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Original Article

Dentistry Section

Evaluation of Anti-inflammatory, Antioxidant and Antimicrobial Activity of Pomegranate Peel Extract: An In-vitro Study

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ABSTRACT

Introduction: Pomegranate fruit contains biologically active compounds that provide anti-inflammatory properties. Byproducts of pomegranate and punicalagins inhibit the growth of pathogens while enhancing the growth of beneficial bacteria. The beneficial effects of phenolic compounds are exhibited in scavenging free radicals.

Aim: To evaluate the anti-inflammatory, antioxidant, and antimicrobial activity of Pomegranate Peel Extract (PPE).

Materials and Methods: The present in-vitro study was designed and carried out at the Nanobiomedicine Laboratort, Department of Pharmacology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from June 2021 to August 2021. The anti-inflammatory activity was evaluated using the Egg Albumin Denaturation assay (EA) and Bovine Serum Albumin Denaturation assay (BSA). The measurement of antioxidant activity was conducted using the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay, Hydrogen peroxide radical scavenging (H2O2) assay, and Ferric Reducing Antioxidant Power (FRAP) assay. Antimicrobial activity was evaluated using the agar well diffusion method. The microorganisms used to evaluate the antimicrobial effect of PPE were *S. mutans*, *S.aureus*, *E.faecalis*, and *C.albicans*. Results were analysed using independent t-tests, Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD).

Results: Anti-inflammatory activity was observed with high concentrations of 40 and 50 µL in EA (70.06%±0.15, 78.08%±0.21) and BSA (75.50%±3.90, 80.82%±3.38) assays. Pronounced antioxidant activity of PPE was seen with higher concentrations of 40 and 50 µL in DPPH (88.17%±0.69, 92.50%±1.23), H2O2 (78.22%±0.94, 88.99%±1.03), and FRAP (78.43%±1.25, 88.49%±0.67) assays. The antimicrobial activity was highest at 100 µL for *S.mutans* (38±2.62 mm), *S.aureus* (36±3.16 mm), *E.faecalis* (21±1.48 mm), and *C.albicans* (23±2.36 mm).

Conclusion: The study concluded that PPE has anti-inflammatory, antioxidant and antimicrobial properties and these properties are concentration-dependent.

Keywords: Assays, Flavonoids, Polyphenols, Punica Granatum L, Punicalgin

INTRODUCTION

There is an increase in demand for phytopharmaceutical products or active molecules contained in Ayurvedic medicines not only in India but also in the Western world. Natural products are considered an alternate source for new active molecules. The healing power of plants has been used in traditional and Indian systems of medicine for quite a long time [1]. In Ayurvedic medicines, extracts of various parts of herbs, namely the bark, roots, leaves, tender stems, fruits, and flowers, were used to cure various chronic ailments. Pomegranate (Punica granatum L) is one such fruit that possesses significant medicinal properties. Many components of the fruit have proven to have effects such as antioxidant, anti-inflammatory, anticarcinogenic, antiatherosclerotic, hypolipidemic, antidiabetic, antiviral, antibacterial, and antifungal activities, and they are attributed to the biological compounds (active molecules) present in the different parts of the fruit [2].

Pomegranate peel contains polyphenols [3-5] like punicalagin, which is a rich source of antioxidants. Other polyphenols include anthocyanins (delphinidin, cyanidin, and pelargonidin 3-glucosides and 3,5-glucosides) [6-8], as well as flavonols [9,10]. Lee CJ et al., have reported the anti-inflammatory properties of compounds present in pomegranate fruit. In-vitro studies have reported the anti-inflammatory properties of compounds present in pomegranate. These compounds showed a dose-dependent inhibition on nitric oxide production [11]. Fabio M et al., reported that PPE was associated with the highest suppression of proinflammatory cytokine expression in the ex-vivo model [12]. It has been reported that pomegranate by-products and punicalagins can inhibit the growth of pathogenic organisms while increasing the growth of beneficial bacteria [13]. Phenolic compounds exhibit their beneficial effects through the scavenging action of free radicals. In the recent past, there has been a renewed interest in determining the relevant dietary sources of antioxidant phenolics [14]. PPE has received much attention in the field of food preservation. PPE gains relevance in the health sector because of its potential in bone regeneration [15]. The anti-inflammatory, antioxidant, and antimicrobial properties of PPE have not been evaluated in a singular research study. In this context, it was decided to prepare PPE and analyse its anti-inflammatory, antioxidant, and antimicrobial activity. The hypothesis is that PPE has significant anti-inflammatory, antioxidant, and antimicrobial activity. The objectives of the present study were to prepare PPE and evaluate its anti-inflammatory, antioxidant, and antimicrobial activity.

