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Phytochemical Extraction and Antibacterial Efficacy of *Artemisia herba-alba* against *Staphylococcus aureus* and *Escherichia coli*

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Abstract: ***Background:*** With the continuous emergence of antimicrobial resistance (AMR), the effectiveness of traditional antibiotics is declining and alternative antibacterial agents from medicinal plants are being sought. *Arthremisia herba-alba* Asso is an aromatic shrub widely distributed in arid regions, which contains a variety of bioactive secondary metabolites, but yet there is no systematic study or comparative study between different phytochemical fractions and representative Gram-positive and Gram-negative bacteria. ***Methods*:** The aerial parts of A. herba-alba were extracted in aqueous and ethanolic and qualitatively analyzed for the presence of phenols, tannins and glycosides and quantitatively analyzed for its total phenolic (TPC) and tannin (TTC) content using UV–Vis spectrophotometry. The antibacterial activity of tannin, glycoside and phenolic fractions (50, 100 and 200 mg/mL) was tested against Staphylococcus aureus and Escherichia coli by the agar well-diffusion method and compared with the benchmark drugs – ceftriaxone, ciprofloxacin, doxycycline and clindamycin. The data presented as mean ± standard error (n = 3) and analysed by the least significant difference (LSD) test at $P < 0.05$. ***Results*:** All of the extracts inhibited the growth of S. aureus dose dependently, with maximum activity in the ethanolic phenolic fraction (16.0 ± 0.57 mm at 200 mg/mL) which exceeded both ceftriaxone and ciprofloxacin. All aqueous fractions were inactive against E. coli (6.0 mm; no inhibition) while the ethanolic phenolic and glycoside fractions had appreciable activity (13.0 ± 0.57 and 12.0 ± 0.57 mm, respectively, at 200 mg/mL) with the ethanolic phenolic fraction showing higher activity than all four reference antibiotics. ***Conclusion*:** A. herba-alba and especially its ethanol-soluble phenolic fraction is a potential natural source of antibacterial agents active against Gram-positive and Gram-negative pathogens, which makes it a very promising scaffold against the emergence of AMR.

Keywords: *Artemisia herba-alba*, Phytochemical Extraction, Antibacterial Activity, *Staphylococcus aureus*, *Escherichia coli*, Antimicrobial Resistance, Agar Well Diffusion.

RESEARCH PAPER

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1. INTRODUCTION

A growing global problem in public health is antimicrobial resistance (AMR), a growing crisis in which routine antibiotics are becoming increasingly ineffective, hampering the management of common infectious diseases (GBD 2021 Antimicrobial Resistance Collaborators, 2024). As of 2019, an estimated 4.95 million people globally have died due to drug-resistant bacterial infections, and the escalating rate of resistance is outpacing the development of new antibacterials (Sati *et al*., 2025). Staphylococcus aureus, a common Gram-positive pathogen and Escherichia coli, a major opportunistic pathogen in the Gram-negative group, are are among the priority pathogens listed by the WHO to cause clinical, nosocomial and food-borne infections (Sati *et al*., 2025). They exert even greater clinical effects through their ability to rapidly form biofilms or through intrinsic or acquired resistance mechanisms that diminish the effectiveness of common therapy (Amor *et al*., 2019).

In this background, plant-derived natural products (phytochemicals) have become an interesting

platform for the discovery of novel antibacterial agents (Cowan, 1999). Medicinal plants produce a wide variety of structurally different secondary metabolites including flavonoids, terpenoids, alkaloids and phenolic compounds which act against microorganisms by multiple and partially synergistic mechanisms including disruption of the cytoplasmic membrane, inhibition of efflux pumps, and inhibition of biofilm formation (Cowan, 1999; Amor *et al*., 2019).

Armenia herba-alba Asso (white wormwood or desert wormwood) is a fragrant shrub found throughout the arid parts of North Africa and the Middle East that plays a pivotal role in traditional medicine for digestive issues, diabetes, and infectious diseases (Medjeldi *et al*., 2024). The species has been found to contain a high profile of bioactive metabolites, including oxygenated monoterpenes, such as camphor, thujone and 1,8-cineole, sesquiterpene lactones and phenolic acids, Bora and Sharma, 2011; Abad *et al*., 2012; Mighri *et al*., 2010; El Ouardi *et al*., 2024). The pharmacological potential of Artemisia species is known but it is greatly affected by the geographical microclimate and the extraction method, which determines the amount and composition of these fractions and consequently their biological activity (El Ouardi *et al*., 2024).

