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GC–MS-Based Phytochemical Profiling, Antioxidant, and Antimicrobial Evaluation of Uterotibb: Scientific Validation of a Traditional Unani Polyherbal Formulation

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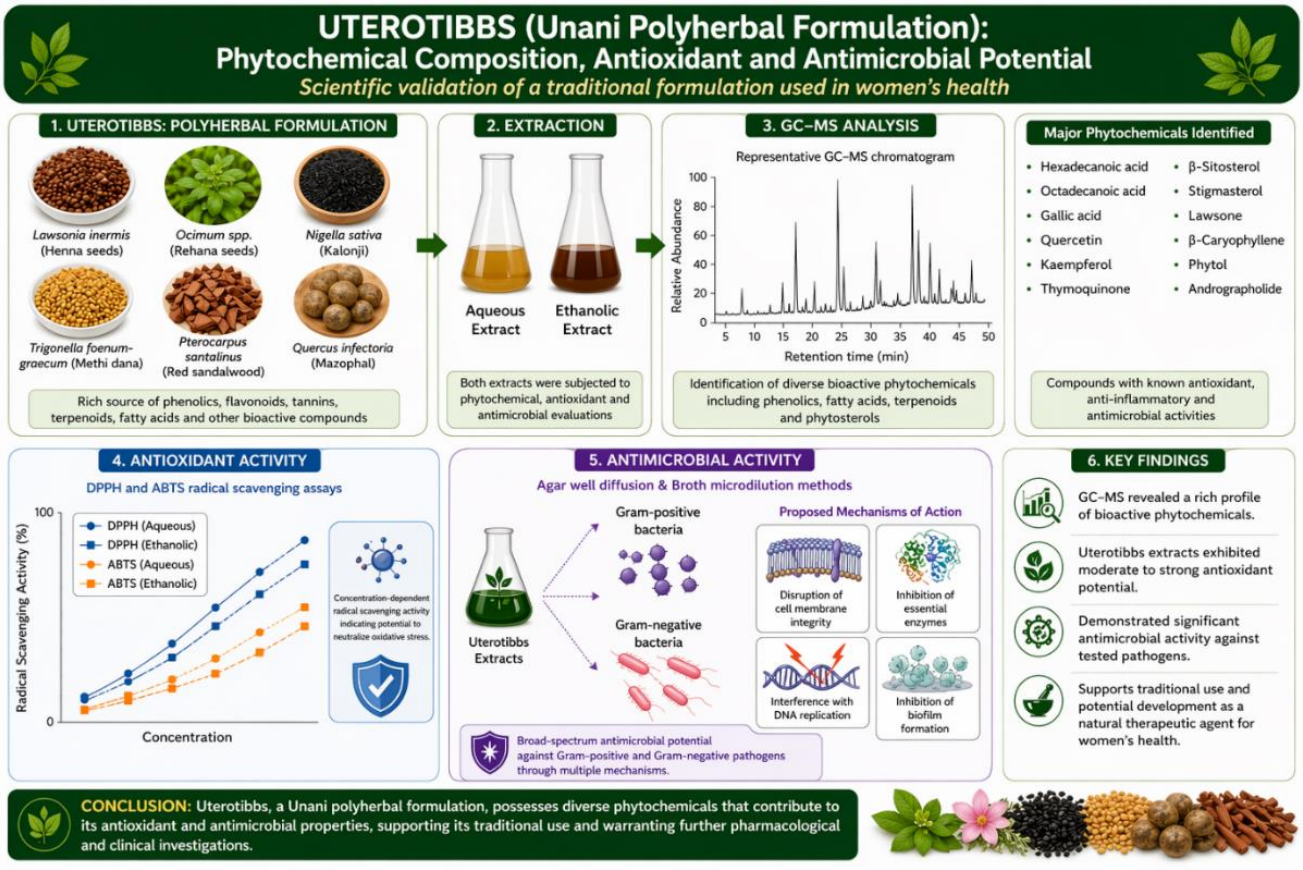
Abstract



Graphical Abstract

Uterotibb is a traditional Unani polyherbal formulation used in women's healthcare. Despite its longstanding traditional use, systematic evidence regarding its phytochemical composition and biological activities remains limited. The current study aimed to characterize the phytochemical constituents of Uterotibb and evaluate the antioxidant and antimicrobial activities of its aqueous and ethanolic extracts to provide scientific support for its traditional use. Aqueous and ethanolic extracts

were subjected to gas chromatography–mass spectrometry (GC–MS) for phytochemical profiling. Antioxidant activity was evaluated using DPPH and ABTS radical-scavenging assays, while antimicrobial activity was assessed against selected Gram-positive and Gram-negative bacterial strains. IC_{50} values and minimum inhibitory concentrations (MICs) were determined. GC–MS identified phenolic derivatives, lipophilic phenolics, fatty acid esters including hexadecanoic acid, terpenoids, aromatic compounds, quinone-related structures, and phytosterols. The ethanolic extract showed 95.7 ± 0.91% scavenging potential at 1.1 mg/mL concentration, where IC_{50} values of 0.68 mg/mL for DPPH and 1.07 mg/mL for ABTS. The aqueous extract showed 86.86 ± 0.30% scavenging at 1.1 mg/mL concentration, with IC_{50} values of 0.821 and 1.133 mg/mL for DPPH and ABTS respectively. Both extracts exhibited an MIC of 1 mg/mL against all tested Microorganisms. The ethanolic extract lowest IC_{50} values against Gram-positive and Gram-negative bacteria were 0.38 and 0.52 mg/mL, respectively, compared with1.62 and 2.15mg/mL for the aqueous extract against tested bacterial strains. The findings support the traditional use of Uterotibb and highlight its potential as a source of bioactive phytochemicals warranting further pharmacological investigation.

Keywords: Uterotibb; Medicinal polyherbal formulation; GC–MS analysis; Antioxidant activity; Antimicrobial activity; Phytochemical profiling.

INTRODUCTION

Unani medicine, rooted in the Greco-Arabic medical tradition, employs complex herbal preparations within a holistic therapeutic framework that includes the management of conditions affecting female reproductive health, including uterine function, menstrual regulation, and abnormal uterine discharge. Previous work on Uterotibb has primarily examined its aqueous alkaline extract, reporting GC–MS-detected constituents together with antioxidant and antimicrobial activities (Aziz et al., 2025). However, comparative evidence for conventionally prepared aqueous and ethanolic extracts of the intact formulation, particularly with respect to quantitative DPPH and ABTS IC_{50} values and antimicrobial dose–response characteristics, remains limited.

The combination of these botanicals is consistent with the complementary therapeutic attributes traditionally ascribed to the individual ingredients, including the astringent and haemostatic properties of oak galls, the antioxidant and anti-inflammatory potential of henna and red sandalwood, the tonic and uterine-supportive roles traditionally associated with fenugreek and nigella, and the demulcent or emmenagogue applications of basil. Such a multi-component formulation reflects the Unani concept of achieving therapeutic balance through combinations of medicinal substances rather than dependence on a single active constituent. Experimental evidence from individual ingredients and composite herbal preparations provides biological plausibility for some of these traditional applications; nevertheless, systematic evidence concerning the intact Uterotibb formulation remains limited, particularly in clinical settings (Table 1 & 2) (Aziz et al. 2025; Batiha et al. 2023; Mohammadi *et al.,* 2024). Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) generation and antioxidant defence, is implicated in several reproductive and gynecological disorders. Plant-derived antioxidants can counter oxidative processes through hydrogen-atom transfer and electron-transfer mechanisms, thereby limiting radical-mediated molecular damage (Azizah *et al.,* 2023; Muheyuddeen, 2023). Uterotibb may therefore possess biological activity arising from the combined contribution of redox-active constituents originating from its different botanical components. Its antimicrobial potential is similarly of interest because several constituent plants contain compounds active against both Gram-positive and Gram-negative bacteria. Hydrolysable tannins from Quercus infectoria have been associated with protein precipitation, membrane effects, and enzyme inhibition, whereas naphthoquinones such as lawsone from Lawsonia inermis have demonstrated broad antibacterial activity 9-10 (Srivastava, 2025; Falakdin *et al.,* 2023). Nigella sativa and thymoquinone have also been reported to affect microbial membranes, biofilm formation, and oxidative processes 11 (Akhtari *et al.,* 2024), while Ocimum spp. and Pterocarpus santalinus contain phenolic and flavonoid constituents with reported antibacterial properties 12-13 (Prakash P and Gupta N, 2005; Manjunatha, 2006). Collectively, these observations provide a rationale for investigating Uterotibb as a complex phytochemical preparation. Accordingly, the present study comparatively evaluates the phytochemical composition and biological activities of its aqueous and ethanolic extracts, with particular emphasis on quantitative antioxidant and antimicrobial responses. The combined presence of these phytochemicals suggests a potential synergistic antimicrobial effect that can target diverse microbial classes.

Accordingly, this study aimed to characterize the phytochemical composition of Uterotibb formulations using gas chromatography–mass

spectrometry (GC–MS) and to comparatively evaluate the phytochemical composition and biological activities antioxidant activities of its aqueous and ethanolic extracts using DPPH and ABTS radical-scavenging assays, together with their antimicrobial activities against selected pathogenic microorganisms. By integrating chemical profiling with quantitative antioxidant and antimicrobial assessment, the study seeks to provide experimental evidence relevant to the traditional use of Uterotibb and establish a basis for subsequent pharmacological investigation.