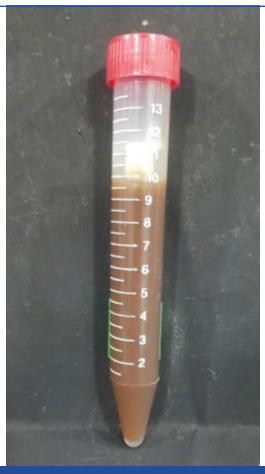
MATERIALS AND METHODS

The present in-vitro study was designed and carried out at the Nanobiomedicine Laboratory, Department of Pharmacology, Saveetha Dental College and Hospitals, Chennai from June 2021 to August 2021. The Ethical Committee of the Institution approved the project (BRULAC/ SDCH/SIMATS/IAEC/05-2022/125). The study evaluated the anti-inflammatory, antioxidant, and antimicrobial activity of the freshly prepared PPE.

Study Procedure

Fresh pomegranate fruits of Ganesh variety were soaked in diluted Koparo Clean vegetable and fruit wash (10 mL in 1 litre of water) for 15 minutes, then washed in running water. The pericarp was then separated and air-dried. The dried peel was coarsely powdered

using a Multi-mill machine (SS 304). A 2 gm of peel powder was mixed with 100 mL of distilled water using a magnetic stirrer (Remi 5 MLH). The mixture was heated for 15-20 minutes using a heating mantle at 60-80 degrees Celsius. The boiled mixture was filtered using Whatman No. 1 filter paper. Then, the filtered extract was further condensed to 5 mL [Table/Fig-1] [16].



[Table/Fig-1]: Pomegranate Peel Extract (PPE).

Anti-inflammatory Activity

Egg Albumin denaturation assay (EA): The EA was done as follows [17]. A 5 mL solution was prepared using 2.8 mL of freshly manufactured pH-6.3 phosphate-buffered saline and 0.2 mL of chicken EA extract. The PPE of different concentrations, namely 10 µL, 20 µL, 30 µL, 40 µL, 50 µL (n=10), were prepared. Diclofenac sodium was used as the positive control. The mixes were then heated in a water bath for 15 minutes at 37°C. The samples were allowed to cool to ambient temperature, and the absorbance at 660 nm was measured.

Bovine Serum Albumin denaturation assay (BSA): The anti-inflammatory activity of PPE was tested using the convention proposed by Mizushima and Kabayashi with modifications (Pratik D et al., 2019) [18]. A 0.05 mL of PPE of various concentrations (10 µL, 20 µL, 30 µL, 40 µL, 50 µL) was added to 0.45 mL of BSA (1% aqueous solution), and the pH of the mixture was adjusted to 6.3 using 1N hydrochloric acid. These samples were incubated at room temperature for 20 minutes and then heated at 55°C for 30 minutes in a water bath. The temperature of the samples was then reduced, and the absorbance was measured by spectrophotometry at 660 nm. Diclofenac Sodium was used as the standard, and Dimethyl Sulphoxide was utilised as a control.

Percentage of protein denaturation was determined by the following equation [18]:

$$\text{Inhibition} = \left(\frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100$$

Antioxidant Activity

Antioxidant activity of PPE was tested using three methods, of which two- DPPH and H2O2- were based on the free-radical scavenging capacity of PPE. The third one, FRAP was based on measuring the iron-reducing capacity.

DPPH Assay: The assay utilised a commercially available free radical DPPH, which is soluble in methanol (Brand-Williams W et al., 1995) [19], and the antioxidant activity was measured by the decrease in absorbance at 515 nm. PPE of various concentrations (10 µL, 20 µL, 30 µL, 40 µL, 50 µL) was prepared and mixed with 1 mL of 0.1 mM DPPH in methanol and 450 µL of 50 mM Tris HCl buffer (pH 7.4) and incubated for 30 minutes. Later, the reduction in the quantity of DPPH free radicals was assessed based on the absorbance at 515 nm. Butylated hydroxytoluene was employed as a control. The percentage of inhibition was determined by the above mentioned equation.

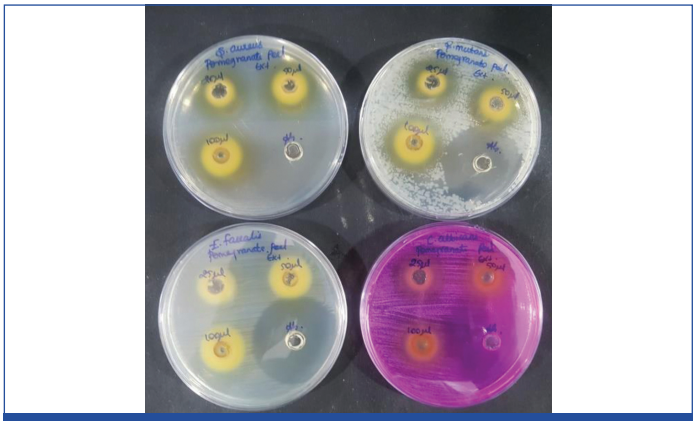
H2O2 Assay

This was performed as described by Halliwell B with minor modifications [20]. All the solutions were prepared freshly. A 1.0 mL of the reaction mixture contained the following: 100 µL of 28 mM of 2-deoxy-2-ribose (dissolved in phosphate buffer 7.4), 500 µL solution of various concentrations of PPE (10 µL, 20 µL, 30 µL, 40 µL, 50 µL), 200 µL of 200 µM FeCl3 and 1.04 mM Ethylenediamine Tetraacetic Acid (EDTA) (1:1 v/v), 100 µL H2O2 (1.0 mM) and 100 µL ascorbic acid (1.0 mM). After incubating the reactant mixture for a period of one hour at 37°C, the extent of deoxyribose degradation was measured by the Thiobarbituric Acid reaction. The absorbance was measured at around 532 nm against the blank solution. Vitamin E was used as the positive control.

