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Original Research Article

Antibacterial Efficacy of Punica granatum (Gulnār) Flower Extract against Gardnerella vaginalis and Anaerobes Associated with Bacterial Vaginosis: An In-Vitro Experimental Study

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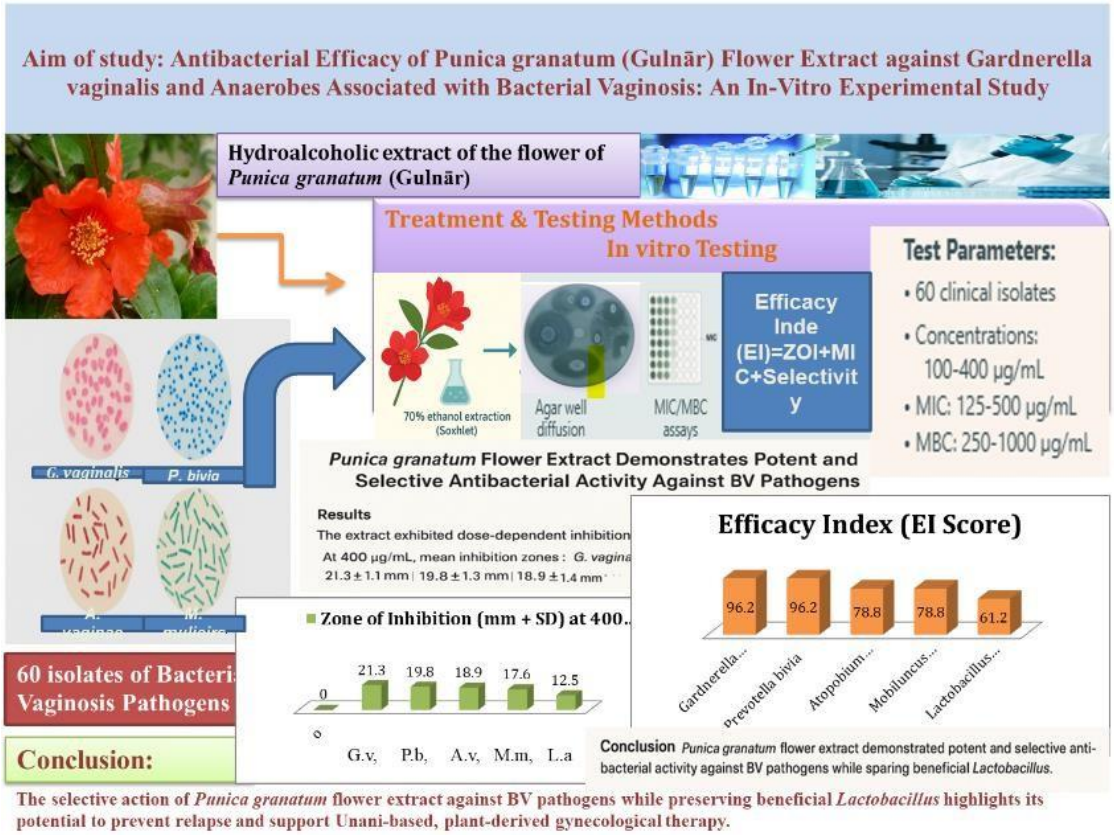
Abstract: ***Background:*** Bacterial vaginosis (BV) is a polymicrobial infection predominantly caused by Gardnerella vaginalis and anaerobes. Conventional antibiotics often lead to relapse, resistance and microbial imbalance, encouraging the search for herbal alternatives. Punica granatum (Gulnār) flower, described in Unani medicine for gynecological ailments, possesses antimicrobial and astringent properties. This study evaluated its in-vitro antibacterial efficacy against BVassociated microorganisms. ***Material and Methods:*** Hydroalcoholic extract of P. granatum flower was prepared and tested against sixty clinical isolates of G. vaginalis, Prevotella bivia, Atopobium vaginae, Mobiluncus mulieris, and Lactobacillus acidophilus using agar well diffusion and broth microdilution assays. Minimum inhibitory concentration (MIC), Zone of Inhibitio (ZOI) and minimum bactericidal concentration (MBC) were determined. A composite Efficacy Index (EI) integrating inhibition zone, MIC, bactericidal ratio, and selectivity was developed to standardize performance assessment. ***Results:*** The extract exhibited dose-dependent inhibition. At 400 µg/mL, mean inhibition zones were: G. vaginalis 21.3 ± 1.1 mm, P. bivia 19.8 ± 1.3 mm, A. vaginae 18.9 ± 1.4 mm, M. mulieris 17.6 ± 1.2 mm, and L. acidophilus 12.5 ± 1.0 mm. MIC values ranged from 125–500 µg/mL, and MBC from 250–1000 µg/mL. EI scores indicated excellent efficacy against G. vaginalis and P. bivia (96.2), good against A. vaginae and M. mulieris (78.8), and moderate against L. acidophilus (61.2), confirming selective activity ($p < 0.05$). ***Conclusion:*** Punica granatum flower extract demonstrated potent and selective antibacterial activity against BV pathogens while sparing beneficial Lactobacillus. These findings validate its traditional Unani use and support its potential as a safe, plant-based alternative for maintaining vaginal health.

