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Assessment of Antimicrobial Properties of *Artemisia annua L.* Solvent Extracts Against Clinical *E. coli* Isolates

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ABSTRACT

Many plants are used to treat specific diseases due to their proven antimicrobial properties. *Artemisia annua*, in particular, has been widely studied for its antimicrobial and antioxidant activities. This research aims to evaluate the antibacterial effects of chloroform, methanol, and ethanol extracts of *A. annua* against twenty clinical bacterial isolates and determine their antimicrobial activity and minimum inhibitory concentration (MIC) using the agar disc diffusion method. The findings revealed that all twenty isolates were identified as *E. coli*. Out of these, six isolates (30%) exhibited multi-drug resistance (MDR) to three or more different classes of antibiotics. The ethanolic extract of A. annua showed a greater effect compared to the methanolic and chloroform extracts on the selected isolates, with isolate E.18 demonstrating a 22 mm zone of inhibition. Gas chromatography-mass spectrometry analysis of the extract revealed the presence of tannins, alkaloids, amino acids, phenolic compounds, and terpenoids. Among the tested extracts, the ethanolic extract showed the most potent MIC results at a concentration of 60 mg/ml. These results indicate that *Artemisia annua* extracts could be useful as medicinal agents or preservatives.

Keywords: Multi-Drug Resistant *E. coli*, *Artemisia annua*, antimicrobial activity

1. Introduction

The escalating global challenge of antibiotic resistance poses grave concerns, leading to adverse patient outcomes and amplified healthcare expenses. Gram-negative bacteria, particularly Enterobacteriaceae, are of particular concern due to the limited therapeutic options available for multidrug-resistant strains and the dearth of new drug development (Ibrahim et al 2020). In Egypt, combating severe bacterial infections attributed to Enterobacteriaceae, including Escherichia coli, has become increasingly challenging due to a substantial upsurge in resistance to commonly prescribed antibiotics like tetracycline and sulfamethoxazole. Furthermore, there has been a noteworthy surge in resistance to ampicillin, chloramphenicol, gentamicin, ciprofloxacin, trimethoprim/sulfamethoxazole, and tobramycin in recent years (Masoud et al 2021; Shabaan et al 2014).

Globally, there is a growing trend in the utilization of traditional medicinal plants to address various health conditions. In Africa, nearly 80% of the population depends on medicinal plants for their primary healthcare needs. Similarly, in China, around 30–35% of traditional herbal plants serve medicinal purposes. The *Artemisia* species is in South Africa, South America, and the United States. Notably,

Artemisia is recognized as one of the largest genera within the Asteraceae family, with widespread distribution across the northern hemisphere (Salmerón et al 2020).

Incorporating medicinal plants with active pharmaceutical and nutritional compounds can improve treatment methods and may even replace conventional therapies (Shaaban et al 2011). The World Health Organization reports that in developed countries, 80 percent of the population now uses traditional medicine. *Artemisia*, known scientifically as Asteraceae annua L. and part of the Asteraceae family, is a key herb genus. It includes many species mainly found in northern temperate regions, where annual rainfall ranges from zero to 50 cm.

Artemisia annua L., commonly known as sweet wormwood, is a plant belonging to the Asteraceae family, found growing wild primarily in Asia, particularly in China, Japan, and Korea. It has also been introduced and cultivated in several other countries. Traditional Chinese herbalists have historically utilized *A. annua* L. for treating a wide array of health conditions. Presently, research efforts are directed towards elucidating the mechanisms of action of *A. annua* L. and the antimalarial properties of artemisinin, its active compound. Furthermore, in recent decades, studies have investigated the potential therapeutic effects of *A. annua* L. on various ailments, including inflammatory and cancerous conditions, as well as infections caused by viruses, *bacteria*, and parasites (Golbarg et al 2021).

Our study aimed to explore the prevalence of antibiotic resistance among *E. coli clinical* isolates from hospitals. We sought to phenotypically characterize these isolates and emphasize the importance of using conventional antimicrobials wisely to combat multidrug resistance (MDR). Additionally, we evaluated the primary biological activity by comparing various extracts from the leaves of *Artemisia annua* using the agar well diffusion method.2. Material and methods

Specimen collection

Between March 2023 and November 2023, a total of 30 clinical isolates were collected from various hospital laboratories in Menoufia main hospitals. The specimens were aseptically transported to the laboratory under cold conditions for analysis. These clinical isolates were obtained from different sources, including urine (12 isolates), pus (8 isolates), and sputum (10 isolates), across various hospitals, units, and infection sites.