Table 1: Comparative Overview of Phytochemical Constituents, Biological Activities, and Existing Research Gaps in Uterotibb Ingredients

S. No.	Ingredient (common name)	Highest-quality evidence	Main findings	Key evidence gaps / safety notes	References
1.	Lawsonia inermis (henna)	Systematic / narrative reviews and multiple preclinical studies.	Reviews report antibacterial, antioxidant, anti-inflammatory activities; lawsone & tannins as major actives. Clinical trials for gynecologic use.	No RCTs for uterine/gynecologic indications; dose-toxicity data (topical vs oral) limited; potential for allergic reactions. Need: standardized extract studies, reproductive toxicology.	Batiha *et al.,* 2023; Muheyuddeen *et al.,* 2023)
2.	Ocimum spp. (basil) basil seeds / Rehana	High-quality narrative/ systematic reviews and clinical trials for non-gynecologic indications.	Antioxidant, nutritive, anti-inflammatory qualities; recent RCTs examine mood/anxiety and other outcomes; limited direct RCT evidence for uterine indications.	Direct clinical data on menstrual/uterine outcomes sparse; mechanisms plausible (mucilage, antioxidants). Need: small RCTs for dysmenorrhea/ emmenagogue claims and PK data.	Azizah *et al.,* 2023; Dahat *et al.,* 2025).
3.	Nigella sativa (kalonji)	Randomized double-blind clinical trial (missed abortion) and multiple preclinical studies.	Improved uterine evacuation outcomes when *N. sativa* oil used adjunctively in missed abortion management, anti-inflammatory, uterotonic potential (thymoquinone).	Single small RCT; safety profile in pregnancy and reproductive endpoints needs further evaluation; interactions and dosing standardization required. Need: larger multicentre RCTs, safety in pregnancy.	(Mohammadi *et al.,* 2024; Dahat *et al.,* 2025).
4.	Trigonella foenum-graecum (fenugreek, methi)	Systematic reviews and consolidated clinical reviews.	Benefits in lactation augmentation, dysmenorrhea and metabolic endpoints; strong preclinical and mechanistic data on saponins and phytoestrogens.	Safety: reproductive-toxicity signals at high dose in animal studies; variable supplement standardization. Need: dose-finding and safety trials for reproductive uses, herb-drug interaction studies.	(Faisal *et al.,* 202; Srivastava *et al.,* 2025; Akhtari *et al.,* 2024)
5.	Pterocarpus santalinus (red sandalwood, Sandal Surq)	Ethno pharmacology reviews; In vitro and animal anti-inflammatory/antioxidant studies.	Antioxidant and anti-inflammatory activity; traditional use as cooling/astringent agent. Clinical human data for uterine use: none.	Lack of clinical trials; uncertain which heartwood constituents are bioavailable orally; conservation and sourcing issues. Need: standardization, toxicity and PK studies.	Dahat *et al.,* 2025; Bulle *et al.,* 2016).
6.	Quercus infectoria (oak galls, Mazophal)	Systematic reviews and in-vitro/in-vivo antimicrobial studies.	Antimicrobial and astringent properties due to gallotannins and phenolics;	Uterine haemostasis or gynecologic outcomes is lacking; tannin-related GI effects and potential	(Amilah *et al.,* 2022; Falakdin *et al.,* 2023;

			beneficial in controlling local infections and secretions in vitro and in animal models.	interactions to be assessed. Need: controlled trials for haemostatic claims and reproductive-toxicity studies.	Kozolowka *et al.,* 2022)

Table 2: Comparison of the most bioactive phytochemical groups of Uterotibb

S. No.	Phytochemical group	Ethanolic extract	Aqueous extract	Biological relevance
1.	Phenolics	High diversity, lipophilic phenols	Simple phenols, tannins	Antimicrobial, antioxidant
2.	Naphthoquinones	Prominent	Minimal	Strong antibacterial, anti-inflammatory
3.	Fatty acid esters	Abundant	Absent or trace	Membrane disruption
4.	Tannins	Moderate	High	Astringent, antibacterial
5.	Sugars/glycosides	Low	High	Nutritional, mucosal support

MATERIALS AND METHODS

Preparation of Uterotibb Aqueous and Ethanolic Extracts

Dried Uterotibb Unani medicine powder was procured from Halal Herbal Remedies, Milath Nagar Colony, Hyderabad – 500005, Telangana State, India, holding license and registration number T584/U, Hyderabad, T.S., India. For comparative extraction, the powdered formulation was separately extracted with double-distilled water and ethanol using a 1:5 (w/v) material-to-solvent ratio. Each preparation was maintained at 25 °C for 24 h under continuous orbital agitation. Following extraction, the aqueous and ethanolic fractions were separated from the residual plant material by filtration and centrifugation. The resulting extracts were concentrated using a rotary evaporator and subsequently stored at 4 °C until further analysis (Nejhad *et al.,* 2023; Aziz *et al.,* 2025).

GC-MS Analysis of Aqueous and Ethanolic Extracts

GC–MS analysis demonstrated clear qualitative and semi-quantitative differences between the ethanolic and aqueous Uterotibb extracts, indicating solvent-dependent selectivity in the recovery of extractable constituents. The ethanolic extract displayed a broader chemical profile, with greater representation of moderately polar and non-polar secondary metabolites, whereas the aqueous extract contained a comparatively greater proportion of highly polar constituents (Do *et al.,* 2014; Azwanida, 2015; Sarker and Nahar, 2012). The ethanolic fraction contained several chemically relevant classes, including phenolic derivatives, fatty acid esters, terpenoids, aromatic compounds, and quinone-related structures. Constituents associated with Nigella sativa, including thymoquinone-related compounds, Lawsonia inermis-derived naphthoquinone-related structures, and lipophilic phenolics were among the detected compounds. These classes have been reported to possess antioxidant and antimicrobial properties. The stronger radical-scavenging response of the ethanolic extract is therefore consistent with its comparatively broader representation of redox-active and lipophilic constituents. However, GC–MS identification alone cannot establish a causal relationship between individual compounds and the measured biological activities; the observed differences should consequently be interpreted as an association between extract composition and bioactivity rather than direct evidence of compound-specific effects.

Antioxidant Activity by DPPH Assay

DPPH Free Radical Scavenging Activity

The radical-scavenging capacity of the aqueous and ethanolic Uterotibb extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay following a previously reported procedure with minor modifications (Nejhad *et al.,* 2023). Aliquots of the aqueous and ethanolic extracts, prepared at concentrations ranging from 100 to 1000 µg/mL. Subsequently, 1 mL of freshly prepared 0.2 mM DPPH solution in ethanol was added to each sample. The reaction mixtures were thoroughly vortexed and maintained in the dark at room temperature for 30 min. Standard was prepared 3 mL of DPPH and 100 L of methanol. Absorbance was subsequently recorded at 517 nm using a UV–visible spectrophotometer. All measurements were performed in triplicate. Radical-scavenging activity was calculated using the following equation (Aziz *et al.,* 2025).

$$\% \text{ of antioxidant activity} = [(Ac - As) \div Ac] \times 100$$

where **Ac** represents the absorbance of the control reaction and as represents the absorbance of the test sample. Vitamin C was used as the positive control.

2.3.2. Determination of Antioxidant Activity Using the ABTS Free Radical Scavenging Method

ABTS radical-scavenging activity was determined according to previously reported procedures

(Aziz *et al.,* 2025; Kozłowska *et al.,* 2022). The ABTS radical solution was generated by mixing ABTS (7 mM) with potassium persulfate (4.95 mM) at a 1:1 (v/v) ratio, followed by incubation overnight in the dark at room temperature. Aliquots (0.2 mL) of the aqueous and ethanolic extracts, prepared at concentrations ranging from 100 to 1000 µg/mL, were added to the assay tubes, followed by 3.9 mL of diluted ABTS solution. The decrease in absorbance was measured at 734 nm using a UV-30 spectrophotometer. An ABTS solution without extract served as the blank. The results were expressed in equivalents of quercetin per milligram of dry weight extract.

Antimicrobial Activity

Antibacterial activity was evaluated using four reference bacterial strains representing both Gram-positive and Gram-negative groups: Staphylococcus aureus ATCC 25923 and Bacillus subtilis ATCC 6051 as Gram-positive organisms, and Escherichia coli ATCC 25922 and Klebsiella pneumoniae ATCC 700603 as Gram-negative organisms. All strains were obtained from the American Type Culture Collection (ATCC), USA. Cultures were initially propagated in trypticase soy broth (TSB) at 37 °C with shaking at 210 rpm for 18 h. The cultures were subsequently subcultured on blood agar, nutrient agar, and MacConkey agar for purity and maintenance, and agar slants were prepared for experimental use. For inoculum preparation, cultures were grown in TSB to an OD600 of 0.5, corresponding to approximately 1–2 × 10^8 CFU/mL or a 0.5 McFarland standard. One-millilitre portions were centrifuged at 3400 × g for 10 min at 4 °C, and the resulting pellets were washed and resuspended in cold phosphate-buffered saline (PBS; pH 7.4). The suspensions were subsequently diluted to approximately 5 × 10^6 CFU/mL according to the method described by (Al Saiqali *et al.,* 2018).

Minimum Inhibitory (MIC)

The minimum inhibitory concentration (MIC) was determined using a broth microdilution procedure based on the National Committee for Clinical Laboratory Standards (NCCLS) methodology. A bacterial inoculum of approximately 5 × 10^6 CFU/mL was prepared, and the extracts were diluted in Mueller–Hinton broth (MHB) with DMSO, with the extract concentration tested up to 1 mg/mL. Bacterial suspensions and extract preparations were dispensed into 96-well microplates, with 125 µL of the extract preparation added to each well. The plates were incubated at 37 °C for 24 h. Following incubation, 25 µL of triphenyltetrazolium chloride (TTC; 5 mg/mL) was added to each well, and microbial growth was assessed based on the development of the characteristic dark-red colour. The lowest extract concentration at which no colour development was observed and considered the MIC. In additional step to assess residual bacterial viability, 100 µL from wells showing no colour change was transferred to Mueller–Hinton agar (MHA) and incubated at 37 °C for 24 h. The minimum concentration preventing visible growth was assessed (Aziz *et al.,* 2025; Kozłowska *et al.,* 2022). IC_{50} values were additionally calculated from the concentration–response data.

Statistical Analysis

All antioxidant experiments were performed in triplicate (n = 3) and the results are presented as mean ± standard deviation (SD). Statistical evaluation of inhibition zone diameters was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to assess differences between concentrations and extracts. The results demonstrated that the increase in inhibition zone diameter with increasing extract concentration was statistically significant ($p < 0.05$) for both ethanol and aqueous extracts across all tested bacterial strains. The same statistical methods were used to evaluate the effect of extract concentration on radical scavenging activity. Differences among the tested concentrations were considered statistically significant at $p < 0.05$. The analysis demonstrated a significant concentration-dependent increase in radical scavenging activity for both DPPH and ABTS assays ($p < 0.05$).

RESULTS AND DISCUSSION

Comparative Phytochemical Profiles of Ethanolic and Aqueous Extracts

Gas chromatography–mass spectrometry (GC–MS) analysis revealed marked qualitative and semi-quantitative differences between the ethanolic and aqueous extracts of Uterotibb, reflecting solvent-dependent extraction selectivity (Fig. 1& 2). The ethanol extract (Fig. 1) exhibited a broader phytochemical spectrum, characterized by a higher abundance of moderately polar to non-polar secondary metabolites, whereas the aqueous extract (Fig. 2) was predominantly enriched with highly polar constituents (Do *et al.,* 2014; Azwanida, 2015; Sarker and Nahar, 2012). In the ethanolic extract, several pharmacologically relevant compound classes were detected, including phenolic derivatives, fatty acid esters, terpenoids, aromatic compounds, and quinone-related structures. Notably, bioactive constituents attributable to *Nigella sativa* (thymoquinone-related compounds), *Lawsonia inermis* (naphthoquinone derivatives such as lawsone analogues), and lipophilic phenolics were prominent. These compounds are well recognised for their antimicrobial, anti-inflammatory, antioxidant, and membrane-disruptive properties, which are mechanistically consistent with the enhanced biological activity observed for ethanolic fractions of polyherbal formulations. The greater radical-scavenging capacity observed for the ethanolic extract is consistent with its enrichment in relatively lipophilic phenolic, terpenoid, quinone-related and fatty-acid-derived constituents identified by GC–MS. Although GC–MS detection alone does not establish biological causality, the differential chemical profiles provide a plausible explanation for the

stronger antioxidant response observed with ethanol extraction.