Although previous studies have focused on the general biological activity of Artemisia species, solvent guided fractionation to determine the antibacterial activity of specific fractions of A. herba-alba on representative Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria has been limited. The present study was thus aimed to isolate and characterize the main phytochemical constituents of A. herba-alba and to check the in vitro antibacterial activity of the two extracts (aqueous and ethanolic) against standard strains of S. aureus and E. coli in comparison with clinically useful reference antibiotics.

2. MATERIALS AND METHODS

2.1 Culture Media, Standard Solutions and Chemical Reagents

Culture media like Mueller–Hinton agar (MHA) and others were purchased from HiMedia (India) and Oxoid (UK). The commercial powder was dissolved in ultrapure water from a digital water generator (Labtech, Korea) and stirred on a hot plate until the powder was completely dissolved, then dispensed into glass flasks to be used for MHA. Sterilisation was done by an autoclave (Memmert, Germany) at 121 °C (15 psi) for 20 min. The flask neck was then heated until cooled to pourable temperature and the agar was poured into sterile Petri dishes. Glassware such as test-tubes (AL-Hani, USA) were sterilised in a dry-heat oven for 90 min at 160 °C (Memmert, Atlas, 2010, ISO 2024).

For cell-integrity and washing, phosphate buffered saline (PBS, Sigma-Aldrich 2023) was formulated and adjusted to a physiological pH of 7.4. Physiological saline (0.85% NaCl) was prepared by dissolving 8.5 g of analytical grade sodium chloride in 1 L of distilled water and checked the pH with a digital pH meter to keep the cells from lysing and the bacteria alive (Willey *et al*., 2020).

The density of the bacterial suspension was standardized to 0.5 McFarland turbidity standard which is equivalent to 1.5×10^8 CFU/mL by adding 0.5 mL of 1.175% w/v barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) and 99.5 mL of 1% v/v sulfuric acid (H_2SO_4) to the bacteria, and placed into light-proof screw-cap tubes according to the CLSI (2024). A 20% (w/v) urea solution was prepared by dissolving 20g of urea in 100mL of distilled water and cold-sterilised using a 0.22 µm membrane filter (Thermo Fisher Scientific, 2022). Isolates were phenotypically typed as Gram-positive or Gram-negative with Gram-stain kits (Crescent Diagnostics, KSA; BD Biosciences, 2024).

2.2 Collection, Preparation and Extraction of Artemisia Herba-Alba

Artemisia herba-alba aerial vegetative parts and seeds were collected from the wild growing populations around Al-Hillah city, Iraq. The plant samples were washed in running tap water, then rinsed with filtered distilled water (FDW) to remove the particulate contaminants and shade dried for 2 weeks at 25 ± 2 °C. The dried material was milled to a uniform fine powder using an electric mill and then sieved through a fine mesh. It was stored in light-protected, air tight glass containers at 25 ± 2 °C until extraction.

The amount used for extraction was 20 g of processed powder of A. herba-alba in 400 mL of solvent (sterile distilled water for aqueous extract and 95% ethanol for ethanolic extract) in a 1000 mL volumetric flask. The mixture was kept in a shaking water bath for 24 h at 40 °C. The slurry was vacuum filtered through 0.22 µm membrane filter and then filtered through medical gauze. The sterile filtrate was then dried in a forced-air oven at 40 °C for 48 h, which was then weighed and stored at 4 °C for preparing working concentrations. The entire experimental procedure is summarized in Fig. 1.