Isolation and biochemical identification

To isolate and characterize E. coli, samples underwent initial culturing in nutrient broth at 37ºC for 18-24 hours. Subsequently, isolates streaked onto MacConkey, brilliant green, and EMB agar plates to facilitate observation of colony morphology. Colonies exhibiting characteristic features of E. coli were further subcultured on EMB agar to obtain pure cultures. Motility was evaluated using the hanging drop technique. Identification of the isolated bacterial strains involved a series of tests, including the Indole Test, Voges-Proskauer Test, Triple Sugar Iron Agar Test, Methyl Red Test,and Catalase Activity Test.

Antibiotic Susceptibility Testing of E. coli Isolates

Antibiotic susceptibility testing was carried out using the disc diffusion method, known as the Kirby-Bauer technique, on Mueller-Hinton agar in accordance with the Clinical and Laboratory Standards Institute guidelines. *E. coli* isolates were tested against 17 different antibiotics spanning six antibiotic classes, utilizing commercially available antimicrobial discs. The susceptibility profiles of the isolates were assessed based on the zones of inhibition, as per the standard database guidelines (Shahid & Umar, 2015) (Shaaban et al,2020)

Plant material

Artemisia annua L. leaves were purchased from market for the food industry and natural products in Egypt.

Methanol Extraction of *Artemisia annua*

Dried plant leaves were mechanically ground, and 2 grams of this material were extracted with 20 milliliters of methanol for 24 hours at room temperature. Subsequently, the extracts were gently heated. After this process, the extracts were filtered. The filtrates were evaporated in an incubator at 30°C. The resulting dried methanol extracts were resuspended in either 10 milliliters or 5 milliliters of distilled water to create different dilutions (Thangjam et al 2020).

Chloroform Extraction of *Artemisia annua*

About 10 grams of *Artemisia annua* plant material was immersed in 100 ml of chloroform (Merck) for two weeks, with the mixture being agitated twice daily. After the two-week period, the mixture was filtered, and the filtrate was evaporated

using a rotary evaporator to yield a gummy residue (Figure 1). The extracts were then stored in a sterile glass bottle at room temperature under standard temperature and pressure (STP) (Khan et al 2022).

Figure 1. E. coli on macconky agar as a model.

Ethanolic extract of Artemisia annua

The leaves underwent cleaning and drying in an oven set at 50°C for three days. Following this, the dried plants were finely powdered using a sterile mortar. Subsequently, 20 grams of A. annua powder was mixed with 100 ml of 70% ethanol alcohol and shaken intermittently at room temperature for 48 hours. The resulting extracts were filtered through Whatman No. 4 filter paper, and the filtrate was then stored at 4°C until its inhibitory effect was evaluated (Shaaban et al 2024).

Assessment of the antibacterial activity of *Artemisia annua* extracts in vitro

The agar well diffusion method, with slight modifications from the protocol by Ullah and Alqahtani (2022), was used to evaluate antimicrobial activities. Mueller Hinton agar was poured into glass plates and left to solidify. The surface of the agar was then streaked with a sterile cotton swab containing the reference bacterial strain. Wells were created in the agar using a sterile 6 mm diameter cork borer. Each well was filled with 100 µl of diluted crude extracts, and the plates were left to stand for 30 minutes before further analysis (Shaaban and El-Sharif 2001).

Determination of minimum inhibitory concentration (MIC) of *Artemisia annua* extracts

The effectiveness of various extracts against the multi-drug resistant (MDR) E.18 isolate was evaluated by applying three treatments at different concentrations: 10, 30, 60, 90, and 120 mg/mL. Fresh culture (0.1 O.D. value at 600 nm) was added to the tubes, which were then incubated for 24 hours at 37°C. The bacterial growth turbidity was measured using a spectrophotometer at 620 nm. The minimum inhibitory concentration (MIC) was identified as the lowest dilution that completely inhibited visible bacterial growth (Navarro et al 2021).

Gas chromatograph (GC-MS) analysis of Artemisia Annua

The ethanolic extract of *A. annua* was analyzed using GC-MS. Components were identified by comparing retention times and spectra with the WILEY 09 and NIST 14 databases (Shaaban et al 2024).