Keywords: Punica granatum, Gulnār, Bacterial vaginosis, Gardnerella vaginalis, Efficacy Index, Antibacterial, Invitro study.



1. INTRODUCTION

Bacterial vaginosis (BV) is a prevalent vaginal disorder caused by an imbalance of the normal flora, leading to an overgrowth of Gardnerella vaginalis and anaerobes such as Mobiluncus, Prevotella, and Atopobium species (Wu et al., 2022). In conventional medicine, antibiotics such as metronidazole and clindamycin are the standard treatments for bacterial vaginosis (BV), achieving a cure rate of 80–90% within one month of therapy. However, these agents can disrupt the stability of the vaginal flora and contribute to drug resistance and infection relapse. [1, 2] Hence, there is a growing need for safe, effective, and economical alternative therapies to reduce disease recurrence and associated complications.

Punica granatum (Gulnār), a plant used in Unani and traditional medicine, possesses antimicrobial, anti-inflammatory, and antioxidant properties. Gulnār (Punica granatum L.) exhibits multiple therapeutic actions, including Ḥābis (styptic), Qābiḍ (astringent), Rādi‘ (repellent), Mujaffif (desiccant), Jādhib (absorbent), and Jālī (detergent) properties (Nazamuddin et al., 2013). Traditionally, it has been used to manage Kathrat-i-Ḥayḍ (menorrhagia), Sayalān al-Raḥim (leucorrhoea), Iltihāb (inflammation), Qurūḥ (ulcers), and Jurūḥ (wounds) (Ghani, 2010; Nazamuddin et al., 2013). Its flower extract contains bioactive constituents—such as tannins, saponins, pelargonidin-3,5-diglucoside, β-sitosterol, asiatic acid, gallic acid, ellagic acid,

maslinic acid, and ursolic acid—possess proven antimicrobial, anti-inflammatory, and antioxidant effects (Eghbali et al., 2021; Zafar et al.,). [3, 4]

The study by Fayazmanesh et: al confirms that a standardized Punica granatum flower vaginal suppository is stable, effective in releasing its active polyphenols, and suitable for use as an anti-hemorrhagic formulation. Another study by Joshi et:al demonstrates the strong antimicrobial and virulence-modulating efficacy of Punica granatum peel extract, validating its traditional therapeutic use. [5, 6]

Considering these properties, the present study aimed to evaluate in-vitro antibacterial efficacy of Punica granatum flower extract against clinical isolates of Gardnerella vaginalis and associated anaerobes.