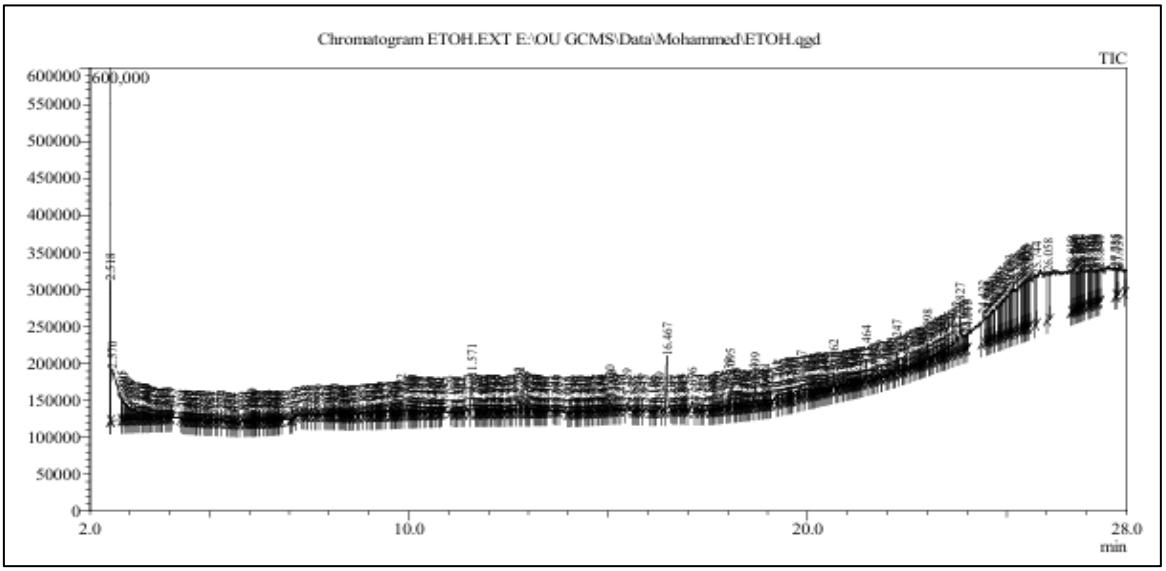


Fig. 1: Chromatogram lustrating the Phytochemical Analysis of Uterotibb Ethanolic Extract

The ethanolic extract showed higher cumulative peak areas for phenolics, quinone-related compounds and fatty acid esters, whereas the aqueous extract was predominantly enriched with tannins and glycosidic constituents (Fig. 1). This distribution reflects the physicochemical properties of the extracted compounds and agrees with previous reports on solvent-selective recovery of bioactive phytochemicals from polyherbal formulations (Azwanida, 2015; Sarker and Nahar, 2012; Dai and Mumper, 2010). In contrast, the aqueous extract displayed a narrower phytochemical profile dominated by polar molecules, including hydrolysable tannins, simple phenols, organic acids, and glycosidic constituents. These compounds are primarily associated with astringent, mucosal protective, and mild antimicrobial effects, consistent with the traditional Unani use of water-based preparations for tissue strengthening and long-term uterine support.

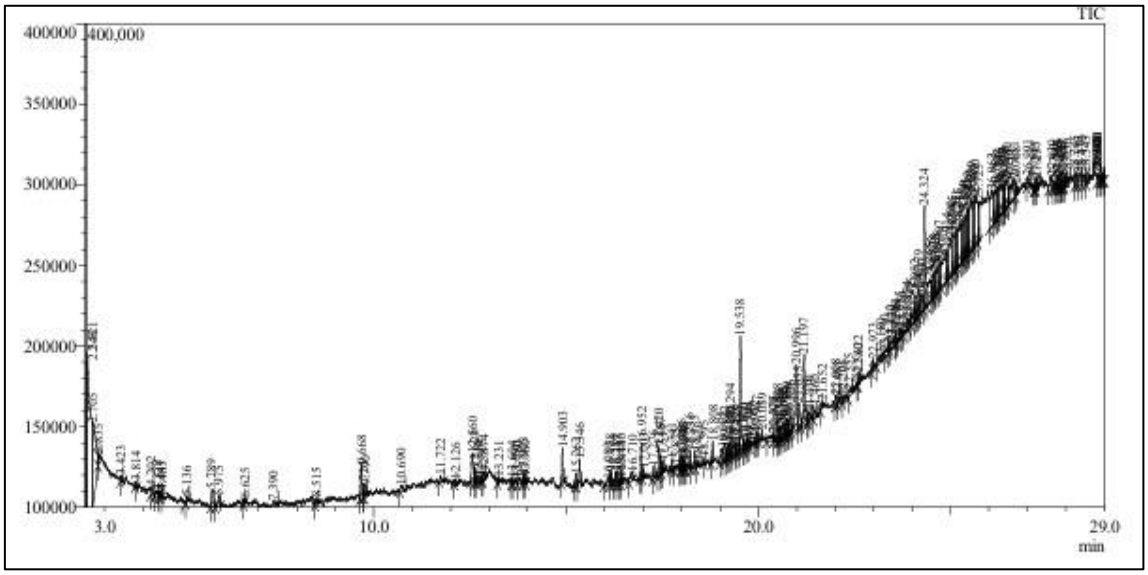


Fig. 2: Chromatogram Showing the Phytochemical Analysis of Uterotibb Aqueous Extract

Differential Bioactive Compound Distribution

Comparison of the major phytochemical groups detected in the two extracts showed a greater representation of compounds with reported antimicrobial properties in the ethanolic fraction. Lipophilic phenolics, naphthoquinone-related constituents, and fatty acid esters were detected at higher relative abundance in the ethanolic extract, whereas the aqueous fraction contained comparatively greater representation of tannin- and glycoside-related constituents (Tables 1 and 2) (Batiha *et al.,* 2020; Nair *et al.,* 2024). This solvent-dependent distribution provides a plausible chemical basis for the differences in biological activity observed between the extracts, although the contribution of

individual constituents requires confirmation through targeted chemical isolation and mechanistic studies.

Hydrolysable tannins, particularly those associated with Quercus infectoria, possess strong protein-binding properties and may contribute to the astringent and antimicrobial characteristics of the aqueous extract. However, their contribution to direct bactericidal activity may differ from that of quinone-related and lipophilic phenolic constituents. The contrasting chemical distributions between the two extracts therefore provide a plausible explanation for their different biological profiles. The relatively greater representation of lipophilic and moderately polar constituents in the ethanolic extract coincided with its stronger antimicrobial response, whereas the predominance of tannin- and glycoside-related constituents in the aqueous extract may contribute to its observed biological activity (Desbois and Smith, 2010; Villanueva *et al.,* 2023; Huang *et al.,* 2024). These associations should nevertheless be regarded as hypothesis-generating because the present study did not individually isolate or experimentally validate the activity of each detected compound.

It is important to note that GC–MS selectively detects volatile and semi-volatile, thermally stable compounds. Consequently, several phytochemical classes known to be present in Uterotibb ingredients such as alkaloids, saponins, flavonoid glycosides, polysaccharides and other high-molecular-weight constituents (Sparkman *et al.,* 2011; Niessen, 2006; Sarker and Nahar, 2012). For example, saponins from *Trigonella foenum-graecum* and mucilage polysaccharides from *Ocimum* seeds are poorly amenable to GC–MS analysis but are pharmacologically relevant for uterine and mucosal health (Wani *et al.,* 2018; Nazir *et al.,* 2021).

Integrated Pharmacological Relevance and Traditional Rationale

The solvent-dependent differences observed in the present study indicate that the aqueous and ethanolic extracts of Uterotibb are chemically and biologically distinct. The ethanolic extract showed stronger in-vitro antioxidant and antibacterial responses, which is consistent with its broader representation of GC–MS-detectable moderately polar and lipophilic constituents. These findings suggest that ethanol may recover a greater proportion of compounds contributing to the measured activities. However, the present experiments do not establish anti-inflammatory efficacy, clinical effectiveness, or long-term tolerability, and such therapeutic implications require dedicated mechanistic, toxicological, and in vivo investigations. The aqueous and ethanolic extracts should therefore be regarded as chemically differentiated preparations rather than interchangeable fractions. Their complementary phytochemical profiles provide a basis for further investigation of the contribution of hydrophilic and lipophilic constituents to the overall biological properties of Uterotibb (Table 2) (Islam *et al.,* 2025).

Antimicrobial Efficacy and Dose–response Potential of Ethanolic Extract

The antibacterial activity of the ethanolic extract of Uterotibb was evaluated against representative Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains using the agar well diffusion method (Table 3). The extract exhibited a clear dose-dependent inhibitory effect, as demonstrated by progressively larger zones of inhibition with increasing extract concentration. At the highest tested concentration (5 mg/mL), the ethanolic extract produced inhibition zones of 16 ± 0.91 mm against *S. aureus* and 17 ± 0.97 mm against *B. subtilis*, indicating substantial activity against Gram-positive bacteria. Slightly lower inhibition zones were observed against Gram-negative organisms, with 15 ± 0.68 mm for *E. coli* and 15 ± 0.84 mm for *K. pneumonia* (Table 3) (Fig. 3). The inhibitory activity decreased gradually with decreasing extract concentrations, reaching 10–11 mm at 1 mg/mL, demonstrating a consistent concentration-dependent antimicrobial response. The minimum inhibitory concentration (MIC) defined as the lowest concentration producing a measurable inhibition zone from 9±0.22 to 11±0.34 mm was 1 mg/mL for *S. aureus*, *B. subtilis*, and *K. pneumoniae*, while 1 mg/mL was required to inhibit *E. coli* (Table 5) (Fig. 5). This pattern is consistent with the well-known higher resistance of Gram-negative bacteria due to the presence of an outer lipopolysaccharide membrane that restricts penetration of antimicrobial compounds (Xu et al. 2026; Silhavy 2010).

To further characterize antimicrobial potency, half-maximal inhibitory concentrations (IC_{50}) were estimated from the concentration–response data. The IC_{50} values were calculated as the concentration required to produce approximately 50% of the maximal inhibition zone observed for each organism. The ethanolic extract exhibited IC_{50} values of 0.38 to 0.42 approximately 0.38–0.42 mg/mL for Gram-positive bacteria, indicating relatively strong antibacterial activity. Higher IC_{50} values were observed for Gram-negative bacteria is with 0.47 mg/mL for *E. coli* and 0.52 mg/mL for *K. pneumoniae* (Table 5) (Fig. 5). These findings confirm that Gram-positive organisms were more susceptible to the ethanolic extract, a phenomenon widely reported for plant-derived antimicrobial compounds (Cushnie and Lamb, 2005).