FRAP Assay: All the reagents were procured from Merck (Germany) company [21]. A 3.6 mL of FRAP solution was added to 0.4 mL of distilled water and incubated at 37°C for five minutes. This solution was then mixed with various concentrations of PPE (10 µL, 20 µL, 30 µL, 40 µL, 50 µL) and incubated at 37°C for 10 minutes. The absorbance of the reaction mixture was measured at 593 nm. A calibration curve was constructed based on five concentrations of FeSO4·7H2O (0.1, 0.4, 0.8, 1, 1.12, 1.5 mM) and the absorbance values were measured for sample solutions.

Antibacterial Activity

The antibacterial activity of PPE was assessed using the agar well diffusion method against pathogenic bacteria, including *Streptococcus mutans, Staphylococcus aureus, Enterococcus faecalis*, and *Candida albicans*. Mueller Hinton agar was prepared and sterilised for 45 minutes under 120 lbs pressure. The medium was poured into sterilised plates and allowed to solidify. Wells were cut using a well cutter, and the test organisms were swabbed onto the plates. PPE with different concentrations of 25 µL, 50 µL, and 100 µL were loaded into the wells, and the plates were incubated for 24 hours at 37°C. After the incubation period, the zone of inhibition was measured and compared with the standard antibiotic Amoxicillin 100 mg/mL [Table/Fig-2].



[Table/Fig-2]: Antimicrobial activity PPE.

Antifungal Activity

The antifungal activity of PPE was evaluated using the agar well diffusion method against *C.albicans*. Sabouraud's dextrose agar was used to prepare the medium, which was sterilised for 45 minutes under 120 lbs of pressure. Wells were cut using a well cutter and then inoculated with the test organism *C.albicans*. PPE was added in different concentrations of 25 µL, 50 µL, and 100 µL. The plates were incubated for 48-72 hours at a temperature of 28°C. The zone of inhibition was measured and compared with the standard antifungal agent Fluconazole [Table/Fig-2].

STATISTICAL ANALYSIS

The results were tabulated and subjected to statistical analysis using Statistical Package for Social Sciences (SPSS) version 23.0 The independent t-test, ANOVA, and Tukey's HSD were used

for statistical analysis. A p-value less than 0.05 were considered significant.

RESULTS

Anti-inflammatory Activity

In EA, the inhibition caused by PPE with a 10 µL concentration was (51.04%±0.145), and for the standard, it was 55.08%±0.252 (10 µL). The difference was statistically significant (p<0.001) [Table/Fig-3,4]. For BSA, the percentage of inhibition was significantly lower for PPE when compared to the standard at 10 µL, 20 µL, and 30 µL (p<0.001) [Table/Fig-3,4].

Concentration	N	EA concentration (Mean±SD)	BSA_Concentration (Mean±SD)
10 µL	10	51.0460±0.14546	43.1300±0.28833
20 µL	10	60.1340±0.24185	55.0980±0.24022
30 µL	10	65.0430±0.13598	68.0570±0.18025
40 µL	10	70.0680±0.15782	75.5040±3.90633
50 µL	10	78.0880±0.21730	80.8200±3.38815

[Table/Fig-3]: Mean values of different concentrations of PPE observed in EA and BSA.

Group Statistics (Independent t-test)						
Concentration	Group	N	Mean	Std. Deviation	Std. error Mean	p-value
EA 10 µL	PPE	10	51.0460	0.14546	0.04600	<0.001
	Standard	10	55.0800	0.25298	0.08000	
EA 20 µL	PPE	10	60.1340	0.24185	0.07648	<0.001
	Standard	10	64.1310	0.30311	0.09585	
EA 30 µL	PPE	10	65.0430	0.13598	0.04300	0.368
	Standard	10	65.1380	0.29551	0.09345	
EA40 µL	PPE	10	70.0680	0.15782	0.04991	0.404
	Standard	10	70.1780	0.37529	0.11868	
EA 50 µL	PPE	10	78.0880	0.21730	0.06872	0.905
	Standard	10	78.1000	0.22725	0.07186	
BSA 10 µL	PPE	10	43.1300	0.28833	0.09118	<0.001
	Standard	10	47.0790	0.18381	0.05813	
BSA 20 µL	PPE	10	55.0980	0.24022	0.07596	<0.001
	Standard	10	60.0880	0.21730	0.06872	
BSA 30 µL	PPE	10	68.0570	0.18025	0.05700	<0.001
	Standard	10	72.0430	0.13598	0.04300	
BSA 40 µL	PPE	10	75.5040	3.90633	1.23529	0.055
	Standard	10	78.0400	0.12649	0.04000	
BSA 50 µL	PPE	10	80.8200	3.38815	1.07143	0.126
	Standard	10	82.8000	1.93218	0.61101	

[Table/Fig-4]: Comparison of Anti-inflammatory activity between different concentrations of PPE and standard.

