrates (Molish's test): A few drops of extract were combined with two milliliters of Molish's reagent. 2 ml of concentrated H_2SO_4 was progressively added along the test tube's sides after the mixture had been well shaken. Due to the formation of a reddish ring at the intersection of two solutions, carbohydrates were present[20].

2.3.2: Test for Alkaloids (Mayer's test): In a test tube, two drops of Mayer's reagent and a few drops of extract were combined. The presence of alkaloids is indicated by a green-colored precipitate[20].

2.3.3: Test for Saponins (Foam test): In the test tube, a few drops of extract were combined with 20 mL of distilled water. For ten minutes, the test tube was shaken. The presence of saponins is confirmed by foam formation[21].

2.3.4: Test for Tannins (Lead Acetate test): In a test tube, 1% lead acetate was mixed with a few drops of extract. Tannins are present when a precipitate has a yellow tint[22].

2.3.5: Test for Flavonoids (Acid test): In the test tube, a small amount of extract and dil. H_2SO_4 were combined. The presence of flavonoids was indicated by the color orange[23].

2.3.6: Test for Terpenoids (Acetic anhydride test): In the test tube, 2 mL of acetic anhydride and extract with concentrated H_2SO_4 were combined. The development of green and blue rings suggested the presence of terpenoids[24].

2.3.7: Test for Proteins (Biuret Test): A small amount of extract was combined with 10% sodium hydroxide. In the same test tube, two drops of copper sulfate were also added. The protein's presence was confirmed by the formation of a violet or pink color[25].

2.3.8: Test for Glycosides (Liebermann's test): Add 2 ml each of acetic anhydride and chloroform to 2 ml of extract. The presence of glycosides is indicated by the formation of a violet, blue, or green reddish brown ring[26].

2.3.9: Test for Coumarins: Add 10% of 3 ml NaOH to 2 ml of extract. The appearance of yellow signals the presence of coumarins[27].

2.3.10: Test for Total Phenols :3% of $FeCl_2$ were mixed with 2ml of extract in the test tube. Deep blue color formation indicated that all of the phenol was present[28].

2.4: Antioxidant Activity

2.4.1: Iron chelating activity

A 0.05% (w/v) methanolic o-phenanthroline solution (1 mL) was prepared and mixed with varying concentrations of the methanolic test compound solution (2 mL, final concentration 2000 mM). This mixture was then combined with 2 mL of methanolic $FeCl_3$ (200 mM) and allowed to incubate at room temperature. Absorbance was measured at 512 nm after 10, 30, 45, 60, and 120 minutes. The percentage of iron chelating activity was subsequently calculated using the appropriate formula[29].

Percentage of iron chelating activity = (Test absorbance - Control absorbance/Test absorbance) ×100.

2.4.2: $FeCl_3$ reducing power activity

An ethanolic solution of each test compound (2 mL, final concentration: 2000 mM) was prepared and mixed with 2.5 mL of an aqueous potassium ferricyanide solution (1%). The mixture was then incubated for 20 minutes at 50°C. After cooling, 2.5 mL of an aqueous trichloroacetic acid solution (10%) was added, and the mixture was centrifuged for 10 minutes at 3000 rpm. The supernatant was combined with 1 mL of freshly made aqueous ferric chloride solution (0.1%) and 2.5 mL of distilled water. Absorbance was measured at 700 nm after 10, 30, 45, 60, and 120 minutes of incubation. Using the following formula, the percentage increase in reducing power was calculated[30].

Percentage increase of reducing power = (Test absorbance/ Control absorbance-1) ×100

2.5: *In vitro* Anti-inflammatory activity

Anti-inflammatory activity was carried out by the Lincy et al., described protocol.

2.5.1: Sample solutions preparation

A stock solution of 100µg/ml of each of the four extracts was made.

2.5.2: Procedure

2 ml of each extract was diluted with 2.8 ml of phosphate buffer (pH=6.5). 0.2 ml of hen's egg albumin was added to each extract. Prepared a control solution by adding 5 ml of double distilled water. Incubated all reaction mixtures for 15 minutes at 37°C. After incubation, heat all the reaction mixtures for 5 minutes at 70°C. Then the reaction mixture to cooled to room temperature. Finally the absorbance of each reaction mixture at 660 nm with the help of a spectrophotometer. For standardization, diclofenac sodium was used as the standard extract with the same dilutions as the target extracts. Repeated the experiment thrice for each chemical. The following formula was used to determine each extract's percentage of age inhibition of protein denaturation.