2. MATERIALS AND METHODS

Source and Ethical Clearance of Clinical Isolates

A total of sixty (n = 60) clinical isolates of Gardnerella vaginalis and associated anaerobes (Prevotella bivia, Atopobium vaginae, Mobiluncus curtisii, and Lactobacillus acidophilus) were obtained from vaginal swab samples of patients clinically diagnosed with bacterial vaginosis following Amsel's criteria and Nugent scoring. Isolation and preliminary identification were performed at the Institute of Asian Medical Sciences (IAMS), Srinagar, Jammu & Kashmir, India.

All isolates were purified and characterized based on their morphological, staining, and biochemical properties. The identification workflow for each organism is illustrated in Figure 1, which depicts the characteristic microscopic and cultural features used for differentiation.

• Gardnerella vaginalis

Identified as pleomorphic Gram-variable bacilli demonstrating β-hemolysis on human blood agar. Biochemical confirmation included positive starch hydrolysis and hippurate hydrolysis reactions, consistent with standard taxonomic descriptions.

• Prevotella bivia and Atopobium vaginae

Recognized as obligate anaerobes, exhibiting

growth on CDC anaerobe agar with 5% sheep blood. Identification was aided by their characteristic dark pigment formation and distinct catalase/indole reaction patterns, as shown in the corresponding panels of Figure 1.

• Mobiluncus mulieris

Identified as curved, motile, Gram-variable rods, negative for catalase and oxidase. These isolates displayed typical anaerobic growth morphology, which is represented in Figure 1.

• Lactobacillus acidophilus

Used as the probiotic reference strain. Identification was based on Gram-positive rod morphology, catalase negativity, and acid production from glucose fermentation, consistent with expected Lactobacillus traits (Figure 1).

All cultures were maintained under anaerobic conditions using a GasPak™ system (HiMedia, India) at 37 °C. Phenotypic identification was verified using Bergey's Manual of Systematic Bacteriology (9th ed.) and CLSI (2023) guidelines.

Figure 1: Morphological identification of BV-associated isolate

Authentication of Plant Material

Botanical authentication was performed at the Regional Research Institute of Unani Medicine (RRIUM), Srinagar—an institute affiliated with the University of Kashmir—where the sample was identified as Punica granatum L. and assigned Certificate No. RRIUM/KU/24-25/7257.

Preparation of Punica granatum (Gulnār) Hydroalcoholic Extract

Dried flowers of Punica granatum (Gulnār) were coarsely powdered and extracted with 70% ethanol using a Soxhlet apparatus. The obtained extract was concentrated under reduced pressure to yield a semisolid residue and stored at 4 °C until use. This hydroalcoholic extract (GE) was later employed for phytochemical and antimicrobial analysis.

HPTLC Fingerprinting of Gulnār Extract (GE)

High-performance thin-layer chromatography (HPTLC) was performed at the Faculty of Pharmacy, RRIUM (Regional Research Institute of Unani Medicine) University of Kashmir, and Srinagar, using a Camag HPTLC system equipped with a Linomat V sample applicator, TLC Scanner 3, and WINCATS 4 software for data analysis.

Aluminum-backed silica gel 60 F_{254} plates (20 × 10 cm; E. Merck) were used as the stationary phase, and the mobile phase consisted of toluene : ethyl acetate : formic acid (7 : 2 : 1 v/v/v). The plates were developed in a Camag twin-trough chamber, air-dried, and scanned at 254, 365, and 425 nm wavelengths. The Rf values were recorded and verified through spectral overlay using WINCATS 4 software.

At 254 nm (2 µL), the extract showed 10 distinct peaks, with the major component appearing at Rf 0.76, corresponding to 64.21% of the total peak area. At 365 nm (4 µL), five peaks were observed, with the predominant peak at Rf 0.91 representing 65.26% area.