Table 3: Antimicrobial Activity of the Ethanolic Extract of Uterotibb Against Bacterial Human Pathogens

Ethanolic Extract					
S. No.	**Concentration of the extract (mg/mL)**	***Staphylococcus aureus* (mm)**	***Bacillus subtilis* (mm)**	***E. coli* (mm)**	***Klebsiella penumonae* (mm)**
1.	1 mg/mL	10±0.10 mm	11±0.34 mm	10±0.97 mm	9±0.22 mm
2.	2 mg/mL	12±0.55 mm	12±0.48 mm	11±0.44 mm	10±0.81 mm
3.	3 mg/mL	13±0.73 mm	14±0.61 mm	13±0.59 mm	11±0.39 mm
4.	4 mg/mL	15 ±0.57 mm	16±0.88 mm	14±0.71 mm	13±0.65 mm
5.	5 mg/mL	16 ± 0.91 mm	17±0.97 mm	15±0.68 mm	15±0.84 mm
6.	Streptomycin 5µg/mL	16±0.89 mm	24±0.99 mm	14±0.69 mm	15±90 mm

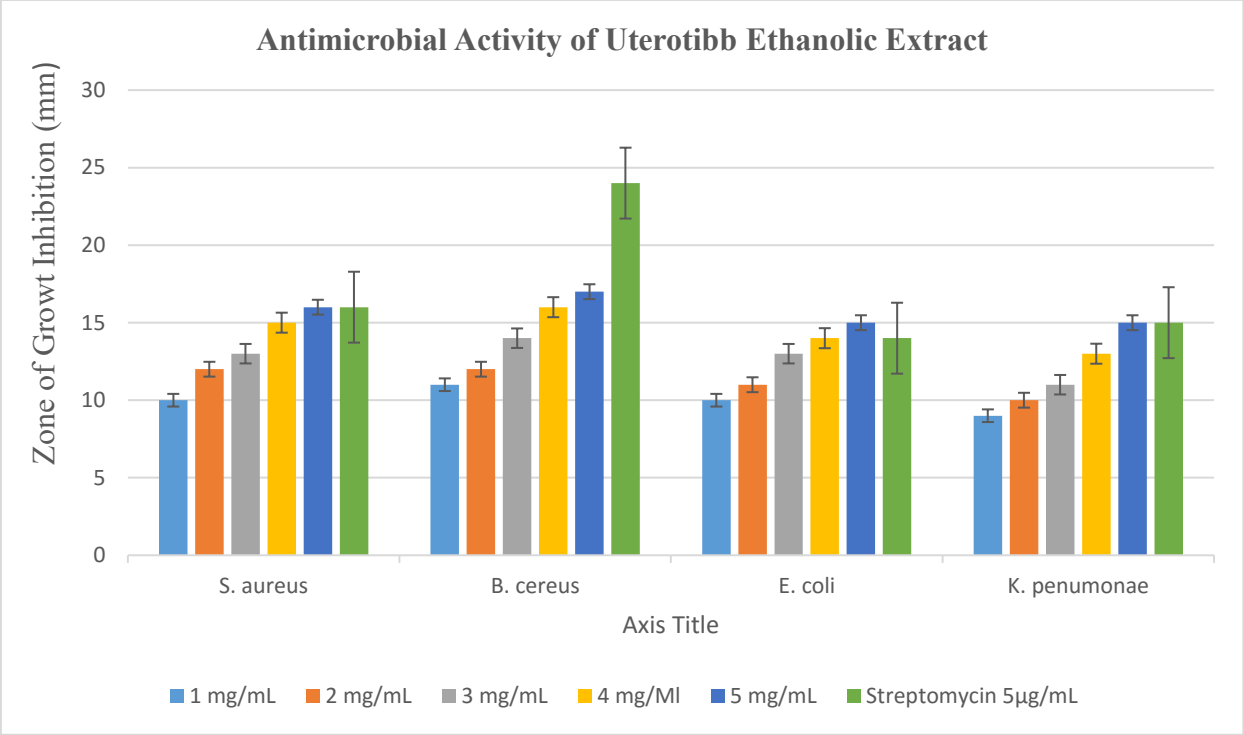


Fig. 3: Antimicrobial Activity of Uterotibb Ethanolic Extract Against Selected Human Pathogens

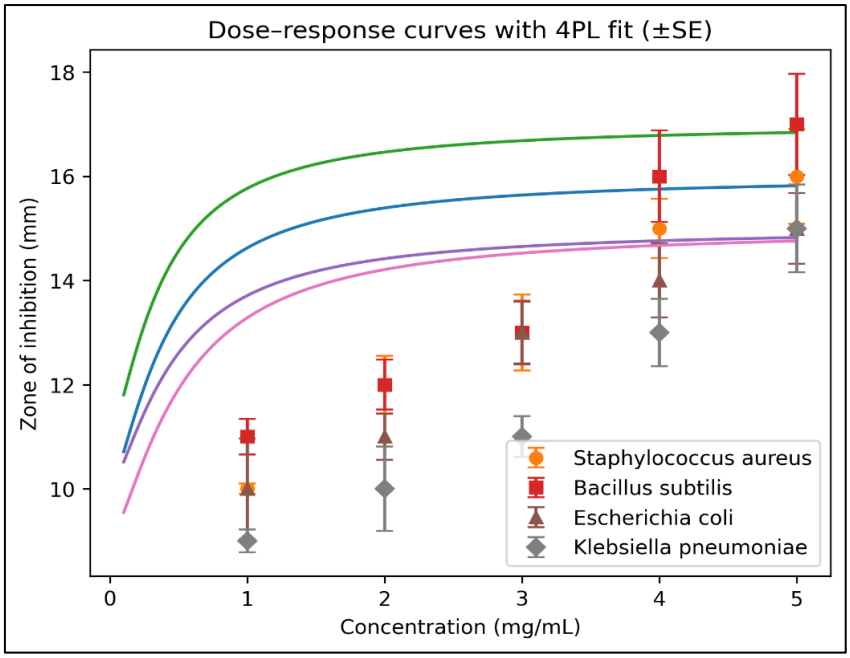


Fig. 5: Dose–Response Curves of Uterotibb Ethanolic Extract Against Bacterial Pathogens.

Data points represent mean zone of inhibition (mm) ± standard error (SE). Curves represent nonlinear regression fits (4-parameter logistic model). Distinct markers indicate different organisms.

Phytochemical Basis of Antimicrobial Activity

The antimicrobial activity of the ethanolic extract can be attributed to bioactive compounds present in the constituent medicinal plants of Uterotibb. Ethanol is particularly effective at extracting phenolic compounds, quinones, flavonoids, and fatty acid derivatives, which are well-known for their antimicrobial properties. Phenolic compounds can disrupt bacterial cell membranes, inhibit essential enzymes, and interfere with nucleic acid synthesis (Azwanida, 2015). The greater antibacterial activity observed for the ethanolic extract may be associated with the more efficient recovery of antimicrobial phytochemical classes from the constituent plants of Uterotibb. Compounds such as thymoquinone from Nigella sativa, lawsone-related constituents from Lawsonia inermis, and tannin-rich constituents of Quercus infectoria have previously been associated with antibacterial effects involving membrane perturbation, interference with microbial enzymes, and oxidative mechanisms (Batiha *et al.,* 2020; Al-Snafi *et al.,* 2019; Villanueva *et al.,* 2023). The detection of such compound classes in the ethanolic extract provides a plausible explanation for its stronger antibacterial response. Nevertheless, because the present experiments were conducted with crude extracts, the individual contribution of these constituents and any potential synergistic interactions cannot be established solely from GC–MS profiling. Overall, the results indicate that the ethanolic extract of Uterotibb possesses broad-spectrum antibacterial activity, with particularly strong effects against Gram-positive bacteria. These findings support the traditional use of this Unani formulation in conditions associated with microbial infections and inflammation and suggest that the formulation may serve as a promising source of natural antimicrobial compounds.

Antimicrobial Activity and Dose–response Relationship of the Aqueous Extract of Uterotibb

The antibacterial activity of the aqueous extract of Uterotibb was evaluated against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) using the agar well diffusion assay. The results demonstrated a clear concentration-dependent antibacterial effect**,** with inhibition zones increasing progressively as the extract concentration increased from 1 - 5 mg/mL. At the lowest tested concentration (1 mg/mL), the aqueous extract exhibited moderate antibacterial activity against Gram-positive bacteria, producing inhibition zones of 9 ± 0.40 mm against *S. aureus* and 10 ± 0.33 mm against *B. subtilis***.** In contrast, 9±0.69 mm and 8±0.23 mm inhibition zones were observed against *E. coli* and *K. pneumonia* respectively, indicating weak inhibitory effect (Table 4) (Fig. 4). This finding is consistent with previous studies showing that Gram-negative bacteria generally exhibit greater resistance to plant-derived antimicrobials due to the protective outer membrane barrier of their cell wall structure (Berida *et al.,* 2024). As the concentration increased, antibacterial activity improved markedly. At 3 mg/mL, inhibition zones reached 12 ± 0.59 mm for *S. aureus* and 13 ± 0.98 mm for *B. subtilis***,** while Gram-negative bacteria showed zones of 12 ± 0.40 mm for *E. coli* and 11 ± 0.39 mm for *K. pneumoniae*. The maximum inhibition was observed at 5 mg/mL**,** where the extract produced zones of 15 ± 0.97 mm, 16 ± 0.96 mm, 14 ± 0.59 mm, and 14 ± 0.73 mm against *S. aureus*, *B. subtilis*,

E. coli, and *K. pneumoniae*, respectively (Table 4) (Fig. 4).

Table 4: Antimicrobial Activity of the Aqueous Extract of Uterotibb Against Bacterial Pathogen Trains

Aqueous extract					
S. No.	**Concentration Of the extract**	***Staphylococcus aureus***	***Bacillus subtilis***	***E. coli***	***Klebsiella penumonae***
1	1 mg/mL	9±0.40 mm	10±0.33 mm	9±0.69	8±0.23 mm
2	2 mg/mL	10±0.63 mm	11±0.49 mm	10±0.22 mm	10±0.61 mm
3	3 mg/mL	12±0.59 mm	13±0.98 mm	12±0.40 mm	11±0.39 mm
4	4 mg/mL	14±0.84 mm	15±0.84 mm	13±0.71 mm	12±0.80 mm
5	5 mg/mL	15±0.97 mm	16±0.96 mm	14±0.59 mm	14±0.73 mm
6	Streptomycin 5 µg/mL	16±0.89 mm	24±0.99 mm	14±0.69 mm	15±0.90 mm