%age inhibition of denaturation of protein= $[Vt/Vc - 1] \times 100$

Vt = Absorbance of target extract

Vc = Absorbance of control

3:Results

3.1: Phytochemical Screening of Pomegranate Extracts

Phytochemical analysis of different extracts revealed the existence of some significant constituents such as proteins, carbohydrates, cumarins, alkaloids, saponins, phenols, glycosides, flavonoids, and terpenoids (Table 4.1). Phytochemical ingredients supply crucial pharmacological capabilities regarding human health. These chemicals could be employed as medications or as dietary supplements to treat or guard against a number of illnesses. The result (Table 1) showed that the aqueous extract of pomegranate peels showed positive detection to all phytochemical tests (carbohydrates, phenols, glycosides, flavonoids, terpenoids, proteins, cumarins, alkaloids, alkaloids, total phenols) except saponins. The Hexane extracts showed positive detection for carbohydrates, flavonoids, terpenoids, and proteins. The Methanol extracts showed positive results in all phytochemical tests except terpenoids. On the other hand, Ethyl Acetate showed positive detection for phytochemical tests except for flavonoids, terpenoids, proteins, and total phenols.

Table 1: Phytochemical screening of various extracts of pomegranate peels

Sr No.	Test for Phytochemicals	Sample Extracts			
		Aqueous	Hexane	Methanol	Ethyl Acetate
1	Carbohydrates	+	+	+	+
2	Alkaloids	+	_	+	+
3	Saponins	_	_	+	+
4	Tannins	+	_	+	+
5	Flavonoids	+	+	+	_
6	Terpenoids	+	+	_	_
7	Proteins	+	+	+	_
8	Glycosides	+	_	+	+
9	Cumarins	+	_	+	+
10	Total Phenols	+	_	+	_

3.2: Fourier Transform Infrared Spectroscopy (FTIR) of Pomegranate peels

The FTIR spectra of powdered pomegranate peel are displayed in Figure 1. Table 2 lists the primary absorption bands for identification. The spectrum demonstrated the presence of a wide range of chemicals and supported the peels' complex character. Numerous investigations have revealed that pomegranate peels include a variety of naturally occurring chemicals with biological properties[31]. Alcoholic substances and carboxylic acids are confirmed to be present by a large peak in the O-H band at 3383.1 cm^{-1}. The carbonyl group C=O is responsible for the sharp mid-intense peak at 1728.22 cm^{-1}, which indicates the presence of aldehydes, ketones, and carboxylic acids. There are unsaturated chemicals present, as indicated by the moderately sharp signal at 1620.21 cm^{-1} (alkenes). The stretching band at 2935.66 cm^{-1} confirms the existence of alkanes. The existence of ethers and esters is confirmed by the absorption peak at 1219 cm^{-1}.

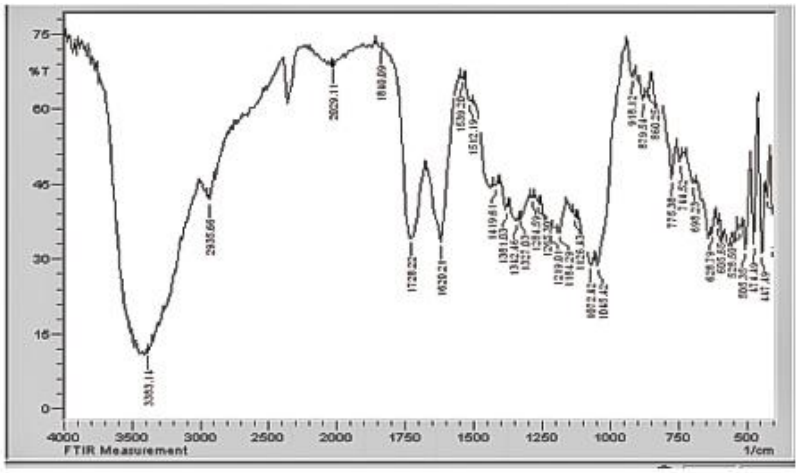

Figure 1*:* FTIR spectrum of pomegranate peel powder

Numerous peaks have interfered with one another because of the peels' complicated chemical makeup; however, the other significant peaks are indicated in the table below.