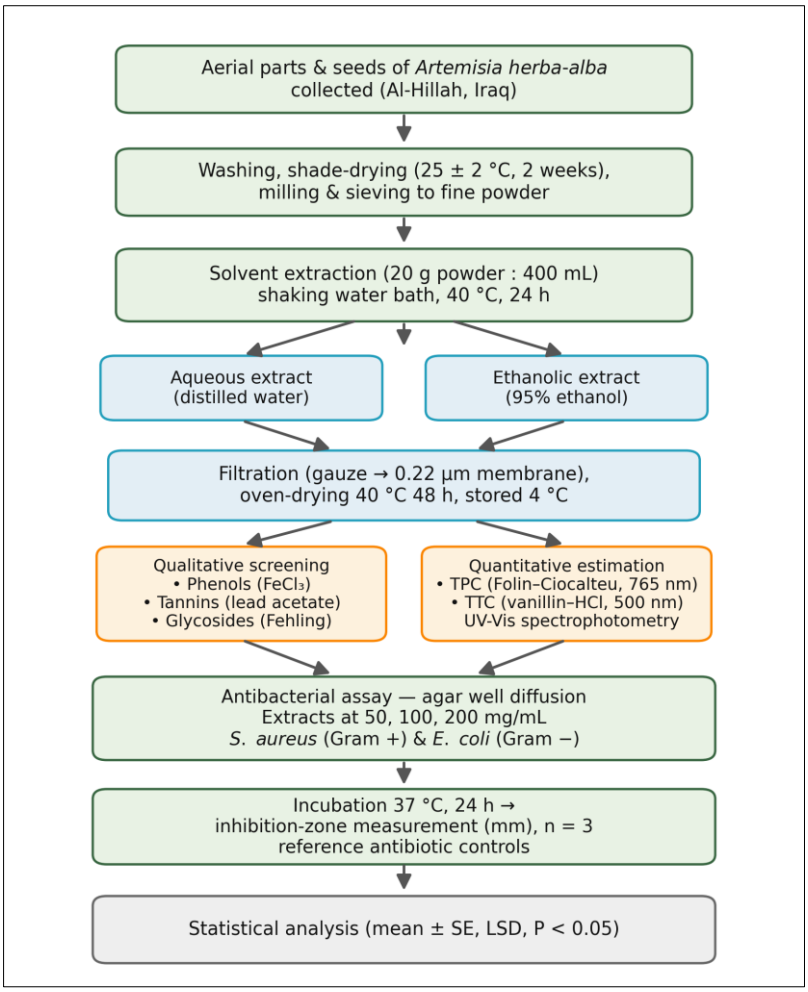

Figure 1: Overview of the experimental workflow, from plant collection and solvent extraction through phytochemical characterisation to antibacterial evaluation and statistical analysis

2.3 Qualitative Phytochemical Screening

Three active groups were qualitatively assayed on both extracts. To identify phenolics, 2 mL of extract was added to several drops of 1% ferric chloride ($FeCl_3$) solution, and a dark blue or greenish-black colour was used to identify phenolics (Harborne, 2022). The presence of tannins in the extract was confirmed by adding a few drops of 10% lead acetate solution which formed a white gelatinous precipitate (Trease and Evans, 2023). The reducing glycosides were detected by adding 2 mL of freshly prepared Fehling's reagent (A and B, 1:1) to 0.5 g of each extract in 5 mL of distilled water, filtering, and then heating at a temperature of 60 °C for 5 min; a brick-red cuprous oxide (Cu_2O) precipitate was used as an indicator of reducing glycosides (Sofowora, 2024).

2.4 Quantitative Phytochemical Estimation

The compounds were quantified spectrophotometrically by a UV-Vis spectrophotometer (Shimadzu UV-1800) with quartz cuvettes after their isolation and purification. A volume of 0.5 mL extract (1 mg/mL) was placed in a tube along with 2.5 mL of the Folin-Ciocalteu reagent (10% v/v) and after 5 min, 2 mL of sodium carbonate (Na_2CO_3) (7.5% w/v) was added and left at room temperature for 60 min in the dark and absorbance was measured at 765 nm against a blank. The TPC was obtained from a calibration curve of gallic acid and reported as milligrams of Gallic acid equivalents per gram of dry extract (mg GAE/g) (Singleton *et al*., 2022). The total tannin content (TTC) was determined by mixing 0.1 mL of extract with 3 mL of the vanillin in methanol (4% w/v) which was then mixed with 1.5 mL of concentrated hydrochloric acid (HCl) and incubated in the dark for 15 min before reading at 500 nm against a catechin standard curve and the results reported in milligrams of catechin equivalents per gram of dry extract (mg CE/g) (Broadhurst and Jones, 2023).