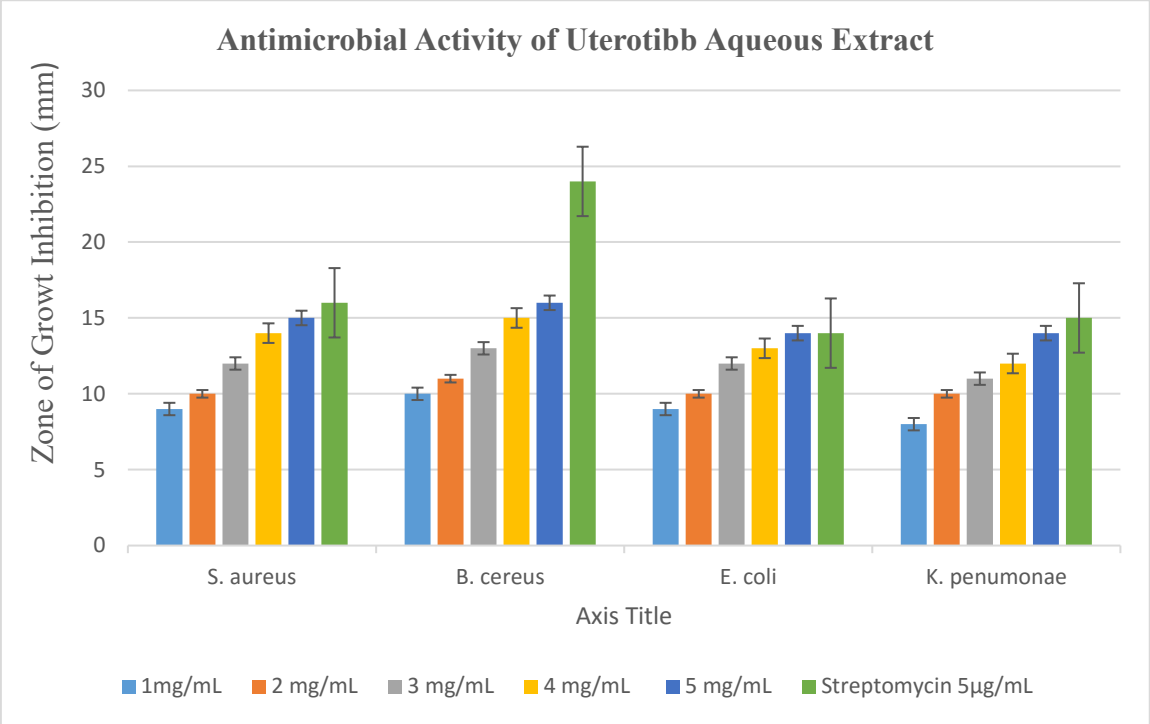


Fig. 4: Antimicrobial Activity of Uterotibb Aqueous Extract Against Selected Pathogens

These results demonstrate that the aqueous extract exhibits broad-spectrum antibacterial activity**,** although Gram-positive bacteria were slightly more susceptible than Gram-negative bacteria. Similar observations have been widely reported in phytochemical antimicrobial studies, where the permeability barrier of Gram-negative bacteria limits the penetration of hydrophilic and hydrophobic plant metabolites (Yan *et al.,* 2024). The reproducibility of the data, supported by low standard error values, confirms the reliability of the observed dose–response relationship. Based on the agar diffusion results, the minimum inhibitory concentration (MIC) defined as the lowest concentration producing a measurable inhibition zone (≥8 mm) was determined. Both the extracts exhibited an MIC of 1 mg/mL for all tested organisms, representing the lowest concentration required to inhibit visible bacterial growth. indicating relatively strong antibacterial activity against Gram-positive bacteria.

For Gram-negative bacteria, the extract showed activity against *E. coli* at 1 mg/mL**,** while a measurable inhibition zone appeared at 2 mg/mL, suggesting an MIC of 1 mg/mL for this organism. Similarly, although a weak inhibition zone was observed for *K. pneumoniae* at 1 mg/mL (Table 5) (Fig. 6). These findings confirm that Gram-negative pathogens require higher extract concentrations for inhibition, which is consistent with their intrinsic structural resistance mechanisms (Zouine *et al.,* 2024).

Nonlinear regression analysis using a four-parameter logistic (4PL) model yielded half-maximal inhibitory concentration (**IC_{50}**) values ranging from 0.38 to 0.42 mg/mL for Gram-positive bacteria to 1.62 - 2.15 mg/mL for Gram-negative bacteria, providing a quantitative measure of extract potency across the concentration gradient. The IC_{50} represents the

concentration required to achieve approximately 50% of the maximal inhibitory effect observed in the assay. The aqueous extract exhibited IC_{50} values of approximately 1.62–1.85 mg/mL for Gram-positive bacteria**,** reflecting relatively strong susceptibility of these organisms to the extract. In contrast, higher IC_{50} values were observed for Gram-negative bacteria**,** estimated at 1.98 mg/mL for *E. coli* and 2.15 mg/mL for *K. pneumonia* (Table 5) (Fig. 6). The higher IC_{50} values for Gram-negative bacteria further support the observation that these organisms possess structural barriers that limit the effectiveness of plant-derived antimicrobial compounds. (Villanueva *et al.,* 2023). The relatively lower IC_{50} values of queues extract can be attributed to the lower efficiency of water in extracting hydrophobic bioactive compounds such as flavonoids and terpenoids. Nonetheless, IC_{50} values within this range still reflect biologically relevant activity, especially for crude aqueous extracts, which often contain lower concentrations of active constituents (Melanie *et al.,* 2017).

Table 5: MIC and IC_{50} of UteroTibb Ethanolic and Aqueous Extracts against Tested Human Pathogens

S. No.	Uterotibb Extract	Micro organism	MIC (mg/mL)	IC_{50} (mg/mL)	Interpretation
1.	Ethanol Extract	*S. aureus*	1	0.42	Highly sensitive
2.	Ethanol Extract	*B. subtilis*	1	0.38	Highly sensitive
3.	Ethanol Extract	*E. coli*	1	0.47	Highly sensitive
4.	Ethanol Extract	*K. pneumoniae*	1	0.52	Moderately sensitive
5.	Aqueous Extract	*S. aureus*	1	1.85	Moderately sensitive
6.	Aqueous Extract	*B. subtilis*	1	1.62	Moderately sensitive
7.	Aqueous Extract	*E. coli*	1	1.98	Moderately sensitive
8.	Aqueous Extract	*K. pneumoniae*	1	2.15	Moderately sensitive

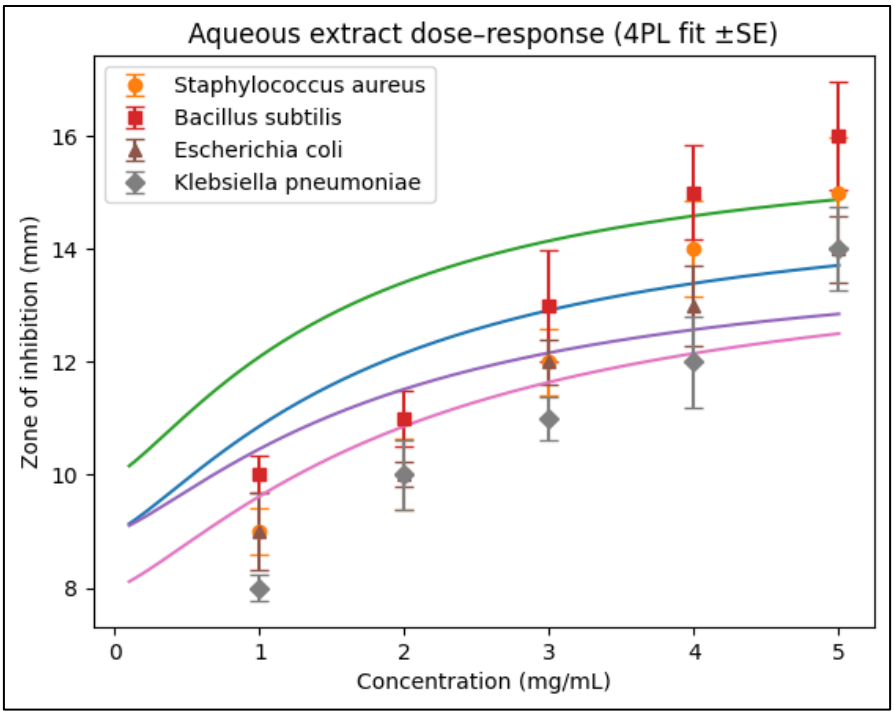


Fig. 6: Dose–Response Curves of the Aqueous Extract of Uterotibb Against Bacterial Pathogens.

Data points represent mean zone of inhibition (mm) ± standard error (SE). Curves represent nonlinear regression fits using a four-parameter logistic (4PL) model.

The antibacterial activity of the aqueous extract was compared with the reference antibiotic streptomycin (5 µg/mL)**.** Streptomycin produced inhibition zones of 16 ± 0.89 mm against *S. aureus*, 24 ± 0.99 mm against *B. subtilis*, 14 ± 0.69 mm against *E. coli*, and 15 ± 0.90 mm against *K. pneumoniae***.** Although the aqueous extract demonstrated lower potency than the standard antibiotic, its inhibition zones at higher concentrations approached those observed for streptomycin against certain organisms, particularly *E. coli*. This indicates that the extract contains bioactive compounds capable of exerting meaningful antibacterial effects.

Overall, the susceptibility of the Gram-positive organisms to the tested extracts may be related to differences in bacterial envelope architecture. Gram-negative bacteria possess an additional outer membrane containing lipopolysaccharide, which can restrict the penetration of certain plant-derived antimicrobial constituents. This structural feature contributes to the higher concentrations required to produce comparable inhibition in Gram-negative organisms.

Pytochemical Basis of Antimicrobial Activity

The antibacterial activity of the aqueous extract can be attributed to the diverse phytochemicals present in the constituent plants of Uterotibb. Aqueous extraction is particularly effective for isolating highly polar phytochemicals such as polysaccharides, glycosides, and certain tannins, flavonoids, glycosides, and polar phenolic compounds (Table 1), many of which possess well-established antimicrobial properties. Tannins can inhibit microbial growth through protein precipitation and enzyme inhibition, while flavonoids may disrupt bacterial membranes and interfere with nucleic acid synthesis. In the context of the Uterotibb formulation, several constituent botanicals contribute antimicrobial phytochemicals. For example, tannins from *Quercus infectoria* exhibit strong antibacterial activity through membrane disruption and enzyme inhibition. Lawsone from *Lawsonia inermis* and thymoquinone from *Nigella sativa* have also been reported to possess broad-spectrum antibacterial effects through oxidative stress induction and inhibition of microbial metabolic pathways. The combined presence of these compounds likely produces synergistic antibacterial effects, resulting in the broad-spectrum antimicrobial activity observed in the present study (Villanueva *et al.,* 2023; Yan *et al.,* 2024; Batiha *et al.,* 2020; Al-Snafi, 2019). Overall, the results indicate that the aqueous extract of Uterotibb exhibits moderate but significant antibacterial activity against both Gram-positive and Gram-negative bacteria, with stronger effects against Gram-positive organisms. The concentration-dependent inhibition pattern, together with the calculated MIC and IC_{50} values, supports the presence of bioactive antimicrobial phytochemicals in the formulation (Zouine *et al.,* 2024)). These findings provide scientific support for the traditional use of this Unani medicine in conditions associated with microbial infections and inflammation (CLSI 2023).

