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EVALUATION OF ANTIMICROBIAL AND HEMOCOMPACTIBILITY ASSAY OF ETHANOLIC EXTRACT OF *PUNICA GRANATUM*

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Abstract
Background: The development of multi-drug resistant microorganisms, as well as the growing demand for biocompatibility of various medical materials, has led pharmaceutical companies to turn their attention to natural products. Punica granatum is recognized as a rich source of various bioactive compounds, including tannins/flavonoids, which possess substantial therapeutic value. Aim: The purpose of the present study was to investigate the antimicrobial properties of Punica granatum ethanolic extract as a potential candidate for biomedical applications.
Materials and Methods: An 80% ethanol extract was prepared from dehydrated Punica granatum. Antimicrobial activities against Staphylococcus aureus and Candida albicans were evaluated by the disk diffusion method at 25, 50, and 100 mg/mL. Hemocompatibility tests, based on ASTM F756-00, involved the determination of the haemolytic percentage of the extract when subjected to blood samples. **Results:** The antimicrobial activities of the extract were very promising. In the case of Staphylococcus aureus, the maximum Zone of Inhibition (ZOI) was 25mm at 25mg/mL. In the case of C. albicans, a very significant Zone of Inhibition was observed, i.e., 35mm, at the same concentration. In the hemocompatibility test, the hemolysis percentage was less than 3%, thus falling into the category of moderately hemolytic and within the permissible limits (less than 5%) for materials to be used. **Conclusion:** The ethanol extract of Punica granatum has very strong antimicrobial activities. It has good hemocompatibility, and thus the ethanol extract of Punica granatum could be used to produce infection-resistant, biocompatible technologies and thus could be a safe, and natural alternative to the use of antibiotics.
Keywords: Punica granatum, Antimicrobial activities, Hemocompatibility, Hemolysis, Staphylococcus aureus, medicine.

1. Introduction

The search for new treatment options from natural sources has increased because of the growing problem of antimicrobial resistance and the urgent need for biocompatible materials in modern medicine.(1),(2). Pomegranate, or *Punica granatum L.*, has become a major potential drug for pharmaceutical development because of its strong biological activities and complex phytochemical profile.(3) (4) . Recent research has shown that the plant's peel, pericarp, and sarcotesta, in particular, have important antimicrobial, anti-inflammatory, and antioxidant effects.(4,5), (4–6). For instance, a lectin-rich protein fraction (PgTel) with significant antigenotoxic effects has been discovered in the sarcotesta, whereas the peel continues to be a concentrated source of polysaccharides (measured at about 315 mg/g) and active small molecules such as ellagic acid and 5-hydroxymethylfurfural (5-HMF).(4–7) (3). The broad-spectrum effectiveness of *Punica granatum* ethanolic and hydroalcoholic extracts against both Gram-positive and Gram-negative bacterial pathogens is well known. These extracts' capacity to alter virulence factors and disturb bacterial homeostasis is the basis of their antimicrobial potential.(1). Pomegranate peel extracts (PGPE) have been shown to effectively suppress pigment production and biofilm formation in pathogens such as *Pseudomonas aeruginosa* at concentrations as low as 5 µg/mL by interfering with quorum sensing-regulated pathways. Additionally, these extracts can dramatically lower pathogen virulence in in vivo models like Caenorhabditis elegans and sensitize bacteria to host defenses like human serum lysis.(1).These extracts exhibit remarkable inhibitory properties according to quantitative evaluations. For example, against *Staphylococcus aureus*, methanolic and ethanolic peel fractions have shown a Zone of Inhibition (ZOI) of 23.7 mm and a Minimum Inhibitory Concentration (MIC) of 0.125 mg/mL.(5). Inhibition zones of 15.6 mm and 14.7 mm have been observed against Gram-negative strains such as *Salmonella typhimurium* and *Escherichia coli*. Pomegranate-derived phytotherapeutic gels have demonstrated high efficacy in preventing oral pathogen adherence beyond simple growth inhibition, with minimum inhibitory concentrations of adherence reaching dilution endpoints of 1:16 for *S. mutans* and 1:64 for *Candida albicans.(5,8)* .
However, the clinical utility of *Punica granatum* depends heavily on its safety and interaction with human physiological systems (5,8,9)Hemocompatibility tests are crucial for confirming that these extracts, when applied topically or systemically, do not result in side effects like erythrocyte lysis. (5,8–10).There is promising evidence; Hemolysis rates for certain preparations of pomegranate peel have reached as low as 0.42%, which falls under limits of biocompatibility. More advanced pomegranate formulations using the dextran polymer have also not reached hemolytic levels during testing.(11).Such safety profiles have paved the way towards innovative applications in biomedicine. Pomegranate peel extracts are being utilized as reducing and stabilizing agents in the green synthesis of selenium nanoparticles, which combine the natural bioactivities of the pomegranate with the improved delivery capabilities of nanoparticles . These advances in nanoparticle synthesis and composite resins reinforce the need to test nanoparticle hemocompatibility to guarantee patient safety in future medical applications(7). This report aims to investigate these different findings in order to provide a comprehensive overview of the anti-microbial effectiveness and a safety index of *Punica granatum* ethanolic extracts. (3).