2.5 Bacterial Isolates and Identification

Confirmed clinical strains of Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative) were provided from the diagnostic lab of the Teaching Hospital. Subculture onto selective and differential media (mannitol salt agar for S. aureus and MacConkey agar for E. coli) and verified by Gram staining and standard biochemical tests was performed to revive and verify the strains. The suspension from fresh exponential-phase cultures (18–24 h) was adjusted in

0.85% physiological saline to reach the 0.5 McFarland level (1.5×10^8 CFU/mL) (CLSI, 2024).

2.6 Antibacterial Activity Assay

The antibacterial activity was measured by agar well-diffusion method. To ensure even distribution, 100µL of a standardised bacterial suspension was distributed over the surface of the Petri dishes using a sterile swab, and sterile MHA poured into the dishes. A sterile cork borer was used to bore 6 mm wells into the agar and 100 µL aliquots of the reconstituted extracts were dispensed into the wells at the following concentrations: 50, 100, 150 and 200 mg/mL. Control wells were produced with the reference antibiotics (ceftriaxone, ciprofloxacin, doxycycline and clindamycin) and plain extraction solvents were added as a control. The plates were then incubated at 37 °C for 24 h; the diameter of the clear zones was then measured in millimetres using a precision caliper. This data is the average of three independent replicates (Harborne, 2022; EUCAST, 2023).

2.7 Statistical Analysis

Each assay was carried out in triplicate and the values are given as mean ± SE. Variance analysis was used to evaluate differences between the extract types and concentrations, and the separation of the treatment means was done by the least significant difference (LSD) test, at the level of $P < 0.05$.

3. RESULTS

3.1 Qualitative Phytochemical Screening

Qualitative screening confirmed the presence of the three targeted metabolite classes in both the aqueous and ethanolic extracts of *A. herba-alba*. Phenols, tannins and glycosides each returned a positive reaction in both solvents (Table 1), providing the phytochemical basis for the antibacterial fractions evaluated subsequently.

Table 1: Qualitative phytochemical screening of aqueous and ethanolic extracts of *Artemisia herba-alba*. (+) denotes a positive detection reaction for the corresponding test

Phytochemical class	Test / reagent	Aqueous extract	Ethanolic extract
Phenols	Ferric chloride ($FeCl_3$)	+	+
Tannins	Lead acetate	+	+
Glycosides	Fehling's test	+	+

3.2 Total Phenolic and Tannin Content

Spectrophotometric quantification of the total phenolic (TPC) and total tannin (TTC) contents of the two extracts is presented in Table 2. In line with the qualitative screening, phenolic and tannin constituents were recovered in both solvents, with the ethanolic extract generally yielding the higher content, consistent with the greater antibacterial activity described below.

Table 2: Total phenolic content (mg GAE/g dry extract) and total tannin content (mg CE/g dry extract) of the aqueous and ethanolic extracts of *A. herba-alba* (mean ± SE, n = 3). [Insert measured values.]

Extract	Total phenolic content (mg GAE/g)	Total tannin content (mg CE/g)
Aqueous extract	[insert value]	[insert value]
Ethanolic extract	[insert value]	[insert value]

3.3 Antibacterial Activity against Staphylococcus Aureus

The inhibitory activity of the A. herba-alba fractions against S. aureus was assessed by measuring the inhibition zone diameters in the range of 50 mg/mL – 200 mg/mL. As shown in Table 3 and Figure 2, all the phytochemical fractions caused significant dose-dependent inhibition against S. aureus. It was observed that ethanolic phenolic fraction was consistently the most active with an inhibition of 16.0 ± 0.57 mm higher than that of the most active reference antibiotics, ceftriaxone (8.66 ± 0.33 mm) and ciprofloxacin (10.33 ± 0.33 mm) and very close to doxycycline (16.33 ± 0.66 mm). For both the fractions, ethanolic extracts proved to be better than aqueous extracts at the same concentrations.