There were significant differences between the groups of different concentrations of PPE in both EA and BSA (p<0.05) [Table/Fig-5]. On pair-wise comparison, a significant difference was found between 10 µL and 40 µL (p=0.002) and 10 µL and 50 µL (p=0.001) in EA [Table/Fig-6]. Similarly, in BSA, a significant difference was found between 10 µL and 50 µL (p=0.028) [Table/Fig-7].

Antioxidant Activity

The antioxidant activity observed for DPPH ranged between 65.44% to 92.50% in the experimental group and 66.25% to 93.18% with the standard. For H2O2, the values ranged from 51.94% to 88.99% for the experimental group and 51.06% to 89.65% for the standard. In the case of FRAP, the values ranged from 53.09% to 88.49% and 51.10% to 88.81%, respectively. [Table/Fig-8] Significant differences were found between PPE concentrations

		Sum of squares	df	Mean square	F	Sig.
EA concentration	Between groups	1272.486	4	318.122	6.409	<0.001
	Within groups	2233.782	45	49.640		
	Total	3506.268	49			
BSA Concentration	Between groups	1985.023	4	496.256	2.897	0.032
	Within groups	7708.735	45	171.305		
	Total	9693.758	49			

[Table/Fig-5]: Comparisons of anti-inflammatory activity between different concentrations of PPE using ANOVA.

(I) Concentration	(J) Concentration µL	Mean Difference (I-J)	Std. Error	Sig.
10 µL	20	-4.49400	3.15086	0.614
	30	-6.87400	3.15086	0.205
	40	-12.77400*	3.15086	0.002
	50	-13.31000*	3.15086	0.001
20 µL	10	4.49400	3.15086	0.614
	30	-2.38000	3.15086	0.942
	40	-8.28000	3.15086	0.082
	50	-8.81600	3.15086	0.055
30 µL	10	6.87400	3.15086	0.205
	20	2.38000	3.15086	0.942
	40	-5.90000	3.15086	0.347
	50	-6.43600	3.15086	0.263
40 µL	10	12.77400*	3.15086	0.002
	20	8.28000	3.15086	0.082
	30	5.90000	3.15086	0.347
	50	-0.53600	3.15086	1.00
50 µL	10	13.31000*	3.15086	0.001
	20	8.81600	3.15086	0.055
	30	6.43600	3.15086	0.263
	40	0.53600	3.15086	1.00

[Table/Fig-6]: Pairwise comparisons of anti-inflammatory activity between different concentrations of PPE obtained in EA.

(I) Concentration	(J) Concentration	Mean difference (I-J)	Std. Error	Sig.
10 µL	20 µL	-4.84200	5.85329	0.921
	30 µL	-8.67700	5.85329	0.579
	40 µL	-13.34900	5.85329	0.170
	50 µL	-18.01100*	5.85329	0.028
20 µL	10 µL	4.84200	5.85329	0.921
	30 µL	-3.83500	5.85329	0.965
	40 µL	-8.50700	5.85329	0.597
	50 µL	-13.16900	5.85329	0.181
30 µL	10 µL	8.67700	5.85329	0.579
	20 µL	3.83500	5.85329	0.965
	40 µL	-4.67200	5.85329	0.930
	50 µL	-9.33400	5.85329	0.508
40 µL	10 µL	13.34900	5.85329	0.170
	20 µL	8.50700	5.85329	0.597
	30 µL	4.67200	5.85329	0.930
	50 µL	-4.66200	5.85329	0.930
50 µL	10 µL	18.01100*	5.85329	0.028
	20 µL	13.16900	5.85329	0.181
	30 µL	9.33400	5.85329	0.508
	40 µL	4.66200	5.85329	0.930

[Table/Fig-7]: Pairwise comparisons of anti-inflammatory activity between different concentrations of PPE obtained in BSA.

of 10 µL, 20 µL, and 30 µL with the standard for all three assays (p<0.05) [Table/Fig-9].

Concentration	N	DPPH (%) Mean±SD	H2O2 (%) Mean±SD	FRAP (%) Mean±SD
10 µL	10	65.4410±0.50711	51.9400±0.09661	53.0910±0.24021
20 µL	10	75.8500±0.49900	57.2460±0.16494	57.4450±0.47603
30 µL	10	84.8610±0.49559	65.6540±0.20998	68.8980±0.46154
40 µL	10	88.1700±0.69921	78.2200±0.94522	78.4380±1.25136
50 µL	10	92.5040±1.23723	88.9900±1.03280	88.4900±0.67495

[Table/Fig-8]: Mean values of different concentrations observed in DPPH, H2O2, FRAP.