3. Results

Out of the 30 samples examined, 20 isolates were identified as *E. coli* as shown in (Table 1) and (Figure 2). The results confirmed the presence of *E. coli* through a series of tests. Isolates Were gram-negative bacilli, short rods, pink, arranged singly or in pairs. The motility test indicated motility, and the TSI agar slant test showed a yellow slant and butt, gas production, and no black precipitate. Carbohydrate fermentation tests demonstrated acid and gas production in various sugars. The catalase test produced bubbles, and the methyl red test yielded a red color, and the indole test showed a red ring, confirming the presence of *E. coli*.

In the study, conventional Beta-lactams, such as ampicillin, exhibited a high resistance rate, with 95% of E. coli clinical isolates showing resistance. In contrast, resistance to cephalosporins and newer generations of Beta-lactams ranged from 15% to 45%. Notably, all isolated E. coli strains were susceptible to imipenem. Resistance to antibiotics from the tetracycline and macrolide classes was observed in approximately 25% to 40% of isolates. A range of 5% to 10% of isolates displayed resistance to quinolones or aminoglycosides. Polymyxin-B showed the lowest resistance, with only one E. coli isolate being resistant. Additionally, multi-drug resistance was identified in 30% of the isolates, specifically in six of the twenty isolates examined. (MDR) to three or more than different classes of antibiotics.

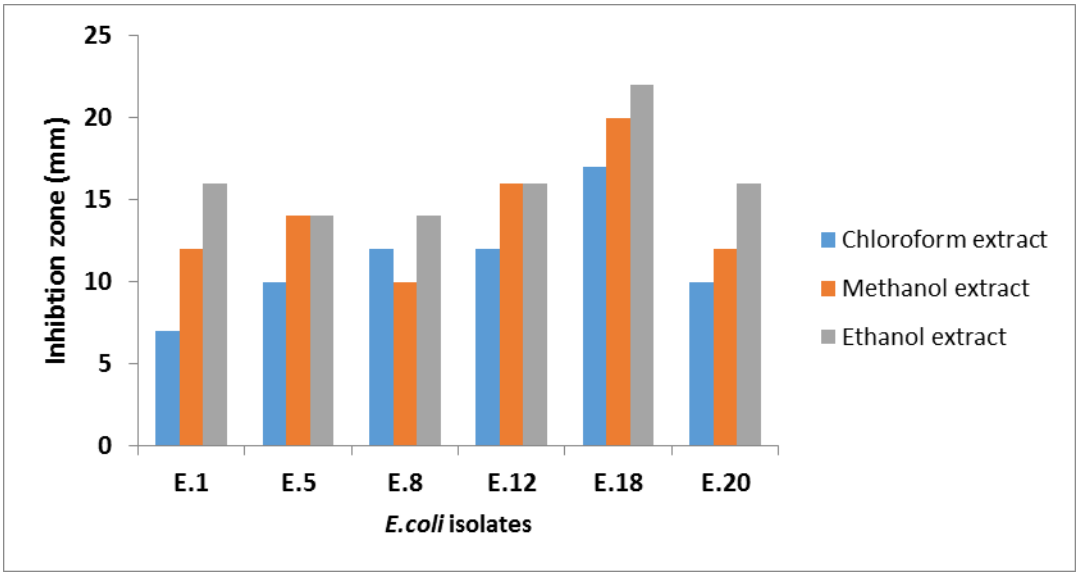

Figure 2. Antimicrobial activity of the organic extracts of *Artemisia annua* using the method of disk diffusion in the agar (inhibitory zone diameter in millimeter)

Table1. Biochemical identification tests of E. coli

Isolate	Gram's Staining	Motility Test	TSI gar Slant Test	Carbohydrate Fermentation Tests	Catalase Test	Methyl Red Test	Voges-Proskauer Test	Indole Test
E.1	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
E.2	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
E.3	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
E.4	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
E.5	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
E.6	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
E.7				+ve	-ve	-ve	-ve	-ve
E.8	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
E.9	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve
E.10	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
E.11	-ve		-ve	-ve	-ve	+ve	+ve	+ve
E.12	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve
E.13	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
E.14	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
E.15	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
E.16	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
E.17	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
E.18	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
E.19	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
E.20	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve

Results in **(Table-3)**, showed the most sensitive MIC value among the examined concentrations of ethanolic extract, and was 60 mg/mL, which reflects the bactericidal activity against the MDR (E.18) isolate within 24 hours. Otherwise, the MIC values of both chloroform and methanolic extract significantly exhibited the same concentration (90 mg/mL).