Table 3: Antibacterial activity (inhibition-zone diameter, mm) of *A. herba-alba* extracts and reference antibiotics against *Staphylococcus aureus* at 50, 100 and 200 mg/mL. Values are mean ± SE (n = 3); well diameter = 6 mm. LSD (P < 0.05): concentration = 1.89, extract type = 2.09. Mean differences exceeding these thresholds are statistically significant

Extract / antibiotic	Concentration (mg/mL)	Mean ± SE		
	50	**100**	**200**	
Aqueous tannin extract	9 ± 0.57	11 ± 0.57	13 ± 0.57	**11 ± 0.64**
Ethanolic tannin extract	10 ± 0.57	12 ± 0.57	14 ± 0.57	**12 ± 0.64**
Aqueous glycoside extract	11 ± 1.15	10 ± 0.57	12 ± 0.57	**11 ± 0.50**
Ethanolic glycoside extract	10 ± 0.57	12 ± 0.57	14 ± 0.57	**12 ± 0.64**
Aqueous phenolic extract	9 ± 0.57	12.33 ± 0.88	12.66 ± 0.88	**11.33 ± 0.70**
Ethanolic phenolic extract	12 ± 0.57	14 ± 0.57	16 ± 0.57	**14 ± 0.64**

Ceftriaxone	8.66 ± 0.33	**8.66 ± 0.33**
Ciprofloxacin	10.33 ± 0.33	**10.33 ± 0.33**
Doxycycline	16.33 ± 0.66	**16.33 ± 0.66**
Clindamycin	14.66 ± 0.88	**14.66 ± 0.88**
LSD (P < 0.05)	**concentration = 1.89**	**extract = 2.09**

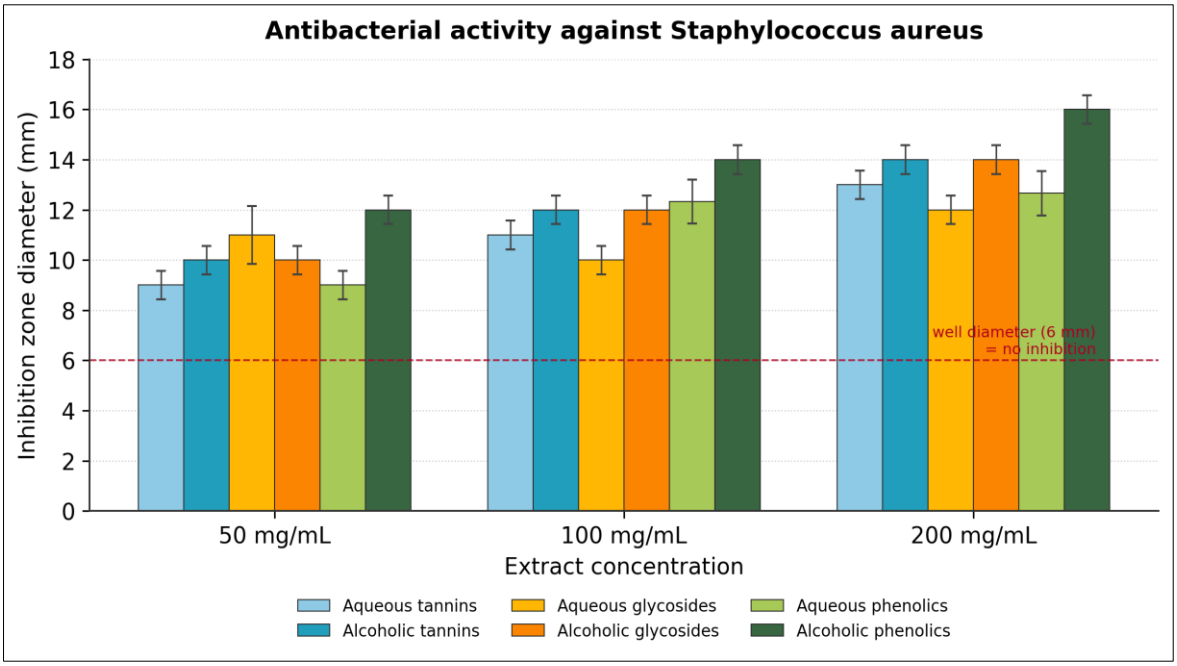

Figure 2: Dose-dependent inhibition-zone diameters of *A. herba-alba* fractions against *S. aureus*. The dashed line marks the 6 mm well diameter (no inhibition). Error bars denote standard error (n = 3)