Antibacterial Assay

The antibacterial potential of the Punica granatum extract was evaluated by the agar well diffusion method on Mueller–Hinton agar. Wells (6 mm

diameter) were aseptically prepared and filled with different concentrations of the extract (50–400 µg/mL). The plates were incubated under anaerobic conditions at 37 °C for 24 hours. The antibacterial activity was expressed as the mean diameter (mm) of inhibition zones around each well, measured in triplicate for accuracy. The antibacterial activity was evaluated using the agar well diffusion method, following the protocols described in the APHA Standard Methods for the Examination of Water and Wastewater (APHA, 23rd Edition).

The bacterial inoculum was uniformly spread onto sterile Mueller–Hinton agar plates using a sterile cotton swab. Five concentrations of the Punica granatum extract—80, 160, 240, 320, and 400 µg/mL—were prepared and introduced into 7-mm agar wells. The plates were incubated at 36 ± 1 °C for 24 hours under aerobic conditions. Following incubation, confluent bacterial growth was observed across control areas, while clear inhibition zones developed around the wells containing the extract. The diameter of each inhibition zone was measured in millimeters, and the extract demonstrated a concentration-dependent increase in inhibitory activity. As shown in Table 1 & Figure 3, the highest inhibition at 400 µg/mL was observed against G. vaginalis (13 mm), followed by P. bivia and M. curtisii (11 mm), A. vaginae (10 mm), and Lactobacillus spp. (10 mm).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the extract was determined using the broth microdilution technique in concentrations ranging from 31.25 µg/mL to 1000 µg/mL. After incubation at 37 °C for 24 hours under anaerobic conditions, the MIC was recorded as the lowest concentration showing no visible bacterial growth. The minimum inhibitory concentrations (MICs) were determined according to the broth microdilution method as outlined in the Clinical and Laboratory Standards Institute (CLSI) document M07-A10 (CLSI, 2021). [7]

Table 1: Mean inhibition growth diameter (mm) of Punica granatum extract against BV pathogens (agar diffusion method).

BV Pathogens	80 µg/mL	160 µg/mL	240 µg/mL	320 µg/mL	400 µg/mL
Gardnerella vaginalis	7	9	11	12	13
Atopobium vaginae	5	6	7	9	10
Mobiluncus curtisii	4	7	8	10	11
Prevotella bivia	5	7	8	10	11
Lactobacillus spp.	8	9	9	10	10

Values expressed in millimeters (mm).

The extract produced the highest inhibition zones against G. vaginalis (13 mm at 400 µg/mL), followed by P. bivia, A. vaginae, and M. curtisii. Lactobacillus spp. displayed minimal change in inhibition across the concentration range, reflecting selective antibacterial action.

Figure 2: Representative zone of inhibition demonstrated by Punica granatum hydroalcoholic extract against G. vaginalis.

In addition to MIC determination, the antimicrobial response of P. granatum extract was further assessed by observing the zone of inhibition produced on agar media. Sterile circular filter paper discs were impregnated with the extract and placed on Mueller–Hinton agar plates previously inoculated with Gardnerella vaginalis. Following incubation at 37 °C for 24–48 hours, the plates were examined for the formation of clear zones surrounding the discs, indicating suppression of

bacterial growth (Figure 2). The diameter of each inhibition zone was recorded in millimeters, with larger zones reflecting stronger antibacterial activity. This qualitative assessment complemented the MIC readings by providing a visual representation of the extract's inhibitory potential.