Table 2: The main FTIR bands in pomegranate peel powder

Signals	Functional Groups	Theoretical values
3383.14	O-H	3350-3450
2915.66	C-H	2850-2925
1728.22	C=O	1650-1850
1512.19	N=O	1350-1550
1019	C-O	1000-1050
1672	C-N	1630-1725

3.3: Antioxidant Activity of Different Extracts Of *Punica granatum*

Aqueous, methanol, ethyl acetate, and n-hexane extracts of Punica granatum were screened for different antioxidant assays. Details are given below:

3.3.1: Iron Chelating Activity

Different pomegranate extracts were found to have chelation activity, whereby they converted Fe^{3+} into Fe^{2+} and consequently produced a chelate with o-phenanthroline. This procedure is used to show how much the extracts have reduced Fe^{3+}[32].

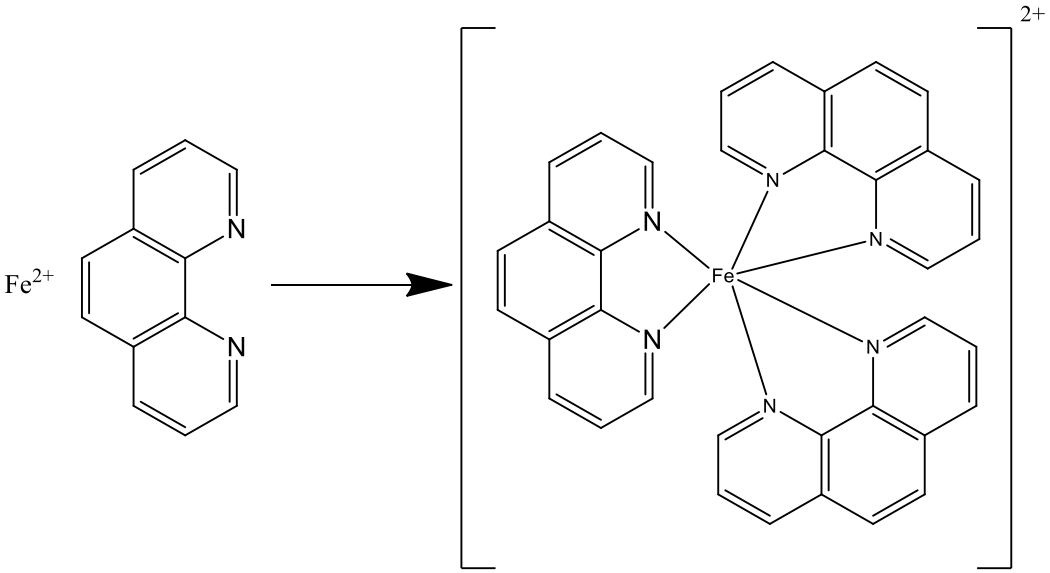

Figure 2: Formation of chelate in iron chelating activity

Table 3: IC_{50} of *Punica granatum* Peels Extract at Different Time Intervals

Sample extracts	10 minutes	30 minutes	45 minutes	60 minutes	120 minute
Ascorbic acid	1.53	6.41	12.5	18.1	36.9
Methanol	1.48	5.5	5.94	10.4	36.7
Ethyl Acetate	2.8	5.1	24.5	43.5	53.1
n- Hexane	41.9	79.07	83.8	139.6	143
Aqueous	1.8	2.3	3	14.5	36.7

Table 3 represents the IC_{50} value of each extract for iron chelating activity. The IC_{50} value of all extracts has increased over time, similar to the standard extract, whose IC_{50} value has also increased with time. Among all the extracts tested for iron chelating, the methanol extract of pomegranate has a lower IC_{50} value which shows its higher chelating capacity. The polarity of the solvent directly affects the type and amount of antioxidant compounds extracted from pomegranate peels. Polar solvents like methanol and aqueous solutions yield extracts with the highest antioxidant activity because they dissolve and extract more phenolic compounds and other polar antioxidants. Non-polar solvents, like n-hexane, result in lower antioxidant activity due to their inability to effectively extract these polar bioactive compounds[33]. The order for increasing activity of extracts was **Methanol > Aqueous > Ethyl acetate > n-Hexane**.