Group Statistics (Independent t-test)						
	Group	N	Mean	Std. Deviation	Std. Error Mean	p-value
DPPH 10 µL	PPE	10	65.4410	0.50711	0.16036	0.002
	Standard	10	66.2570	0.46928	0.14840	
DPPH 20 µL	PPE	10	75.8500	0.49900	0.15780	<0.001
	Standard	10	78.5100	0.49542	0.15667	
DPPH 30 µL	PPE	10	84.8610	0.49559	0.15672	0.006
	Standard	10	85.5910	0.55065	0.17413	
DPPH 40 µL	PPE	10	88.1700	0.69921	0.22111	0.103
	Standard	10	88.6100	0.40838	0.12914	
DPPH 50 µL	PPE	10	92.5040	1.23723	0.39125	0.131
	Standard	10	93.1800	0.54579	0.17259	
H2O2 10 µL	PPE	10	51.9400	0.09661	0.03055	<0.001
	Standard	10	51.0600	0.44272	0.14000	
H2O2 20 µL	PPE	10	57.2460	0.16494	.05216	<0.001
	Standard	10	56.8900	0.03162	.01000	
H2O2 30 µL	PPE	10	65.6540	0.20998	0.06640	<0.001
	Standard	10	66.0600	0.12649	0.04000	
H2O2 40 µL	PPE	10	78.2200	0.94522	0.29891	0.101
	Standard	10	78.7920	0.44892	0.14196	
H2O2 50 µL	PPE	10	88.9900	1.03280	0.32660	0.086
	Standard	10	89.6560	0.52475	0.16594	
FRAP 10 µL	PPE	10	53.0910	0.24021	0.07596	<0.001
	Standard	10	51.1000	<0.00100	<0.00100	
FRAP 20 µL	PPE	10	57.4450	0.47603	0.15053	0.002
	Standard	10	56.9000	<0.00100	<0.00100	
FRAP 30 µL	PPE	10	68.8980	0.46154	0.14595	<0.001
	Standard	10	68.1000	<0.00100	<0.00100	
FRAP 40 µL	PPE	10	78.4380	1.25136	0.39572	0.432
	Standard	10	78.7600	0.18950	0.05993	
FRAP 50 µL	PPE	10	88.4900	0.67495	0.21344	0.174
	Standard	10	88.8110	0.24223	0.07660	

[Table/Fig-9]: Comparison of antioxidant activity of different PPE concentrations and standard obtained in DPPH, H2O2 and FRAP.

There were significant differences between the groups of different concentrations of PPE in both DPPH, H2O2, and FRAP (p<0.05) [Table/Fig-10]. On pair-wise comparison, significant differences were found between 10 µL and 50 µL (p=0.01) and 20 µL and 50 µL (p=0.028) in DPPH [Table/Fig-11]. However, in H2O2 and FRAP, significant differences were found between 10 µL and 50 µL (p<0.05) [Table/Fig-12,13].

Antibacterial Activity

The antibacterial activity was assessed against *S.mutans*, *S.aureus*, and *E.faecalis* using three different concentrations of PPE. The zone of inhibition was measured, and the highest

ANOVA						
		Sum of squares	Df	Mean square	F	Sig.
DPPH_PPE	Between groups	1233.578	4	308.394	4.014	0.007
	Within groups	3457.329	45	76.830		
	Total	4690.906	49			
H2O2_PPE	Between groups	2092.292	4	523.073	3.288	0.019
	Within groups	7158.713	45	159.083		
	Total	9251.005	49			
FRAP_PPE	Between groups	1827.570	4	456.893	2.913	0.032
	Within groups	7058.222	45	156.849		
	Total	8885.792	49			

[Table/Fig-10]: Comparisons of Antioxidant activity between different concentrations of PPE.

(I) Concentration	(J) Concentration µL	Mean difference (I-J)	Std. error	Sig.
10 µL	20	-1.58000	3.91994	0.994
	30	-7.00400	3.91994	0.394
	40	-9.02300	3.91994	0.163
	50	-13.61400*	3.91994	0.010
20 µL	10	1.58000	3.91994	0.994
	30	-5.42400	3.91994	0.641
	40	-7.44300	3.91994	0.333
	50	-12.03400*	3.91994	0.028
30 µL	10	7.00400	3.91994	0.394
	20	5.42400	3.91994	0.641
	40	-2.01900	3.91994	0.985
	50	-6.61000	3.91994	0.452
40 µL	10	9.02300	3.91994	0.163
	20	7.44300	3.91994	0.333
	30	2.01900	3.91994	0.985
	50	-4.59100	3.91994	0.767
50 µL	10	13.61400*	3.91994	0.010
	20	12.03400*	3.91994	0.028
	30	6.61000	3.91994	0.452
	40	4.59100	3.91994	0.767

[Table/Fig-11]: Pairwise comparisons of Antioxidant activity between different concentrations of PPE obtained in DPPH.

value was observed for Streptococcus mutans (38±2.62 mm) at a concentration of 100 µL. Similarly, Staphylococcus aureus exhibited a zone of inhibition of 36±3.16 mm at a concentration of 100 µL. When assessing the antifungal activity against C. albicans, a zone of inhibition of 23±2.36 mm was observed at a concentration of 100 µL [Table/Fig-14].