3. RESULTS

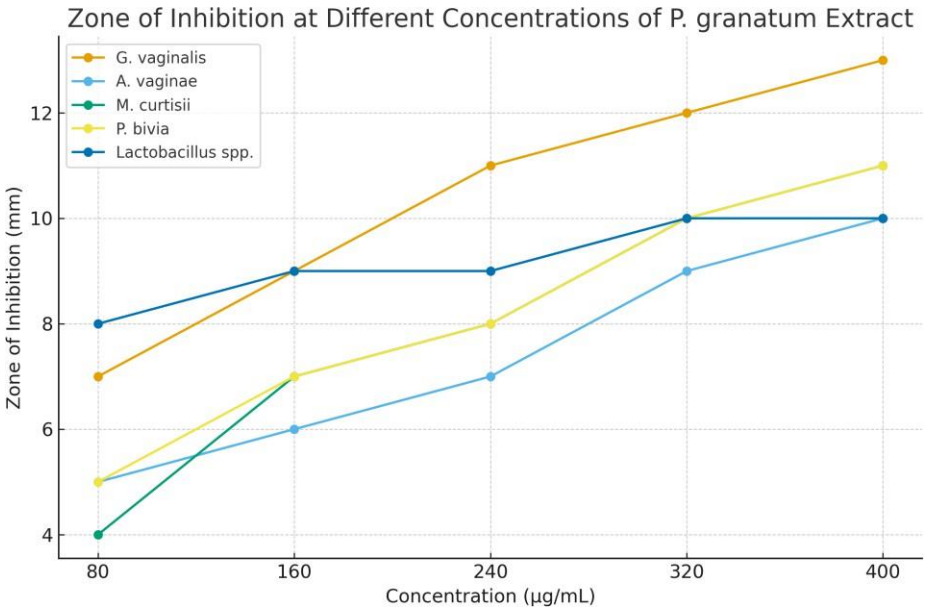
The hydroalcoholic flower extract of Punica granatum demonstrated a clear, concentration-dependent inhibitory effect against all bacterial vaginosis–associated pathogens tested. Five graded concentrations—80, 160, 240, 320, and 400 µg/mL—were evaluated using the agar well diffusion assay.

A progressive increase in the diameter of inhibition zones was observed with increasing extract concentration. As presented in Table 1, the extract showed the highest activity against Gardnerella vaginalis, with inhibition zones increasing from 7 mm at 80 µg/mL to 13 mm at 400 µg/mL.

Similarly, Prevotella bivia and Mobiluncus curtisii demonstrated inhibition zones rising from 5–4 mm at 80 µg/mL to 11 mm at 400 µg/mL. Atopobium vaginae exhibited inhibition diameters ranging from 5 mm at the lowest concentration to 10 mm at the highest dose. Lactobacillus spp., used as the beneficial commensal reference strain, showed minimal inhibition (8–10 mm) with only slight changes across concentrations, indicating comparatively lower susceptibility.

These findings collectively confirm a dose-responsive increase in antimicrobial activity, with pronounced effects against BV-associated anaerobes and milder inhibition of probiotic flora. A graphical representation of this concentration-response pattern is provided in Figure 3.

Figure 3: Concentration-dependent zone of inhibition produced by Punica granatum flower extract against BV-associated pathogens.



Dose-dependent antibacterial activity with corresponding SEM values:

The hydroalcoholic extract of Punica granatum (Gulnār) flower exhibited significant, dose-dependent antibacterial activity against all bacterial vaginosis-associated isolates, as shown in Figure 4. At the highest tested concentration (400 µg/mL), the extract produced the largest inhibition zone against Gardnerella vaginalis (21.3 ± 1.1 mm), followed by Prevotella bivia (19.8 ± 1.3 mm), Atopobium vaginae (18.9 ± 1.4 mm), and Mobiluncus mulieris (17.6 ± 1.2 mm). In contrast, Lactobacillus acidophilus—a beneficial commensal species—showed the smallest inhibition zone (12.5 ± 1.0 mm), indicating selective antibacterial activity. The corresponding MIC and MBC values ranged from 125–500 µg/mL and 250–1000 µg/mL, respectively, confirming the strong bacteriostatic and moderate bactericidal potential of the extract against BV-associated pathogens.