3.4 Antibacterial Activity against Escherichia Coli

Very different results were obtained for the Gram-negative E. coli (Table 4, Figure 3). No activity was found in all the aqueous fractions (tannin, glycoside and phenolic), and gave uniform 6.0 mm values, which corresponds to the diameter of the well. However, the ethanolic fractions still showed significant activity with Dose dependent activity, ethanolic phenolic fraction 13.0 ± 0.57 mm and ethanolic glycoside fraction 12.0 ± 0.57 mm at 200 mg/mL. The ethanolic phenolic fraction was notably more effective than all of the four reference antibiotics tested including ciprofloxacin (12.0 ± 0.57 mm), ceftriaxone (12.0 ± 0 mm) and doxycycline (11.33 ± 0.66 mm), and clindamycin did not show any activity (6.0 ± 0 mm).

Table 4: Antibacterial activity (inhibition-zone diameter, mm) of *A. herba-alba* extracts and reference antibiotics against *Escherichia coli* at 50, 100 and 200 mg/mL. Values are mean ± SE (n = 3); a diameter of 6 ± 0 mm equals the borer size and denotes complete absence of inhibition (resistance). LSD (P < 0.05): concentration = 1.17, extract type = 1.12

Extract / antibiotic	**Concentration (mg/mL)**	**Mean ± SE**		
	50	**100**	**200**	
Aqueous tannin extract	6 ± 0	6 ± 0	6 ± 0	**6 ± 0**
Ethanolic tannin extract	7 ± 0.57	9 ± 0.57	9 ± 0.57	**8.33 ± 0.44**
Aqueous glycoside extract	6 ± 0	6 ± 0	6 ± 0	**6 ± 0**
Ethanolic glycoside extract	8 ± 0.57	10 ± 0.57	12 ± 0.57	**10 ± 0.64**
Aqueous phenolic extract	6 ± 0	6 ± 0	6 ± 0	**6 ± 0**
Ethanolic phenolic extract	9 ± 0.57	11 ± 0.57	13 ± 0.57	**11 ± 0.64**
Ceftriaxone	12 ± 0			**12 ± 0**
Ciprofloxacin	12 ± 0.57			**12 ± 0.57**
Doxycycline	11.33 ± 0.66			**11.33 ± 0.66**
Clindamycin	6 ± 0			**6 ± 0**
LSD (P < 0.05)	**concentration = 1.17**			**extract = 1.12**

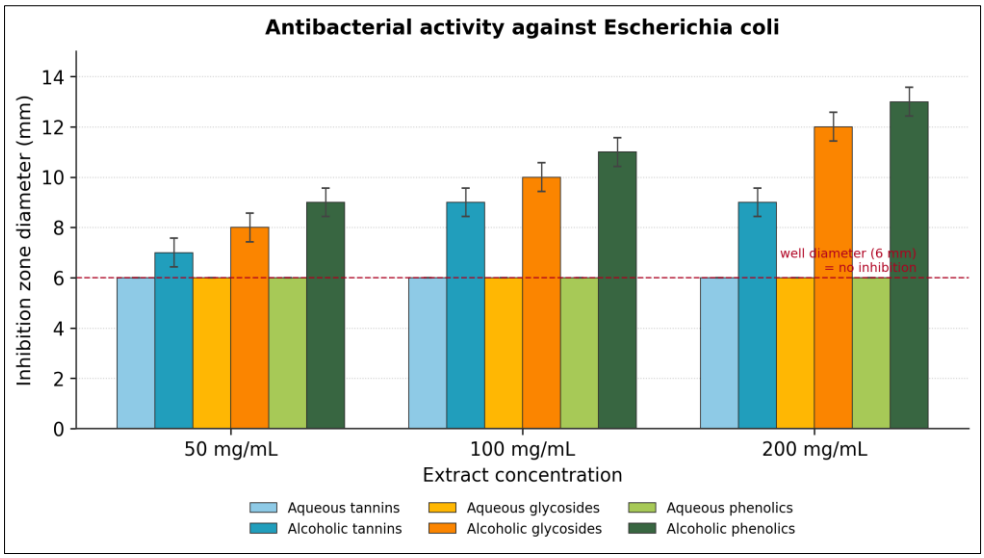

Figure 3: Inhibition-zone diameters of *A. herba-alba* fractions against *E. coli*. All aqueous fractions remained at the 6 mm well diameter (dashed line), indicating no inhibition; only the ethanolic fractions were active. Error bars denote standard error (n = 3)