2. Materials and methods:

2.1 Collection and Preparation of Plant Material. *Fresh Punica granatum fruits were obtained from local sources. The fruits were cleaned with tap water and then distilled water to remove any impurities that may be present on their surface. The edible parts of the fruits were cut into smaller pieces and then dried under the sun for 3-4 days to completely dehydrate them. The dehydrated material was then ground into a fine powder using an electric grinder and stored in an airtight container at room temperature for future use.*

2.2 Extraction Procedure. *Preparation of ethanolic extract: 10 grams of the powdered pomegranate material was taken and mixed with 100 ml of 80% ethanol in a beaker. It was stirred with the help of a magnetic stirrer and heated at 60°C for 2 hours to facilitate the extraction process. It was then cooled and filtered with the help of a Buchner funnel and Whatman filter paper. It was then concentrated with the help of a rotary evaporator and stored at 4°C for further assays.*

2.3 Antimicrobial Activity Assay. *Antimicrobial activity of ethanolic extract was carried out against Staphylococcus aureus and Candida albicans using the disk diffusion method. Different concentrations of ethanolic extract (25, 50, and 100 mg/mL) were screened for their activity. Sterile filter paper discs were impregnated with ethanolic extract and placed on agar plates containing test organisms. Plates were incubated at 37°C for 24 hours, and inhibition zones were measured in millimeters.*

2.4 Hemocompatibility Assay. *Hemocompatibility of the extracts was evaluated following the guidelines provided by the ASTM standards. This involved collecting blood samples and mixing them with the extracts to check for hemolysis. A hemolysis percentage below 3% showed that the extracts were hemocompatible.*

3. Results:

3.1 Antimicrobial Activity Against *S.Aureus* and *C. Albicans*

Table-1- Antimicrobial Activity of ethanolic extract against *S.Aureus* and *C.albicans* under various concentrations.

Pomegranate	Antibiotics	25	50	100
S.Aureus	25mm	-	-	15mm
C.Albicans	35mm	11mm	12mm	17mm

Fig-1: Antimicrobial Activity of ethanolic extract against *S.Aureus* and *C.albicans* under various concentrations.

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The ethanolic extract of Punica granatum exhibited notable antimicrobial activity against both *Staphylococcus aureus* and *Candida albicans* at different concentrations. The extract showed the highest inhibitory effect against *C. albicans*, producing a zone of inhibition of 35 mm, whereas *S. aureus* exhibited a 25 mm zone of inhibition. At concentrations of 50 µL and 100 µL, the extract demonstrated measurable antimicrobial effects against *C. albicans* with inhibition zones of 11 mm and 12 mm, respectively, while a 15 mm inhibition zone was observed against *S. aureus* at 100 µL concentration. These findings indicate that the ethanolic extract possesses broad-spectrum antimicrobial properties, with comparatively stronger antifungal activity against *C. albicans* than antibacterial activity against *S. aureus*.

3.2 Hemocompatibility of *Punica granatum*:

Figure 2: Hemocompatibility assay results indicating less than 3% lysis according to ASTM Standards.

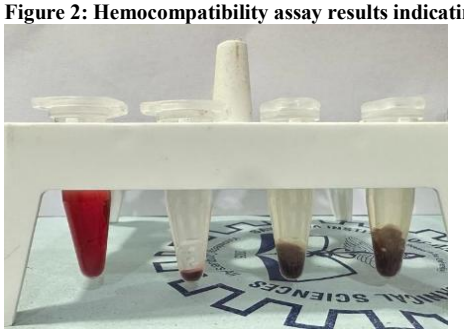
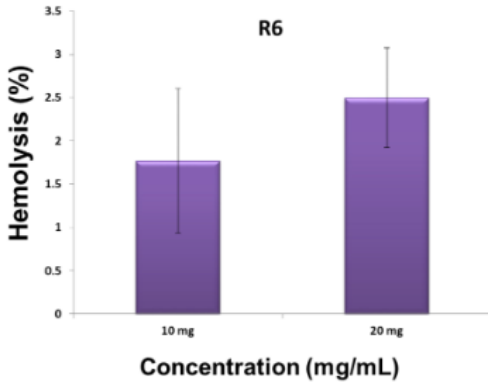


Figure 3: Comparative analysis of hemolytic activity showing the extract remains within permissible limits.