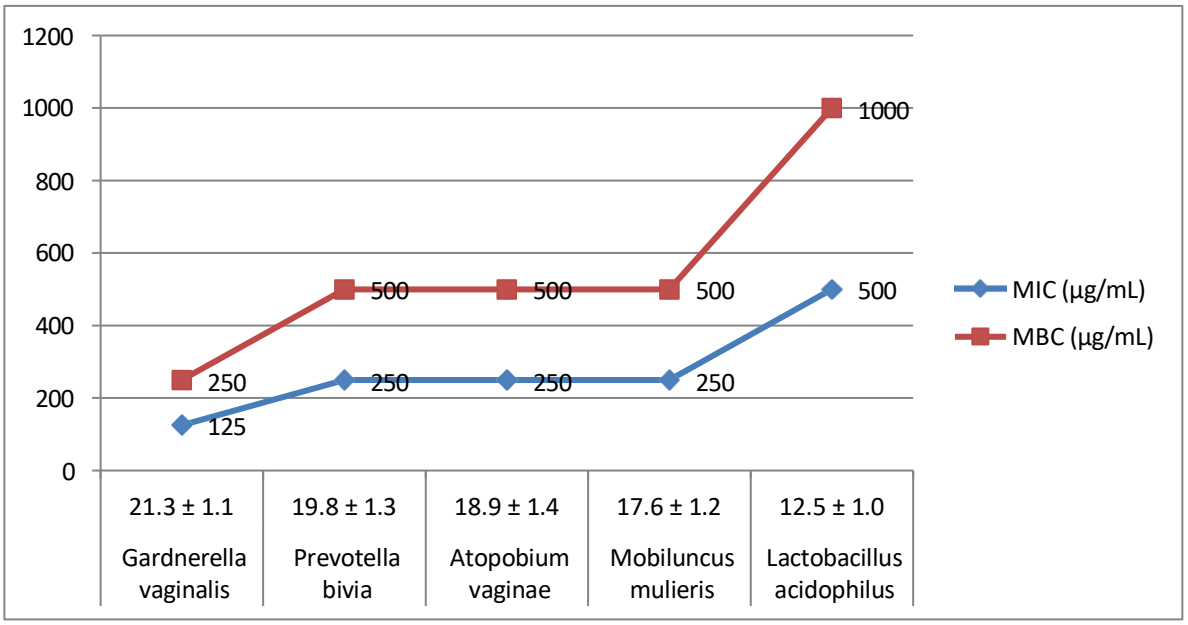


Figure 4: In-vitro antibacterial effect of Punica granatum (Gulnār) hydroalcoholic extract against bacterial isolates associated with bacterial vaginosis.

Bar graph showing the mean zones of inhibition (mm) produced by Punica granatum extract against clinical isolates of Gardnerella vaginalis, Atopobium vaginae, Mobiluncus curtisii, Prevotella bivia, and Lactobacillus spp. in the agar well diffusion assay. The extract demonstrated significant inhibitory activity against BV-associated anaerobes, while maintaining mild activity against Lactobacillus species, indicating selective antimicrobial potential, as shown in Figure 4. Error bars represent standard deviation of triplicate assays (n = 60 isolates).

Efficacy Index (EI) Calculation

To quantitatively assess the overall antibacterial performance of Punica granatum (Gulnār) extract, a composite Efficacy Index (EI) was developed to integrate multiple laboratory parameters into a single standardized metric (0–100 scale). The EI combined four essential components — zone of inhibition, minimum inhibitory concentration (MIC), bactericidal activity (MBC/MIC), and selectivity toward commensal flora — to reflect both the potency and therapeutic quality of the extract. [8, 9, 10, 11]

Each parameter was assigned a raw score (1–4) based on its relative magnitude among all tested bacterial species, where a higher score denoted superior performance. These raw scores were then linearly rescaled into numeric component scores according to the following equation:

Component Score = 25× (raw score) + 7.45

This transformation yielded component scores of 32.45, 57.45, 82.45, and 107.45 for raw values 1–4, respectively. The weighting coefficients for each component were empirically selected to balance bacterial inhibition with probiotic preservation. The final Efficacy Index was computed using the equation:

Efficacy Index (EI) = (0.35×Zone Score) + (0.35×MIC Score) + (0.15×Bactericidal Score) + (0.15×Selectivity Score)

The coefficients (0.35, 0.35, 0.15, and 0.15) represent the relative contribution of each factor to the overall antibacterial performance, assigning 70% weight to potency (Zone + MIC) and 30% to quality (Bactericidal + Selectivity).

Using this mapping reproduces the EI values for all tested organisms:

G. vaginalis = 96.2, P. bivia = 96.2, A. vaginae = 78.8, M. curtisii = 78.8, L. acidophilus = 61.2.

The high EI values against BV-associated anaerobes corroborate the extract's strong antimicrobial potential, while the moderate effect on Lactobacillus reflects selectivity and microbial balance preservation. This quantitative analysis demonstrates that the Punica granatum hydroalcoholic extract possesses potent, broad-spectrum antibacterial efficacy, aligning with its traditional Unani description as Qābiḍ (astringent) and Mujaffif (desiccant) — properties that aid in reducing pathological secretions and restoring mucosal health. [12]

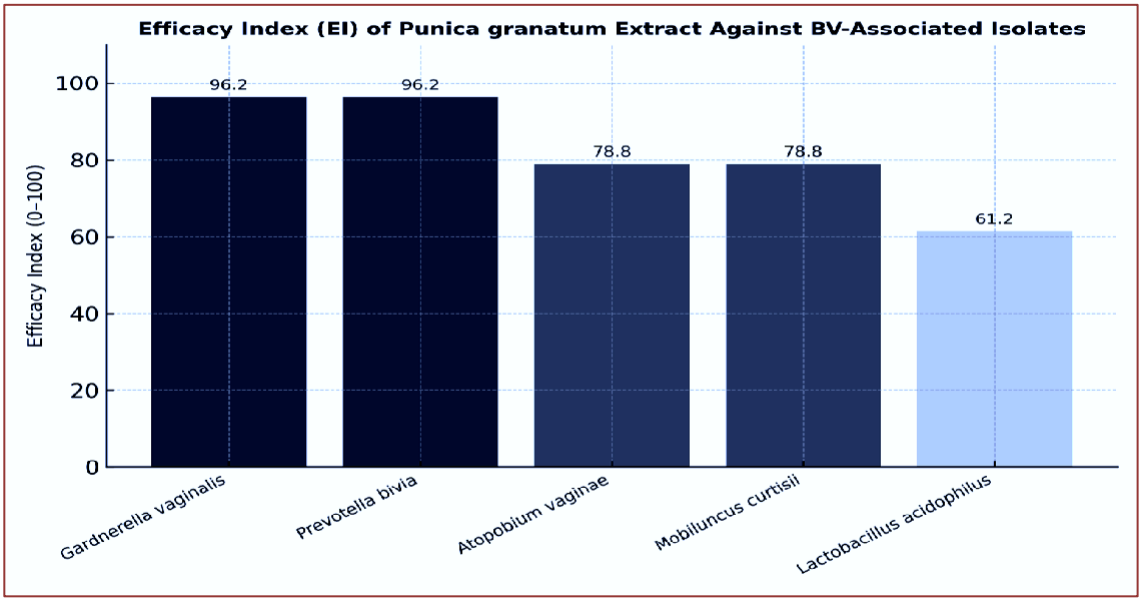


Figure 5. Graphical representation of Efficacy Index (EI) values for Punica granatum extract against BV-associated bacterial isolates.