3.5 Comparative Efficacy and Dose–Response

A comparison of mean inhibition between organisms (Table 5) showed two clear trends: ethanol was the more effective extraction solvent than water for all phytochemical class and the most effective phytochemical fraction against both pathogens was the phenolic fraction. The dose–response behaviour of the ethanolic phenolic fraction was close to linear over the tested range for both organisms (Figure 4), and the activity of the ethanolic phenolic fraction was relative to the reference antibiotics is presented in figure 5. On an overall basis, S. aureus was more susceptible than E. coli, consistent with the differences in the structure of the cell envelope of Gram-negative bacteria, as described below.

Table 5: Comparative mean inhibition-zone diameters (mm) of the six *A. herba-alba* fractions against *S. aureus* and *E. coli* (pooled across concentrations). The ethanolic phenolic fraction (highlighted) was the most active against both pathogens

Extract fraction	Mean zone vs S. aureus (mm)	Mean zone vs E. coli (mm)
Aqueous tannin	11.0	6.0 (no inhibition)
Ethanolic tannin	12.0	8.33
Aqueous glycoside	11.0	6.0 (no inhibition)
Ethanolic glycoside	12.0	10.0
Aqueous phenolic	11.33	6.0 (no inhibition)
Ethanolic phenolic	**14.0**	**11.0**

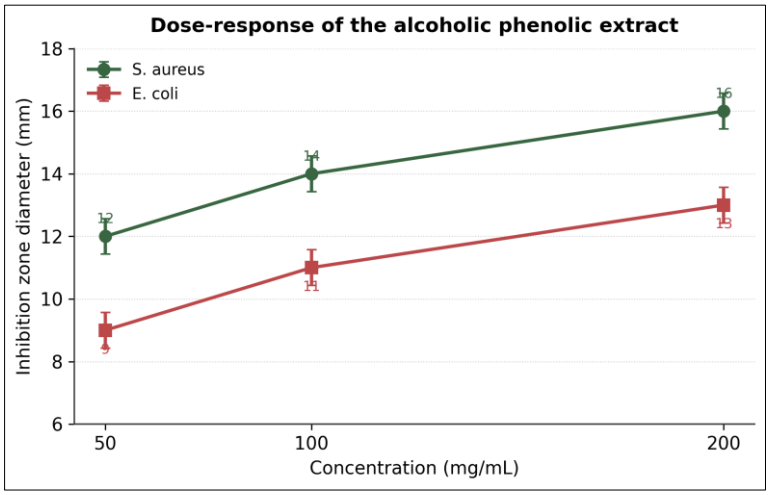

Figure 4: Dose–response of the ethanolic phenolic fraction against *S. aureus* and *E. coli* over the 50–200 mg/mL range (mean ± SE, n = 3)

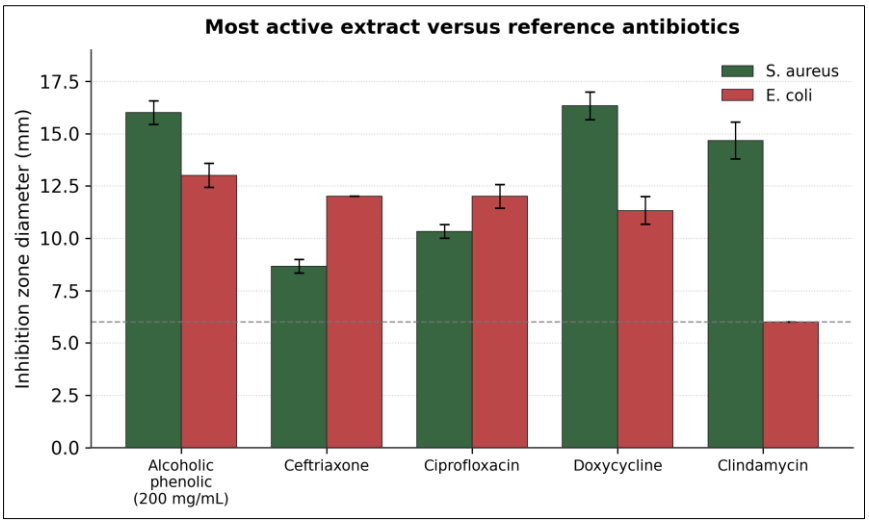

Figure 5: Activity of the most potent fraction (ethanolic phenolic, 200 mg/mL) relative to the four reference antibiotics against *S. aureus* and *E. coli*. The dashed line marks the 6 mm well diameter (no inhibition)