(I) Concentration	(J) Concentration µL	Mean difference (I-J)	Std. error	Sig.
10 µL	20	-5.99100	5.64061	0.825
	30	-9.18100	5.64061	0.488
	40	-14.12700	5.64061	0.108
	50	-18.73100*	5.64061	0.015
20 µL	10	5.99100	5.64061	0.825
	30	-3.19000	5.64061	0.979
	40	-8.13600	5.64061	0.604
	50	-12.74000	5.64061	0.178
30 µL	10	9.18100	5.64061	0.488
	20	3.19000	5.64061	0.979
	40	-4.94600	5.64061	0.904
	50	-9.55000	5.64061	0.448

40 µL	10	14.12700	5.64061	0.108
	20	8.13600	5.64061	0.604
	30	4.94600	5.64061	0.904
	50	-4.60400	5.64061	0.924
50 µL	10	18.73100*	5.64061	0.015
	20	12.74000	5.64061	0.178
	30	9.55000	5.64061	0.448
	40	4.60400	5.64061	0.924

[Table/Fig-12]: Pairwise comparisons of antioxidant activity between different concentrations of PPE obtained in H2O2.

(I) Concentration	(J) Concentration µL	Mean difference (I-J)	Std. error	Sig.
10 µL	20	-4.53700	5.60088	0.926
	30	-8.08600	5.60088	0.603
	40	-12.08200	5.60088	0.215
	50	-17.54500*	5.60088	0.024
20 µL	10	4.53700	5.60088	0.926
	30	-3.54900	5.60088	0.969
	40l	-7.54500	5.60088	0.664
	50	-13.00800	5.60088	0.157
30 µL	10	8.08600	5.60088	0.603
	20	3.54900	5.60088	0.969
	40	-3.99600	5.60088	0.952
	50	-9.45900	5.60088	0.451
40 µL	10	12.08200	5.60088	0.215
	20	7.54500	5.60088	0.664
	30	3.99600	5.60088	0.952
	50	-5.46300	5.60088	0.865
50 µL	10l	17.54500*	5.60088	0.024
	20l	13.00800	5.60088	0.157
	30l	9.45900	5.60088	0.451
	40	5.46300	5.60088	0.865

[Table/Fig-13]: Pairwise comparisons of Antioxidant activity between different concentrations of PPE obtained in FRAP.

Organisms	PPE 25 µL	PPE 50 µL	PPE100 µL	Control 100 mg/mL
S.mutans	24±1.23 mm	28±2.06 mm	38±2.62 mm	38±0.42 mm
S.aureus	31±2.18 mm	34±2.94 mm	36±3.16 mm	43±0.62 mm
E.faecalis	16±0.12 mm	19±0.84 mm	21±1.48 mm	43±2.56 mm
C.albicans	18±0.56 mm	21±1.62 mm	23±2.36 mm	34±2.98 mm

[Table/Fig-14]: Zone of inhibition at different concentrations of PPE and control.

Upon comparison of the antibacterial and antifungal activity of different concentrations of PPE with the standard used, a statistically significant difference was found at all concentrations against S. mutans, *S.aureus*, *E.faecalis*, and *C.albicans* (p<0.01), except for PPE at 100 µL for *S.mutans* (p=0.995) [Table/Fig-15]. There were significant differences between the groups of different concentrations of PPE (p<0.05) [Table/Fig-16]. On pair-wise comparison, significant differences were found between all three concentrations used for *S.mutans* and *E.faecalis* (p<0.01). However, for *S.aureus*, the difference between only 25 µL and 100 µL was statistically significant (p=0.003) [Table/Fig-17].

DISCUSSION

A number of biologically active compounds present in pomegranate provide anti-inflammatory, antioxidant, and antibacterial properties [14]. Parts of the fruit, especially the peel, are abundant in biologically active compounds; therefore, peel extract was selected for this study. The study has proven that PPE has definite anti-inflammatory, antioxidant, and antibacterial activity.

Anti-inflammatory Activity

Inflammation is caused by the denaturation of proteins, and PPE can inhibit that process, thereby reducing inflammation. The anti-inflammatory property was measured by EA and BSA using different concentrations of PPE. At higher concentrations of PPE, the anti-inflammatory activity was similar to that of the standard, with no statistically significant difference (p>0.05). The results indicate that both the experimental and standard groups have similar anti-inflammatory potential. At lower concentrations, the anti-inflammatory potential was slightly inferior. It can be stated that with the increase in concentration, the anti-inflammatory property also enhances. Based on the present observations, PPE can be considered a successful substitute for popularly used anti-inflammatory drugs.