The hemocompatibility assessment of the Punica granatum coating demonstrated a hemolysis ratio of less than 3%, confirming its acceptable blood compatibility according to the ASTM F756-00 standard. Since materials exhibiting less than 5% hemolysis are considered hemocompatible, the coating falls within the permissible safety range. Furthermore, based on the ASTM classification, materials with hemolysis values below 2% are categorized as non-hemolytic, while those between 2% and 5% are considered moderately hemolytic. Therefore, the *Punica granatum* coating can be classified as moderately hemolytic, indicating minimal erythrocyte damage and suggesting its potential suitability for biomedical applications requiring blood-contacting materials.

4. Discussion

The results obtained revealed that the ethanolic extract of pomegranate has significant antimicrobial activity against *Staphylococcus aureus*, as it showed an inhibition zone of 25 mm at a concentration of 25 mg/ml, whereas at a concentration of 100 mg/ml, it showed an inhibition zone of 15 mm. On the other hand, the ethanolic extract of pomegranate showed inhibition zones of 35 mm at a concentration of 25 mg/ml, 11 mm at a concentration of 50 mg/ml, 12 mm at a concentration of 100 mg/ml, and 17 mm at the highest concentration against *Candida albicans*, revealing that the maximum inhibition is at the concentration of 100 mg/ml. This is consistent with the previous research, which revealed that the extracts of pomegranate exhibit significant antibacterial activity, especially against pathogenic bacteria such as *Staphylococcus aureus*. Further research would be required to reveal the hemocompatibility of the extracts, which would be required for therapeutic use(12)(13).

Previous studies have emphasized the antimicrobial potential of the extracts obtained from the fruit of the pomegranate tree, attributing the same to the presence of bioactive compounds like flavonoids, tannins, and other phytochemicals that not only enhance the antimicrobial potential but also contribute to the therapeutic potential of the extracts(13–15). The findings obtained from the current study that show a considerable inhibition zone of 25 mm against *Staphylococcus aureus* at a concentration of 25 mg/ml confirm the findings obtained from previous studies that emphasized the effectiveness of the extracts against a number of bacterial strains. Furthermore, the considerable effect against *Candida albicans* with an inhibition zone of 35 mm at the same concentration shows the potential of the extracts to fight a number of diseases caused by both bacteria and fungi(16)(13,14).

An investigation into the antimicrobial activity of *Punica granatum,* especially in terms of its ethanolic extract, initiates a dialogue into its possible use as an alternative to conventional antibiotics(17). Inhibitory activity against microorganisms like *Staphylococcus aureus* and *Salmonella typhi* is noteworthy, especially in the context of the rising global alarm over the menace of antibiotic resistance. The rise of multi-drug resistant bacteria has called for an investigation into new antimicrobial agents, and natural products like pomegranate extracts can play a pivotal role in this context(18)(19).
In addition, a comparison of the antimicrobial activity of ethanolic extract with existing antibiotics presents a promising area in the creation of alternative treatments. Although existing antibiotics have unique mechanisms of action, a multi-pronged approach of natural extracts may provide a "synergistic effect" that could prevent the possibility of drug resistance(20)(13–15,21). The ability of pomegranate extract to inhibit pathogens through various mechanisms, including the disruption of cell membrane function or the interference with metabolic processes, implies that pomegranate extract could be an effective adjunct to the conventional methods of treatment.(22)
According to the ASTM F756-00 standard, the *Punica granatum* coating has less than 3% hemolysis (lysis) and thus can be said to be hemocompatible as it falls within the acceptable limit of less than 5% for a hemolytic material(23). Materials that have a hemolysis ratio of less than 2% are said to be non-hemolytic, those with a ratio between 2% and 5% are said to be moderately hemolytic, while those with a ratio of over 5% are said to be hemolytic. Thus, the less than 3% ratio of

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the *Punica granatum* coating makes it moderately hemolytic. However, this does not mean that the coating does not work well with blood-based biomedical applications, as confirmed in previous studies(24).
This can be said to be a better performance compared to that of previous studies. For example, Kaiser et al. reported that the hemolysis rates for pristine PU, PUChCo, and PUChCoHp patches were 3.00%, 2.61%, and 2.33%, respectively. Among these materials, the one coated with heparin was the most compatible and thus the most appropriate for the creation of cardiac patches. Wang et al.(25)

5. Conclusion:

The results obtained in the present study clearly indicate that the ethanolic extract of *Punica granatum* has dual functional therapeutic potential with potent broad-spectrum antimicrobial activity as well as hemocompatibility. Large zones of inhibition against *Staphylococcus aureus* and *Candida albicans* attest to the efficacy of the bioactive constituents of the ethanolic extract of *Punica granatum*, such as tannins and flavonoids, in inhibiting the growth of the causative agents of disease and thus present a promising lead in the search for drugs against antibiotic-resistant microorganisms. *Punica granatum* thus presents itself as a promising lead in the search for drugs against antibiotic-resistant microorganisms as an adjuvant or an alternative to conventional antimicrobial drugs in the development of infection-resistant biocompatible technologies.

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CONFLICT OF INTEREST: Nil

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