3.3.2: $FeCl_3$ Reducing Power Activity

The potassium ferrocyanide (Fe^{3+}) that is produced when extracts with reduction potential react with potassium ferricyanide (Fe^{2+}) in the reducing power test method subsequently combines with ferric chloride to form ferric–ferrous complex ($KFe^{III}[Fe^{II}(CN)_6]$, which has an absorbance maximum at 700 nm[34].

Potassium Ferricyanide (Fe^{+3}) ⟶ Potassium Ferrocyanide (Fe^{+3}) ⟶ Ferric Ferrous Complex

$$K + Fe^{+3} + [Fe^{II}(CN)_6]^4 \longrightarrow KFe^{III}[Fe^{II}(CN)_6]$$

Table 4: IC_{50} of Punica *granatum* Peels Extract at Different Time Intervals

Sample extracts	10 minutes	30 minutes	45 minutes	60 minutes	120 minute
Ascorbic acid	4.7	6.4	8.9	15.4	24.4
Methanol	0.2	0.6	3.75	12.6	16.6
Ethyl Acetate	5	9.3	20	24.9	31
n- Hexane	39.4	48.9	55	71.3	153
Aqueous	1.3	7.3	19.8	37.6	55.4

Table 4 represents the IC_{50} value of each extract for FeCl3-reducing power activity. The IC_{50} value of all extracts has increased over time, similar to the standard extract, whose IC_{50} value has also increased with time. Among all the extracts tested for FeCl3-reducing power activity, the methanol extract of pomegranate has a lower IC_{50} value which shows its higher reducing power capacity. The polarity of the solvent directly affects the type and amount of antioxidant compounds extracted from pomegranate peels. Polar solvents like methanol and aqueous solutions yield extracts with the highest antioxidant activity because they dissolve and extract more phenolic compounds and other polar antioxidants. Non-polar solvents, like n-hexane, result in lower antioxidant activity due to their inability to effectively extract these polar bioactive compounds[33]. The order for increasing activity of extracts was **Methanol > Aqueous > Ethyl acetate > n-Hexane**.

3.4: In vitro Anti-inflammatory activity

The "percentage inhibition of denaturation of protein" protocol was used to test the anti-inflammatory efficacy of extracts *in vitro*. The stabilization or inhibition of denaturation of albumin by target compounds and standard compound diclofenac sodium is shown by the rise in absorbance value of tested compounds and in turn high percentage value when compared to control[35].DMF was used as a control and it has shown the absorbance value 0.4788.

Table 5: In vitro anti-inflammatory activity of Diclofenac Sodium

Sr. No.	Dilutions (µg/ml)	Absorbance (nm)	% Inhibition of Protein Denaturation
1	6.25µg/ml	0.8711	82
2	12.5µg/ml	1.4671	206
3	25µg/ml	1.8732	291
4	50µg/ml	2.1321	345
5	100µg/ml	2.0251	323

In the case of the different dilutions of diclofenac sodium tabulated in Table 5, which is used as a standard drug value of absorbance increases as the concentration of the compound increases and in turn %age inhibition of protein denaturation also increases same as in the case of extracts.

Table 6: In vitro anti-inflammatory activity of Aqueous Extract

Sr. No.	Dilutions (µg/ml)	Absorbance (nm)	% Inhibition of Protein Denaturation
1	6.25µg/ml	0.3211	−33
2	12.5µg/ml	0.4675	−2.4
3	25µg/ml	0.7632	59.4
4	50µg/ml	1.8721	290
5	100µg/ml	2.0114	320

In the case of the above tabulated Aqueous Extract different dilutions were made from the stock solution. These dilutions showed different absorbance values depending on the concentration of the compound. Starting from a low value of concentration, the value of absorbance increases from top to bottom as the concentration rises [36] and in turn %age inhibition of protein denaturation also increases. A higher concentration of 100µg/ml of the aqueous extract has shown a %age inhibition value of 320.

Table 7: In vitro anti-inflammatory activity of Methanol Extract

Sr. No.	Dilutions (µg/ml)	Absorbance (nm)	% Inhibition of Protein Denaturation
1	6.25µg/ml	0.2401	−50
2	12.5µg/ml	0.3456	−28
3	25µg/ml	0.6510	36
4	50µg/ml	1.6377	242
5	100µg/ml	2.1451	348

In the case of the above tabulated methanol Extract different dilutions were made from the stock solution. These dilutions showed different absorbance values depending on the concentration of the compound. Starting from a low value of concentration, the value of absorbance increases from top to bottom as the concentration rises [36] and in turn %age inhibition of protein denaturation also increases. A higher concentration of 100µg/ml of the methanol extract has shown a %age inhibition value of 348.