The polyphenols contained in PPE are converted to urolithins in the gut by the activity of microbiota. Urolithins have shown significant anti-inflammatory activity [22]. The individually fractionated biomolecules of pomegranate influence the expression of inflammatory cell signalling protein in cancer cells [23]. Pomegranate juice, tannins, and punicalagin reduce the expression of Cyclooxygenase-2 (COX-2), responsible for the production of prostanoids that induce inflammation [24]. Ellagic acid found in PPE can control intestinal inflammation by downregulating inflammation-mediating compounds and blocking cell signalling pathways [25]. The polyphenol-rich pomegranate fruit extract or compounds derived from it can be used for the treatment of inflammatory diseases, possibly by suppressing basophils and mast cell activation [26].

	t-test for Equality of Means						
	t	df	Sig. (2-tailed)	Mean difference of PPE and standard	Std. error difference	95% Confidence interval of the difference	
						Lower	Upper
*S.mutans*_PPE_25	-29.889	18	<0.001	-14.03800	0.46968	-15.02475	-13.05125
*S.mutans*_PPE_50	-11.822	18	<0.001	-9.78300	0.82750	-11.52152	-8.04448
*S.mutans*_PPE_100	.006	18	0.995	0.00600	0.97277	-2.03771	2.04971
*S.aureus*_PPE_25	-13.579	18	<0.001	-11.62000	0.85573	-13.41782	-9.82218
*S.aureus*_PPE_50	-8.704	18	<0.001	-8.64800	0.99358	-10.73543	-6.56057
*S.aureus*_PPE_100	-6.112	18	<0.001	-6.69200	1.09495	-8.99241	-4.39159
*E.Faecalis*_PPE_25	-30.763	18	<0.001	-26.76800	0.87014	-28.59610	-24.93990
*E.Faecalis*_PPE_50	-26.211	18	<0.001	-23.76200	0.90655	-25.66659	-21.85741
*E.Faecalis*_PPE_100	-21.821	18	<0.001	-21.78000	0.99810	-23.87693	-19.68307
*C.albicans*_PPE_25	-15.247	18	<0.001	-15.82400	1.03784	-18.00442	-13.64358
*C.albicans*_PPE_50	-10.899	18	<0.001	-12.82000	1.17628	-15.29127	-10.34873
*C.albicans*_PPE_100	-8.550	18	<0.001	-10.92200	1.27747	-13.60587	-8.23813

[Table/Fig-15]: Comparison of antibacterial activity and antifungal activity of difference concentration of PPE and standard.

	Sum of squares	df	Mean square	F	Sig.
S.mutans					
Between groups	122.187	2	61.093	15.588	<0.001
Within Groups	105.820	27	3.919		
Total	228.006	29			
	Sum of squares	**df**	**Mean square**	**F**	**Sig.**
S.aureus					
Between groups	123.146	2	61.573	6.642	0.005
Within groups	250.310	27	9.271		
Total	373.456	29			
	Sum of squares	**df**	**Mean square**	**F**	**Sig.**
E.faecalis					
Between groups	126.148	2	63.074	51.299	<0.001
Within groups	33.198	27	1.230		
Total	159.346	29			
	Sum of squares	**df**	**Mean square**	**F**	**Sig.**
C.albicans					
Between groups	122.187	2	61.093	15.588	<0.001
Within groups	105.820	27	3.919		
Total	228.006	29			

[Table/Fig-16]: Comparisons of Antibacterial and antifungal activity of different concentrations of PPE using ANOVA.

(I) Concentration	(J) Concentration	Mean difference (I-J)	Std. error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
Dependent variable: *S.mutans*						
25	50	-4.25500*	1.02763	0.001	-6.8029	-1.7071
	100	-14.04400*	1.02763	<0.001	-16.5919	-11.4961
50	25	4.25500*	1.02763	0.001	1.7071	6.8029
	100	-9.78900*	1.02763	<0.001	-12.3369	-7.2411
100	25	14.04400*	1.02763	<0.001	11.4961	16.5919
	50	9.78900*	1.02763	<0.001	7.2411	12.3369
Dependent variable: *S.aureus*						
25	50	-2.97200	1.36167	0.092	-6.3481	0.4041
	100	-4.92800*	1.36167	0.003	-8.3041	-1.5519
50	25	2.97200	1.36167	0.092	-0.4041	6.3481
	100	-1.95600	1.36167	0.337	-5.3321	1.4201
100	25	4.92800*	1.36167	0.003	1.5519	8.3041
	50	1.95600	1.36167	0.337	-1.4201	5.3321
Dependent variable: *E.faecalis*						
25	50	-3.00600*	0.49589	<0.001	-4.2355	-1.7765
	100	-4.98800*	0.49589	<0.001	-6.2175	-3.7585
50	25	3.00600*	0.49589	<0.001	1.7765	4.2355
	100	-1.98200*	0.49589	0.001	-3.2115	-0.7525
100	25	4.98800*	0.49589	<0.001	3.7585	6.2175
	50	1.98200*	0.49589	0.001	0.7525	3.2115
25	50	-3.00400*	0.88535	0.006	-5.1992	-0.8088
	100	-4.90200*	0.88535	<0.001	-7.0972	-2.7068
50	25	3.00400*	0.88535	0.006	0.8088	5.1992
	100	-1.89800	0.88535	.100	-4.0932	0.2972
100	25	4.90200*	0.88535	<0.001	2.7068	7.0972
	50	1.89800	0.88535	0.100	-0.2972	4.0932

[Table/Fig-17]: Pairwise comparisons antibacterial and antifungal activity between different concentrations of PPE.
*The mean difference is significant at the 0.05 level

Inflammation caused by free radicals can be eliminated by PPE. Due to the antimicrobial properties imparted by the phytochemicals of pomegranate, inflammation caused by microbes can also be eliminated. The phytochemicals present in pomegranate have multiple activities and can affect more than one inflammatory factor, resulting in enhanced healing [27-31]. The outcomes of various researchers, as referenced earlier, align with the findings of the present study. The concentration of active components within PPE plays a crucial role in augmenting its anti-inflammatory properties.