3.6. Comparative Antimicrobial Activity of Ethanol and Aqueous Extracts

A comparative evaluation of the ethanolic and aqueous extracts of Uterotibb revealed notable differences in antibacterial potency and spectrum of activity. Overall, the ethanolic extract exhibited stronger antimicrobial effects, producing larger zones of inhibition and lower apparent MIC and IC_{50} values across the tested bacterial strains compared with the aqueous extract. This observation is consistent with numerous phytochemical studies reporting that organic solvents such as ethanol are more efficient than water in extracting broader bioactive antimicrobial compounds, including phenolics, flavonoids, quinones, terpenoids, and lipophilic flavonoids which contribute significantly to antimicrobial activity (Fig. 7) (Berida *et al.,* 2024; Do *et al.,* 2014).

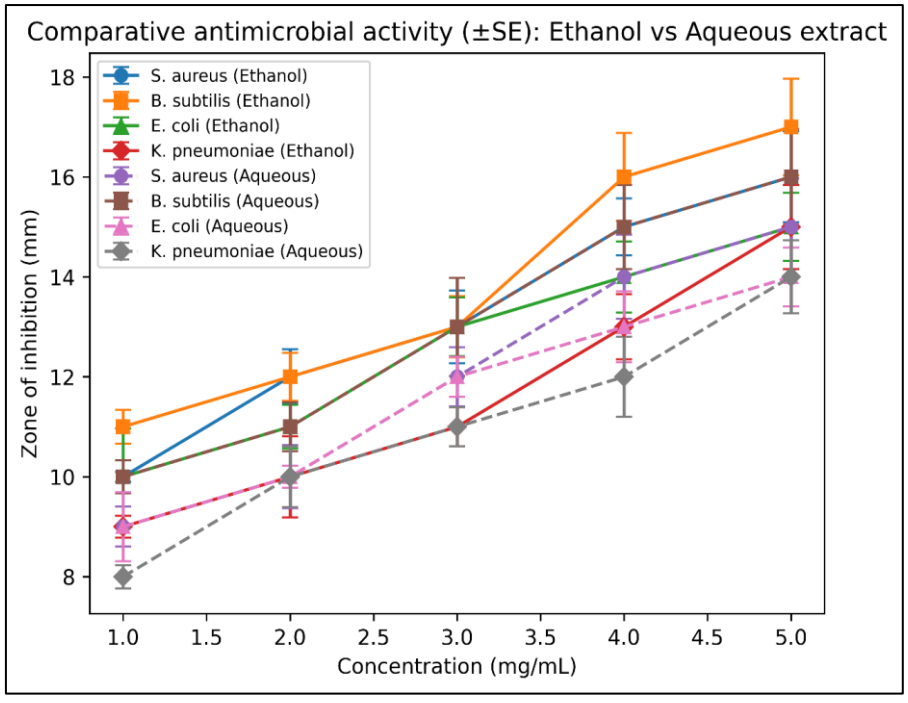


Fig. 7: Comparative Dose–Response Curves of Uterotibb Ethanol and Aqueous Extracts Against Bacterial Pathogens

Data points represent mean zone of inhibition (mm) ± standard error (SE). Solid lines indicate ethanol extract, while dashed lines indicate aqueous extract. The ethanol extract demonstrated consistently higher antibacterial activity across all tested concentrations.

In the present study, the ethanolic extract demonstrated higher antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria, indicating the presence of relatively non-polar or moderately polar antimicrobial constituents. In contrast, the aqueous extract showed moderate inhibitory activity, particularly against Gram-positive bacteria. At the highest concentration (5 mg/mL), the ethanol extract produced larger inhibition zones (16–17 mm for Gram-positive and 15 mm for Gram-negative bacteria) relative to the aqueous extract (14–16 mm), indicating enhanced antibacterial potency (Fig. 7). The reproducibility of the data, supported by low standard error values, confirms the reliability of the observed dose–response relationship. The reduced activity of the aqueous extract may be attributed to the fact that water primarily extracts highly polar phytochemicals such as polysaccharides, glycosides, and certain tannins, which may exhibit weaker antibacterial effects compared with the more diverse phytochemical profile obtained through ethanol extraction (Azwanida, 2015).

The greater antibacterial activity observed for the ethanolic extract may be associated with the more efficient recovery of antimicrobial phytochemical classes from the constituent plants of Uterotibb. Compounds such as thymoquinone from Nigella sativa, lawsone-related constituents from Lawsonia inermis, and tannin-rich constituents of Quercus infectoria have previously been associated with antibacterial effects involving membrane perturbation, interference with microbial enzymes, and oxidative methods (Batiha *et al.,* 2020; Al-Snafi *et al.,* 2019; Villanueva *et al.,* 2023). The detection of such compound classes in the ethanolic extract provides a plausible explanation for its stronger antibacterial response. Nevertheless, because the present experiments were conducted with crude extracts, the individual contribution of these constituents and any potential synergistic interactions cannot be established solely from GC–MS profiling. Furthermore, the slightly greater susceptibility of Gram-positive bacteria compared with Gram-negative bacteria observed for both extracts is consistent with well-established differences in bacterial cell wall structure. The greater susceptibility of the Gram-positive organisms to the tested extracts related to differences in bacterial envelope architecture. Gram-negative bacteria possess an additional outer membrane containing lipopolysaccharide, which can restrict the penetration of certain plant-derived antimicrobial constituents. This structural feature may contribute to the higher concentrations required to produce comparable inhibition in Gram-negative organisms. The observed difference should, however, be interpreted in the context of the specific bacterial strains and extract compositions examined in the present study.

Collectively, these findings suggest that while both extracts possess measurable antibacterial activity, the ethanolic extract exhibits superior antimicrobial potency, likely due to its richer and more diverse phytochemical composition. The complementary phytochemical profiles of the ethanolic and aqueous extracts may nevertheless contribute synergistically to the overall therapeutic efficacy of Uterotibb in traditional medicinal applications.

The MIC for both extracts was determined to be 1 mg/mL for all tested organisms, representing the lowest concentration required to inhibit visible bacterial growth. It serves as a benchmark for comparing the efficacy of plant-derived compounds (Woo *et al.,* 2023). According to recent classifications, crude plant extracts with MIC values below 8 mg/mL are considered biologically active, although higher potency is associated with lower MIC values in purified compounds (Zouine *et al.,* 2024). Thus, the observed MIC indicates that both Uterotibb extracts possess biologically relevant antibacterial activity, with potential for further optimization. Despite identical MIC values, nonlinear regression analysis revealed marked differences in pharmacological potency between the extracts. The ethanolic extract exhibited significantly lower IC_{50} values (0.38–0.52 mg/mL) compared to the aqueous extract (1.62–2.15 mg/mL), demonstrating higher inhibitory efficiency across the concentration gradient. Unlike MIC, which represents a single endpoint, IC_{50} reflects the overall dose–response behavior and is considered a more sensitive parameter for assessing antimicrobial potency and pharmacodynamic performance. The lower IC_{50} values of the ethanolic extract indicate that effective bacterial inhibition is achieved at substantially lower concentrations, highlighting its superior therapeutic potential. Recent studies confirm that extraction solvent polarity is a critical determinant of antimicrobial efficacy, with ethanol and other organic solvents generally yielding extracts with higher activity due to improved solubilisation of active phytochemicals. This is consistent with the present findings, where the ethanolic extract demonstrated approximately 3–4-fold greater potency based on IC_{50} values (Zouine *et al.,* 2024).

Mechanistically, the observed sigmoidal dose–response relationship reflects the cumulative and multi-target actions of phytochemicals present in the extracts. At lower concentrations, partial disruption of bacterial cell membranes and mild inhibition of metabolic enzymes may occur, resulting in modest antibacterial effects. As concentration increases, these effects intensify, leading to increased membrane permeability, leakage of intracellular constituents, inhibition of

enzymatic systems, and interference with nucleic acid and protein synthesis. Such multi-target mechanisms are characteristic of plant-derived antimicrobials and contribute to their effectiveness against diverse bacterial species. Additionally, the synergistic interactions among phytochemicals may enhance antibacterial efficacy and reduce the likelihood of resistance development ((Woo *et al.,* 2023). From a clinical perspective, these findings are highly relevant in the context of the growing global burden of antimicrobial resistance (AMR). Recent reports emphasize that plant-derived antimicrobial agents represent a promising source of novel therapeutics due to their diverse chemical composition and multi-target mechanisms, which can overcome or reduce bacterial resistance. Moreover, phytochemicals have been shown to enhance the efficacy of conventional antibiotics, reduce resistance pathways, and inhibit biofilm formation, which is a major contributor to chronic and persistent infections (Alinaghi *et al.,* 2025).

Overall, the comparative analysis demonstrates that while both Uterotibb's ethanolic and aqueous extracts exhibit biologically significant antibacterial activity, the ethanolic extract possesses markedly higher potency and pharmacodynamic efficiency. The combination of a low MIC (1 mg/mL) and significantly lower IC_{50} values underscores its potential for further development alternative antimicrobial therapy or may serve as a potential candidate for adjunct. These findings highlight the importance of solvent selection in phytochemical extraction and support the continued exploration of plant-based antimicrobials as viable strategies for addressing antibiotic resistance and improving clinical outcomes. Finally, the inhibition zones produced by the ethanolic extract at higher concentrations than that produced by reference antibiotic streptomycin against certain strains, suggesting the presence of potent antimicrobial phytochemicals within the formulation.

Phytochemical Basis of Antimicrobial Activity

The stronger antibacterial response of the ethanolic extract is consistent with its greater representation of phenolic, quinone-related, terpenoid, and other moderately lipophilic constituents detected by GC–MS (Pérez-Flores *et al.,* 2025; Cushnie *et al.,* 2005). Ethanol can facilitate extraction of compounds with intermediate polarity and lipophilic characteristics, several of which have previously demonstrated antimicrobial activity (Desbois and Smith, 2010). Thymoquinone from Nigella sativa, lawsone from Lawsonia inermis, and hydrolysable tannins associated with Quercus infectoria have each been reported to affect bacterial viability through mechanisms including membrane perturbation, protein interactions, enzyme inhibition, and redox-associated effects (Nair *et al.,* 2024; Batiha *et al.,* 2020; Al-Snafi *et al.,* 2019; Villanueva *et al.,* 2023). The presence of these phytochemical classes may therefore contribute to the broad antibacterial response observed in the present study. However, the proposed mechanisms remain inferential because individual compounds were not isolated or tested separately. Overall, the results support the presence of measurable antibacterial activity in Uterotibb, with greater potency observed for the ethanolic extract (Balouiri *et al.,* 2016; Delcour *et al.,* 2009). The higher antibacterial activity of the ethanolic extract can be correlated with its phytochemical profile, which is enriched in phenolics, quinones, fatty acid derivatives and other moderately lipophilic compounds known for antimicrobial effects. Phenolic compounds disrupt bacterial membranes and inhibit essential enzymes, while quinones such as lawsone and thymoquinone exert bactericidal effects through redox cycling and oxidative stress induction (Pérez-Flores *et al.,* 2025; Cushnie *et al.,* 2005). Fatty acid esters further contribute by increasing membrane permeability, particularly in Gram-positive bacteria (Desbois and Smith 2010).