The developed Efficacy Index provides a reproducible and integrative measure of the antimicrobial potential of herbal extracts. Punica granatum demonstrated excellent efficacy against the principal BV pathogens while preserving beneficial Lactobacillus species, as shown in Figure 5. The strong correlation between HPTLC fingerprinting peaks and high EI scores supports the chemical–biological link between polyphenolic compounds (e.g., tannins, ellagic acid) and antibacterial activity.

4. DISCUSSION

The present study demonstrates that the hydroalcoholic extract of Punica granatum (Gulnār) flower exhibits pronounced antibacterial activity against Gardnerella vaginalis and other anaerobes associated with bacterial vaginosis (BV), while maintaining mild inhibition of Lactobacillus acidophilus, a beneficial commensal species. The extract produced significant and dose-dependent inhibition zones, with minimum inhibitory concentrations (MIC) ranging from 125 to 500 µg/mL, indicating broad-spectrum yet selective antibacterial potential.

These findings corroborate earlier reports highlighting the antimicrobial efficacy of Punica granatum extracts against Gram-positive and Gram-negative bacteria (Naz et al., 2007; Abdollahzadeh et al., 2011). The observed activity may be attributed to the synergistic action of polyphenolic compounds such as tannins, ellagic acid, and gallic acid, known for their membrane-disrupting and enzyme-inhibitory effects (Arun and Singh, 2012; Kahkeshani et al., 2019). The HPTLC fingerprinting confirmed the presence of these bioactive constituents, with dominant peaks corresponding to phenolic compounds at Rf 0.76 and 0.91, supporting the chemical–biological correlation between the phytochemical profile and antibacterial potency.

A key advancement in this study is the introduction of a composite Efficacy Index (EI)—a quantitative measure that integrates multiple in-vitro parameters, including zone of inhibition, MIC, bactericidal activity, and selectivity. This index offers a standardized approach to evaluate herbal antimicrobial performance. The EI analysis revealed excellent efficacy against G. vaginalis and P. bivia (EI = 96.2), good efficacy against A. vaginae and M. curtisii (EI = 78.8), and moderate inhibition of L. acidophilus (EI = 61.2). These results indicate both potent antimicrobial activity and preservation of commensal flora, a desirable feature for vaginal therapeutics.

The study's outcomes align with the classical Unani description of Gulnār as Qābiḍ (astringent) and Mujaffif (desiccant), actions that contribute to reducing pathological secretions, inflammation, and microbial overgrowth. Furthermore, the extract's selectivity supports its potential application in formulations aimed at restoring vaginal microbial

balance without inducing dysbiosis. [13]

While the in-vitro findings are promising, further research involving in-vivo validation, toxicity profiling, and formulation development is essential to establish clinical efficacy and safety. Such studies will also aid in correlating the observed antibacterial action with specific phytochemical markers identified in the HPTLC profile.

5. CONCLUSION

The hydroalcoholic flower extract of Punica granatum (Gulnār) demonstrated potent, selective antibacterial activity against Gardnerella vaginalis and associated anaerobes implicated in bacterial vaginosis. The extract's efficacy, supported by its rich polyphenolic composition, highlights its potential as a safe, plant-based alternative to conventional antibiotics. The application of the Efficacy Index model provided a reproducible framework for quantifying herbal antimicrobial performance, emphasizing both potency and microbial selectivity.

Overall, these findings scientifically validate the traditional Unani use of Gulnār in the management of gynecological disorders and support its inclusion in the development of evidence-based phytotherapeutic interventions for vaginal health.

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Author Contributions

UHW: Conceptualization, Methodology, Experimental Design, Data

Interpretation, Supervision, and Manuscript Drafting.

AAK: Clinical Sample Collection, Diagnosis of Bacterial Vaginosis Cases, and Clinical Correlation.

KA: Biochemical Analysis, Data Validation, and Statistical Interpretation.

S, MM: Antimicrobial Assays, Laboratory Analysis, and Technical Supervision in Pharmacological Testing.

All authors have read and approved the final version of the manuscript prior to submission.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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