Antioxidant Activity

The antioxidant property of all concentrations of PPE and the standard was similar across the three assays. When comparing the concentrations of 40 µL and 50 µL, there was no significant difference between the test material and the standard. This establishes the fact that at higher concentrations, the antioxidant activity of PPE was similar to that of the standard, indicating that the antioxidant activity is dose-dependent. The presence of punicalagin and hydrolysable tannins in pomegranate extract provides very high antioxidant activity compared to the antioxidant properties of green tea and red wine, with the potency of PPE being three times higher than the others [32]. Pomegranate peel is a reservoir of biologically active compounds that provide excellent antioxidant properties [33]. The presence of phenolic compounds is influenced by the extraction methods and cultivar, and the activity increased with the concentration of PPE [34].

Lamiae Benchagra compared PPE and extracts of arils and reported a higher concentration of phenolic compounds in peels than in arils. DPPH, FRAP, and H2O2 assays revealed that the antioxidant activity was dose-dependent, with higher concentrations giving higher antioxidant activity [35]. Shalini M et al., made extracts of the Ganesh variety of pomegranate peel in water, methanol, and ethanol, as well as combinations with water. The antioxidant activity and total phenolic content were evaluated. The 70% ethanol: 30% water and 100% aqueous extract had higher phenolic content and showed higher antioxidant activity [36]. Singh RP et al., prepared methanol, ethyl acetate, and water extracts of pomegranate seeds and peels. The methanol extract of pomegranate peel showed the highest antioxidant activity among all the extracts [37]. The dried methanolic extract of pomegranate peel protected hepatic cells from the toxic effects of Carbon Tetrachloride, mainly due to the antioxidant function of the biologically active compounds found in the peel [38].

The findings of the large number of research workers mentioned above and the results obtained from the present study are comparable. PPE contains effective antioxidant elements which have established therapeutic potential. The concentration of the active components contained in PPE plays a significant role in enhancing the antioxidant property.

Antimicrobial Effect

When the antimicrobial efficacy was assessed, the maximum antibacterial effect of PPE was against staphylococcus aureus, followed by streptococcus mutans, and E.Faecalis. For all the organisms tested, the zone of inhibition increased with the increase in concentration of PPE. The methanolic extract of pomegranate showed an antibacterial effect. The antibacterial activity was attributed to the phenolic structure contained in the extract, specifically Gallic acid and other phenolics [39,40]. Ether, chloroform, methanol, and water extracts of Punica granatum had an antibacterial effect, with the methanolic extract being the most effective [41]. Cruz-Valenzuela MR et al., reported that the peel extract showed antimicrobial activity against bacterial and fungal cultures, specifically against Staphylococcus and Aspergillus [42]. The mechanism behind the antimicrobial activity has been reported by various investigators. The phytoneutrients are toxic to the bacterial cell wall and form complexes with enzyme cofactors and sulfhydryl groups of proteins. This alters the cell membrane permeability and disturbs the respiratory chain [43].

Antifungal Activity

The PPE showed definite antifungal activity, which is due to the presence of polyphenols. These polyphenols cause precipitation of cell membrane proteins, resulting in cell leakage. This cell leakage leads to alterations in the composition of cytoplasm and cell membrane, inhibiting fungal growth [44].

Both the crude extract and the isolated punicalagin compound from pomegranate demonstrated a significant inhibitory effect against both Trichophyton and Microsporum genera. The isolated punicalagin compound exhibited a similar minimum inhibitory concentration value as the crude extract [45]. PPE, rich in gallic acid, strongly inhibited the growth of fungus and reduced its drug resistance. This extract is a promising natural antifungal agent for clinical use [46]. The PPE exhibits high concentrations of polyphenols, specifically punicalagin and ellagic acid, attributing to its antifungal properties. The peel demonstrates effectiveness against a wide range of fungi, encompassing both pathogenic and opportunistic pathogens [47]. The antimicrobial activity observed in the current study closely resembles that reported in previous research.

Limitation(s)

The specific biologically active component responsible for the anti-inflammatory, antioxidant, and antibacterial activity was not ascertained.

CONCLUSION(S)

The PPE has a definite anti-inflammatory, antioxidant, and antibacterial effect, which depends on the concentration of the active ingredients contained in the PPE. PPE can be used as a therapeutic agent where an anti-inflammatory, antioxidant, and antibacterial effect is required. In the Indian context, natural products like pomegranate can be considered preferentially to improve affordability and accessibility among economically weaker sections of society without sacrificing therapeutic quality.

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