Comparative Interpretation and Pharmacological Relevance

The comparative results demonstrate that the ethanolic extract generally produced stronger antibacterial responses, whereas the aqueous extract also showed measurable activity against both Gram-positive and Gram-negative organisms. These differences are consistent with solvent-dependent recovery of chemically distinct phytochemical groups and provide a possible explanation for the variation in antimicrobial potency between the two preparations. The observed concentration-dependent inhibition supports a biological effect associated with the extracts; however, the present in-vitro findings should not be interpreted as evidence of clinical efficacy. Further mechanistic and in vivo studies are required to determine whether the antibacterial activity of Uterotibb contributes to any therapeutic effects associated with its traditional use (Nostro *et al.,* 2012; Islam et al ,2025).

Antioxidant Activity of the Ethanolic Extract of Uterotibb

The antioxidant activity of the ethanolic extract of Uterotibb was evaluated using two widely accepted free radical scavenging assays, namely the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and the ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolourization assay. These assays are commonly used to assess the hydrogen-donating and electron-transfer capacity of plant-derived antioxidants. The ethanolic extract demonstrated concentration-dependent antioxidant activity in both assays. As the extract concentration increased from 0.2 to 1.1 mg/mL, the percentage of radical scavenging increased significantly. In the DPPH assay, the scavenging activity increased from 6.97 ± 0.18% at 0.2 mg/mL to 95.7±0.91% at 1.1 mg/mL. Similarly, the ABTS assay showed an increase in radical scavenging activity from 3.14 ± 0.18% at 0.2 mg/mL to 50.82±0.79% at 1.1 mg/mL (Table 6). The progressive increase in radical

scavenging activity with increasing concentration indicates that the ethanolic extract contains compounds capable of donating hydrogen atoms or electrons to neutralize free radicals, thereby terminating radical chain reactions. The antioxidant potency of the extract was further quantified by calculating the IC_{50} value, defined as the concentration required to scavenge 50% of the free radicals present in the assay system. The ethanolic extract exhibited an IC_{50} value of approximately 0.68 mg/mL in the DPPH assay, indicating relatively strong antioxidant activity. In contrast, the IC_{50} value for the ABTS assay was approximately 1.07 mg/mL (Table 8) (Fig. 8), suggesting moderate scavenging activity toward ABTS radicals.

The lower IC_{50} value observed for the DPPH assay indicates that the extract has greater efficiency in reducing DPPH radicals compared with ABTS radicals. Such differences are frequently observed in antioxidant studies due to variations in the chemical nature and reaction mechanisms of the two radicals. DPPH radicals are primarily reduced through hydrogen atom transfer mechanisms, whereas ABTS radicals can be neutralized through both electron transfer and hydrogen donation mechanisms (Ilyasov *et al.,* 2020; Prior *et al.,* 2005).

Table 6: Antioxidant Activity of the Uterotibbs Ethanolic Extract Determined by DPPH and ABTS Assays

Concentration (mg/mL)	% DPPH scavenging assay	% ABTS Free radical scavenging activity
0.2	6.97±0.18	3.14±0.18
0.4	16.75±0.24	7.01±0.43
0.6	40.22±0.37	22.27±0.59
0.8	64.36±0.29	31.73±0.88
1.0	85.51±0.25	45.20±0.67
1.1	95.7±0.91	50.82±0.79

Table 7: Antioxidant Activity of the Uterotibbs Aqueous Extract Determined by DPPH and ABTS Assays

S. No.	Concentration (mg/mL)	% DPPH scavenging assay	% ABTS scavenging assay
1.	0.2	5.81 ± 0.16	4.51 ± 0.21
2.	0.4	12.18 ± 0.19	8.25 ± 0.74
2.	0.6	25.14 ± 0.25	14.10 ± 0.45
4.	0.8	47.27 ± 0.33	26.15 ± 0.54
5.	1.0	73.66 ± 0.20	40.48 ± 0.62
6.	1.1	86.86± 0.30	47.65 ± 0.72

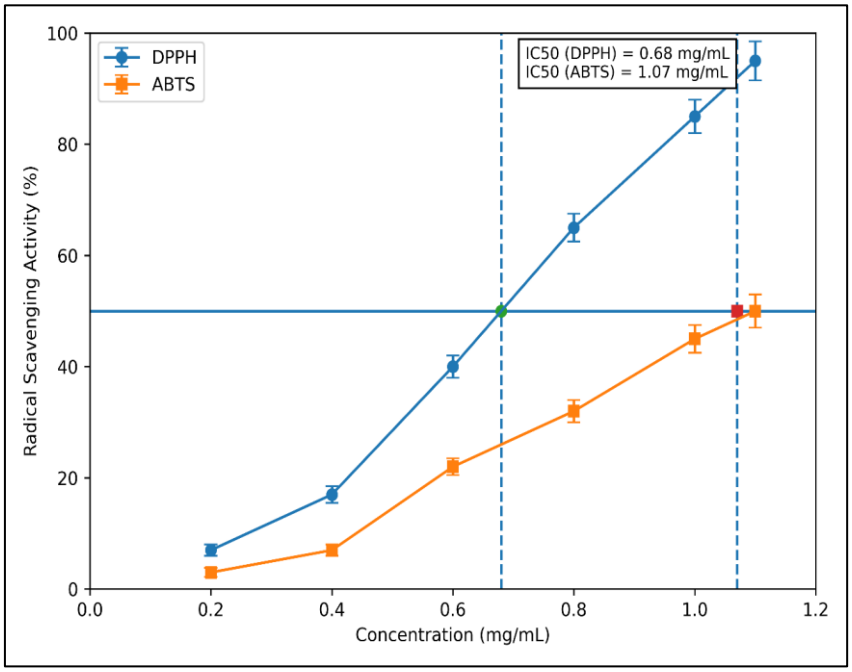


Fig. 8: Combined Dose–Response Curves for DPPH and ABTS Radical Scavenging Activities of the Ethanolic Extract of Uterotibb

Data are expressed as mean ± SD (n = 3) and ± standard error (SE). The extract exhibited concentration-

dependent antioxidant activity in both assays. The estimated IC_{50} values were 0.68 mg/mL for the DPPH assay and 1.07 mg/mL for the ABTS assay.

Antioxidant Activity of the Aqueous Extract of Uterotibb

The antioxidant activity of the aqueous extract of Uterotibb was evaluated using two widely accepted free radical scavenging assays, namely the DPPH assay and the ABTS radical assay. Dose–response curves for the antioxidant activity of the aqueous extract of Uterotibbs determined using DPPH and ABTS radical scavenging assays. Values represent mean ± SD (n = 3). The aqueous extract exhibited concentration-dependent antioxidant activity in both assays. As the extract concentration increased from 0.2 to 1.1 mg/mL, the percentage of radical scavenging increased significantly. In the DPPH assay, the scavenging activity increased from 5.81 ± 0.16% at 0.2 mg/mL to 86.86 ± 0.30% at 1.1 mg/mL. Similarly, the ABTS assay showed an increase in radical scavenging activity from 4.51 ± 0.21% at 0.2 mg/mL to 47.65 ± 0.72% at 1.1 mg/mL (Table 7). The calculated IC_{50} values were approximately 0.821 mg/mL for the DPPH assay and 1.133 mg/mL (Table 8) (Fig. 9) for the ABTS assay, indicating moderate free-radical scavenging activity of the aqueous extract.

Table 8: IC_{50} values of Antioxidant Activity of Uterotibbs Ethanolic and Aqueous Extracts

S. No.	Uterotibb ethanolic extract		
	Assay	**IC50 value**	**Interpretation**
1.	DPPH	0.68 mg/mL	Strong antioxidant activity
2.	ABTS	1.07 mg/mL	Moderate antioxidant activity
Uterotibb aqueous extract			
1.	DPPH	0.821 mg/mL	Strong antioxidant activity
2.	ABTS	1.133 mg/mL	Moderate antioxidant activity

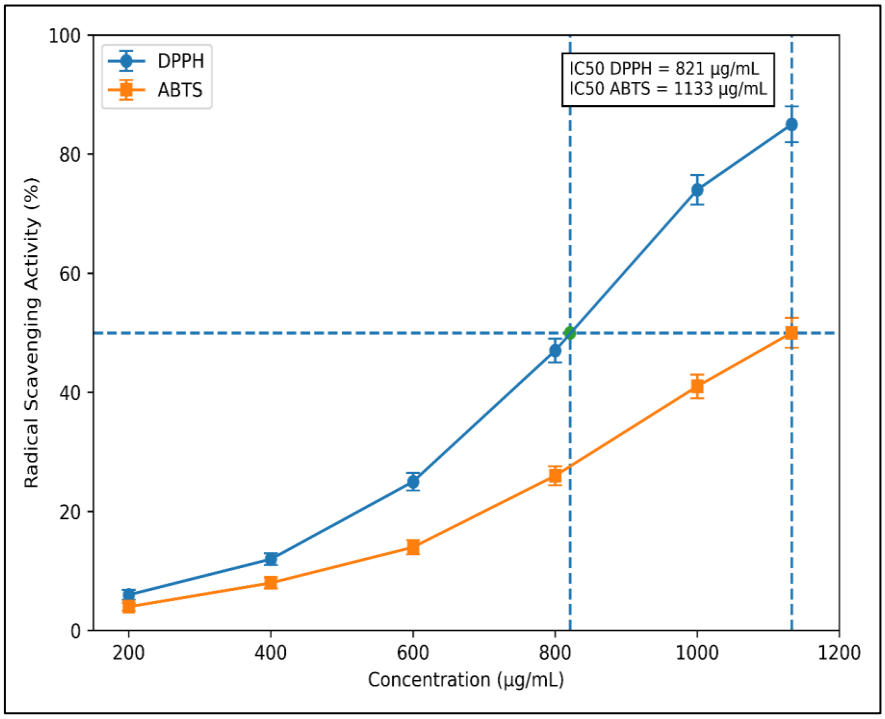


Fig. 9: Dose-Response Dependent Antioxidant Activity of DPPH and APTS Uterotibbs Aqueous Extract

Values represent mean ± SD (n = 3) and ± standard error (SE). The extract exhibited concentration-dependent antioxidant activity. The calculated IC_{50} values were approximately 821 µg/mL for the DPPH assay and 1133 µg/mL for the ABTS assay, indicating moderate free-radical scavenging activity of the aqueous extract.