4. DISCUSSION

The present results confirm the solvent and organism dependent antibacterial activity of Artemisia herba-alba which was the most effective against both Gram-positive and Gram-negative pathogen when used as ethanolic phenolic fraction. All the fractions inhibited S. aureus in a dose-dependent manner, which is in accordance with the known susceptibility of Gram-positive bacteria, which have a comparatively less complex outer membrane barrier that allows the phenolics and other amphiphilic metabolites to penetrate to their intracellular and membrane targets (Cowan, 1999; Mohammed *et al*., 2021).

A significant result from the E. coli assays was the full resistance to all aqueous fractions (tannin, glycoside and phenolic) with a 6.0 mm value being obtained for all concentrations. The ethanolic phenolic fraction, however, showed remarkable suppression of 13.0 ± 0.57 mm at 200 mg/mL, compared to the activities of ciprofloxacin (12.0 ± 0.57 mm), ceftriaxone (12.0 ± 0 mm) and doxycycline (11.33 ± 0.66 mm), with clindamycin being inactive (6.0 ± 0 mm).

The resistance of E. coli to the aqueous fractions is intrinsic, which is related to the selective permeability of the outer membrane of Gram-negative bacteria (Aldhaher, 2021). The outer membrane of gram-negative bacteria is tightly packed with lipopolysaccharide (LPS) which is cross-linked by divalent cations to create a hydrophilic barrier, which prevents entry of foreign molecules, and is supplemented by active efflux mechanisms that pump polar molecules out of the cell (Aldhaher, 2021; Mohammed *et al*., 2021). This leaves behind a large amount of polar, high molecular weight constituents which remain in the aqueous fractions and are therefore not able to reach the cytoplasmic membrane.

The antimicrobial activity of ethanolic phenolic fraction against E. coli is attributed to its higher content of low molecular weight (LMW), amphiphilic flavonoids and phenolic acids which are more easily extracted in organic solvent. These compounds are permeabilising agents which disrupt LPS layer and allow bioactive compounds to reach the intracellular targets (Mohammed *et al*., 2021). The enrichment of total polyphenols, flavonoids and tannins in ethanolic and methanolic extracts of A. herba-alba compared to aqueous and non-polar extracts is in agreement with previous reports that these extracts possess superior antibacterial and antioxidant activity, which is correlated with the content of total polyphenols and flavonoids (Bora and Sharma, 2011; Abad *et al*., 2012; El Ouardi *et al*., 2024). The fact that an extract containing a crude phenolic fraction may be as effective or more effective than standard antibiotics under in vitro conditions shows that A. herba-alba has the potential to be a source of lead compounds against resistant pathogens (Cowan, 1999).

All these results make A. herba-alba and especially its ethanol-soluble phenolic components a promising agent for the development of antibacterial formulations of plant origin. However, there are some caveats to be noted. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) as well as chromatographic identification of individual active constituents, and testing against a larger set of clinical and multi-drug resistance (MDR) isolates, are important steps to be taken in the future in the development of the activity reported here. In vivo efficacy and cytotoxicity assessment will also be necessary prior to therapeutic translation.

5. CONCLUSION

In this study, Artemisia herba-alba has been fractionated using solvents, with the representative Gram-positive and Gram-negative bacteria being

evaluated for comparative purposes. All the fractions inhibited the growth of S. aureus in a dose dependent manner whilst E. coli was not inhibited by all of the aqueous fractions and only the ethanolic fractions. The most active treatment was the ethanolic phenolic fraction in both organisms, with equal or greater activity than several reference antibiotics. The results obtained in this study proved that ethanol is an efficient extraction solvent to release the antibacterial components of A. herba-alba compared with water, and that the phenolic fraction of this plant is a source of natural antibacterial agents. More research is justified to isolate and characterize the compounds that could be responsible for the bioactivity, determine their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and test their safety and efficacy in vivo for further development of these fractions to a promising product to combat antibacterial resistance.

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