Table 8: In vitro anti-inflammatory activity of Ethyl acetate Extract

Sr. No.	Dilutions (µg/ml)	Absorbance (nm)	% Inhibition of Protein Denaturation
1	6.25µg/ml	0.0231	−95
2	12.5µg/ml	0.2345	−51
3	25µg/ml	0.4213	−12
4	50µg/ml	0.7662	60
5	100µg/ml	1.8321	283

In the case of the above tabulated ethyl acetate extract different dilutions were made from the stock solution. These dilutions showed different absorbance values depending on the concentration of the compound. Starting from a low value of concentration, the value of absorbance increases from top to bottom as the concentration rises [36] and in turn %age inhibition of protein denaturation also increases. A higher concentration of 100µg/ml of the ethyl acetate extract has shown a %age inhibition value of 283.

Table 9: In vitro anti-inflammatory activity of N-hexane Extract

Sr. No.	Dilutions (µg/ml)	Absorbance (nm)	% Inhibition of Protein Denaturation
1	6.25µg/ml	0.0076	−98.4
2	12.5µg/ml	0.0897	−81.2
3	25µg/ml	0.2984	−38
4	50µg/ml	0.3761	−21.4
5	100µg/ml	1.7435	264

In the case of the above tabulated n-hexane extract different dilutions were made from the stock solution. These dilutions showed different absorbance values depending on the concentration of the compound. Starting from a low value of concentration, the value of absorbance increases from top to bottom as the concentration rises [36] and in turn %age inhibition of protein denaturation also increases. A higher concentration of 100µg/ml of the n-hexane extract has shown a %age inhibition value of 264.

Table 10: Comparison of different extracts of pomegranate with standard drug diclofenac sodium

Extracts	% inhibition of protein denaturation of different Dilutions				
	6.25µg/ml	**12.5µg/ml**	**25µg/ml**	**50µg/ml**	**100µg/ml**
Diclofenac sodium	82	206	291	345	323
Aqueous	−33	−2.4	59.4	290	320
Methanol	−50	−28	−36	242	348
Ethyl Acetate	−95	−51	−12	60	283
n-Hexane	−98.4	−81.2	−38	21.4	264

Diclofenac sodium has shown a higher %age inhibition value of 323 for its higher concentration of 100µg/ml.N-Hexane extract exhibited the least activity with a %age inhibition value of 264% for its higher concentration of 100µg/ml, the rest of its dilutions have shown minimum values. Ethyl acetate extract has shown better activity with a %age inhibition value of 283% for its higher concentration of

100µg/ml. The aqueous extract has shown a good %age inhibition value as compared to the other two compounds. Its higher concentration 100µg/ml has shown a higher value of %age inhibition of 320. Methanol extract has shown the highest %age inhibition value of 348% for its higher concentration of 100µg/ml. The order for increasing % inhibition of protein denaturation of extracts was **Methanol > Aqueous > Ethyl acetate > n-Hexane**.

Conclusion

This study showed that pomegranate is a rich source of antioxidants and anti-inflammatory agents, making it valuable in modern medicine. Extracts like aqueous, methanol, ethyl acetate, and n-hexane were tested in various experiments FTIR analysis confirmed that pomegranate peel extracts contain various bioactive compounds, including carbohydrates, alkaloids, tannins, flavonoids, terpenoids, proteins, glycosides, coumarins, and phenols, contributing to their antioxidant capacity. The extract of methanol showed the highest level of antioxidant activity, with the lowest IC50 values at different time intervals, indicating its superior reducing or antioxidant potential. It was observed that as time progressed, the IC_{50} values decreased, further confirming the robust antioxidant properties of the methanol extract. Moreover, the methanol extract demonstrated notable anti-inflammatory activity by effectively inhibiting protein denaturation, a key marker of inflammation. The abundance of beneficial compounds in pomegranate, particularly in the methanol and aqueous extracts, underscores its therapeutic potential against various diseases. Therefore, extracts from Punica granatum could be effectively utilized to combat oxidative stress and inflammation-related conditions.

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