Comparison of DPPH and ABTS Antioxidant Systems

The aqueous extract of Uterotibbs demonstrated concentration-dependent antioxidant activity in both DPPH and ABTS radical scavenging assays. The radical scavenging activity increased progressively with increasing extract concentration, reaching 86.86% inhibition in the DPPH assay and 47.65 % inhibition in the ABTS assay at 1.1 mg/mL. The

calculated IC_{50} values were approximately 0.821 mg/mL for DPPH and 1.133 mg/mL for ABTS, indicating that the extract exhibits a stronger scavenging capacity toward DPPH radicals compared with ABTS radicals. The relatively higher IC_{50} value observed in the ABTS assay suggests a moderate antioxidant potential of the aqueous extract. This activity may be attributed to the presence of phenolic compounds, tannins, and flavonoids derived from the constituent medicinal plants of the Uterotibb formulation, which are known to donate hydrogen atoms or electrons to neutralize free radicals and inhibit oxidative chain reactions. The stronger activity observed in the DPPH assay compared with the ABTS assay suggests that the major antioxidant constituents in the aqueous extract may be compounds capable of efficient hydrogen atom donation. Many plant-derived phenolic compounds exhibit this behaviour due to the presence of hydroxyl groups capable of stabilizing free radicals through resonance structures. The moderate ABTS scavenging activity may reflect the presence of compounds with selective radical-scavenging mechanisms or limited solubility under ABTS assay conditions. Differences between DPPH and ABTS activities have been widely reported in phytochemical studies and are often attributed to the structural diversity and polarity of antioxidant compounds present in plant extracts **(**Batiha *et al.,* 2020; Al-Snafi, 2019).

Phytochemical Basis of Antioxidant Activity

The observed antioxidant activity of the ethanolic extract can be attributed to the presence of phenolic compounds, flavonoids, tannins, and quinones derived from the constituent botanicals of the Uterotibb formulation. Ethanol extraction is particularly effective in isolating these phytochemicals due to its ability to dissolve both moderately polar and non-polar compounds. For example, thymoquinone from *Nigella sativa* has been widely reported to possess potent antioxidant activity through free radical scavenging and inhibition of lipid peroxidation. Similarly, **l**awsone from *Lawsonia inermis* and hydrolysable tannins from *Quercus infectoria* contribute significantly to antioxidant activity (Table 1) through hydrogen donation and metal chelation mechanisms. Flavonoids present in the formulation may further enhance antioxidant activity by stabilizing reactive oxygen species and inhibiting oxidative chain reactions. Overall, the ethanolic extract of Uterotibb demonstrated substantial free radical scavenging activity, with stronger activity observed in the DPPH assay than in the ABTS assay. The relatively low IC_{50} value obtained for the DPPH assay indicates the presence of potent antioxidant compounds capable of neutralizing free radicals. These findings support the hypothesis that the traditional Unani formulation contains bioactive phytochemicals with significant antioxidant potential (Fig. 10), which may contribute to its therapeutic efficacy by protecting biological systems against oxidative stress (Villanueva *et al.,* 2023; Shahidi, 2015). Major classes of phytochemicals including phenolic compounds, fatty acid derivatives, terpenoids, and phytosterols may contribute to the observed antioxidant and antimicrobial activities through mechanisms such as hydrogen atom donation, reactive oxygen species scavenging, inhibition of lipid peroxidation, and disruption of microbial cell membranes. The GC-MS analysis of Uterotibbs extracts revealed the presence of several classes of phytochemicals including phenolic compounds, fatty acid derivatives, terpenoids, and phytosterols (Fig. 10), which are widely recognized for their antimicrobial and antioxidant activities. These compounds may collectively contribute to the bioactivity of the formulation through multiple biochemical activities (Batiha *et al.,* 2020; Al-Snafi *et al.,* 2019; Villanueva *et al.,* 2023; Villanueva *et al.,* 2023).

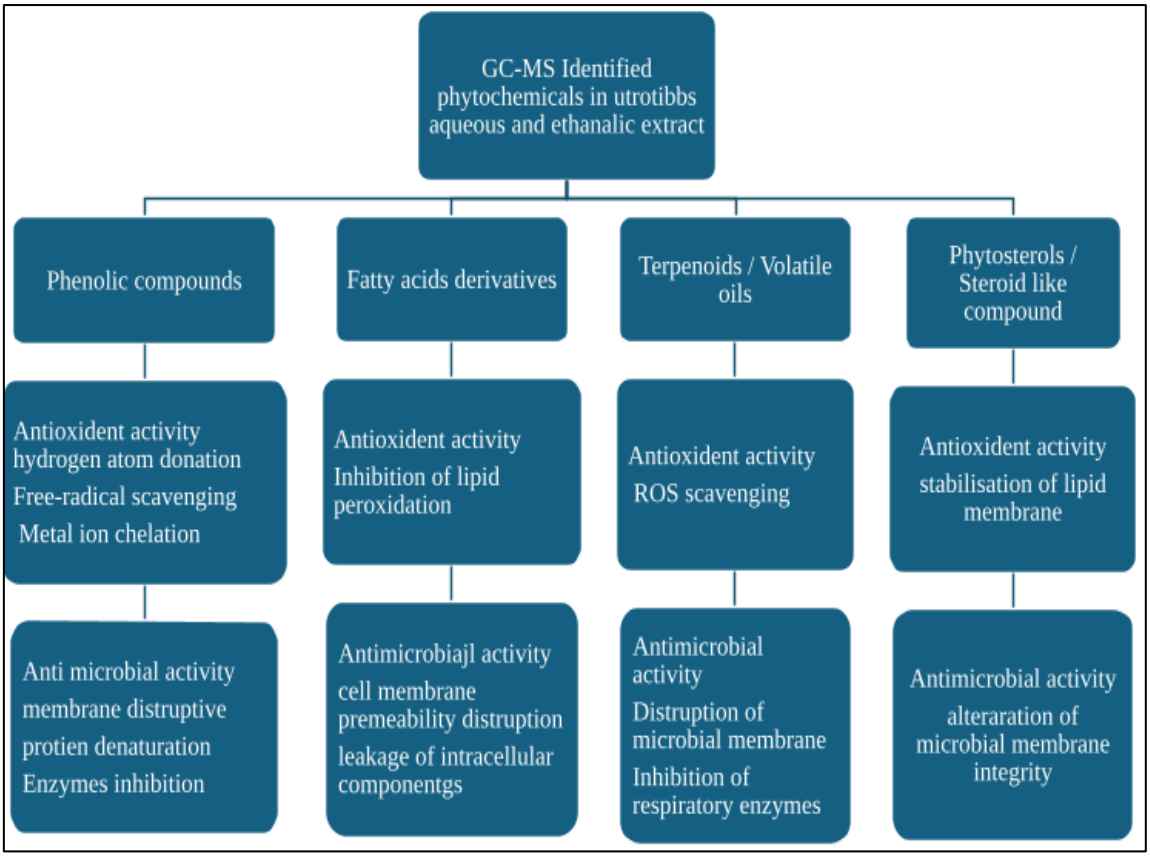


Fig. 10: Phytochemical–Bioactivity Correlation Identified by GC-MS Analysis of Uterotibbs Extracts and their Reported Biological Activities

Phenolic compounds represent one of the most important natural antioxidants and are capable of neutralizing reactive oxygen species through hydrogen atom transfer and single-electron transfer mechanisms. The presence of hydroxyl groups in phenolic structures allows these compounds to donate electrons or hydrogen atoms, thereby stabilizing free radicals and preventing oxidative chain reactions (Shahidi & Ambigaipalan, 2015). Fatty acid derivatives detected by GC-MS are also known to exhibit antimicrobial properties (Table 1). The greater antibacterial response of the ethanolic extract may partly reflect its enrichment in lipophilic and moderately polar constituents capable of interacting with bacterial envelopes. Nevertheless, because the present study evaluated crude extracts, the contribution of individual compounds cannot be assigned solely from GC–MS detection.

Therefore, Long-chain fatty acids can interact with microbial cell membranes, increasing membrane permeability and causing leakage of intracellular components, ultimately leading to microbial cell death (Desbois & Smith, 2010). Terpenoids and volatile oil constituents identified in medicinal plant extracts have been reported to possess strong antimicrobial effects. These compounds disrupt microbial cell membranes and interfere with respiratory enzymes and energy metabolism, thereby inhibiting microbial growth (Bakkali *et al.,* 2008). Additionally, phytosterols and steroid-like compounds may contribute to antioxidant defense by stabilizing biological membranes and reducing lipid peroxidation. The combined presence of these phytochemical classes may therefore explain the synergistic antioxidant and antimicrobial activities observed in the Uterotibb extracts **(**Batiha *et al.,* 2020). Overall, the results suggest that the pharmacological activity of the Uterotibbs formulation is likely due to the synergistic action of multiple phytochemicals rather than a single active compound, a phenomenon commonly observed in traditional herbal medicines.

CONCLUSION

Uterotibb demonstrated measurable antioxidant and antimicrobial activities that were associated with a chemically diverse phytochemical profile identified by GC–MS. Both aqueous and ethanolic extracts showed concentration-dependent radical-scavenging activity, with IC_{50} values of 0.821 and 1.133 mg/mL for DPPH and ABTS, respectively, for the aqueous extract, compared with 0.68 and 1.07 mg/mL for the ethanolic extract. Both extracts exhibited an MIC of 1 mg/mL, while the ethanolic extract showed lower antimicrobial IC_{50} values than the aqueous extract. The greater activity of the ethanolic preparation was consistent with its broader representation of phenolic, quinone-related, terpenoid, fatty-acid-derived, and other moderately lipophilic constituents. Collectively, these findings provide experimental support for the biological potential

of Uterotibb and contribute to the scientific documentation of its traditional use. Further studies involving compound isolation, mechanism-based assays, toxicological evaluation, and in vivo validation are warranted to establish the contribution and safety of individual constituents and to determine the pharmacological relevance of these in-vitro findings.

DECLARATIONS

Conflict of Interest: The author declares no competing interests.

Authors Contributions

MOHD, and SSA, designed the concept, carried out the experiments and MN, contributed to manuscript writing and proof reading. The authors read and approved the final manuscript.

Highlights of the study:

1. Uterotibb, a traditional Unani polyherbal formulation used in women's healthcare, was systematically evaluated for its phytochemical and biological properties.
2. GC–MS profiling revealed diverse bioactive constituents, including phenolic derivatives, fatty acid esters, terpenoids, aromatic compounds, quinone-related structures, and phytosterols.
3. Both aqueous and ethanolic extracts exhibited concentration-dependent antioxidant activity in DPPH and ABTS radical-scavenging assays.
4. The ethanolic extract demonstrated astonishing antioxidant and antimicrobial potential, with DPPH and ABTS IC_{50} values of 0.68 and 1.07 mg/mL, respectively, and antimicrobial IC_{50} values of 0.38 mg/mL against *B. subtilis* and 0.52 *K. pneumoniae* mg/mL.
5. The findings provide experimental support for the traditional use of Uterotibb and highlight its potential as a source of bioactive phytochemicals for further pharmacological investigation.

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