Phytochemical component of *Artemisia annua ethanolic* extract

The phytochemical analysis of *A. annua* ethanolic extract using GC 1300 mass spectrometer revealed the presence of various compounds including stigmasterol, 1-heptatriacotanol, oleic acid, eicosyl ester, 9-octadecenoic acid, 2-phenyl-1,3-dioxolan-4-yl methylester, cis 9, 9-octadecenoic acid 1,2,3-propanetriyl ester (E,E,E), 1(22),7(16)-diepoxy-, 1,3-benzenedicarboxylic acid bis(2-ethylhexyl) ester, tetracosanoic acid methyl ester, octadecanoic acid 2-hydroxy-1,3-propanediyl ester, 2,3-dihydroxypropyl elaidate, linoleic acid ethyl ester, ethyl iso-allocholate, cyclopropanebutanoic acid methyl ester, oleic acid, docosanoic acid methyl ester, (E)-13-docosenoic acid, glycidyl oleate, stearic anhydride, 1-heptatriacotanol, 2,3-dihydroxypropyl elaidate, oleic acid 3-hydroxypropyl ester, isopropyl linoleate, ethyl oleate, linoleic acid ethyl ester, qinghaosu C, 7-hydroxy-6-methoxy-N-hexadecanoic acid, hexadecanoic acid methyl ester, valerenic acid, and 2,6-diethenyl-1,4-benzenediol.

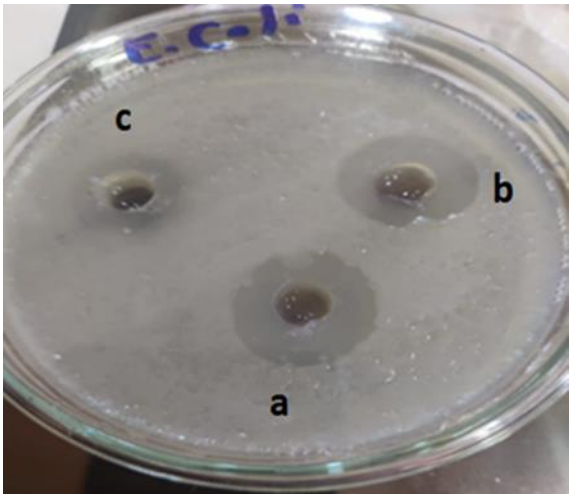

Figure 3. Bacterial growth inhibition zone achieved in agar well diffusion against MDR E.18 isolate a) ethanolic extract b) methanolic extract c) chloroform.

Table 2: Antimicrobial susceptibility pattern of *E. coli* strains isolated from clinical

Antibiotic class	Tested members (conc. µg ml)	Clinical Isolates (20)	
		Sensitive	Resistant
Beta-lactams	Ampicillin (AMP) (30)	10%(2)	95%(19)
	Cefixime (CFM) (30), Cefoxitin (CX) (30),	50%(10)	45%(9)
	Cefotaxime (CTX) (30), Cefuroxime (CXM) (30)	60%(12)	35%(7)
	Imipenem (IMP) (15), Azetronam (AT) (30)	90%(18)	15%(3)
Quinolones	Ciprofloxacin (CIP) (5), Gatifloxacin (GAT) (5), Levofloxacin (LE) (5), Lomefloxacin (LOM) (10),	60%(12)	10%(2)
Aminoglycosides	Amikacin (AK) (30), Kanamycin (K) (30),	85%(17)	5%(1)
Tetracycline	Doxycycline (DO) (5), Tetracycline (TE) (5)	65%(13)	40%(8)
Macrolides	Azithromycin (AZM) (30)	70%(14)	25%(5)
Polymxins	Polymyxin-B (PB) (30)	95%(19)	5%(1)

Table 3. Minimum Inhibition Concentration (MIC)

Treatments	Control	10mg/ml	30mg/ml	60mg/ml	90mg/ml
Optical density (OD) of E18 isolate					
Ethanolic extract	0.61±0.003[a]	0.45±0.008[a]	0.37±0.003[a]	0.23±0.008[a]	0.30±0.02[a]
Methanoic extract	0.61±0.003[a]	0.50±0.017[b]	0.50±0.003[c]	0.45±0.003[b]	0.42±0.003[b]
Chloroform extract	0.61±0.003[a]	0.65±0.006[c]	0.62±0.012[b]	0.59±0.05[c]	0.58±0.001[b]

4. Discussion

In recent times, the emergence of antimicrobial resistance (AMR) in E. coli has emerged as a significant public health issue, leading to treatment failures. The escalation in multi-drug resistance (MDR) within this bacterium primarily stems from the excessive and inappropriate use of antibiotics by healthcare providers. This study delves into the AMR patterns of E. coli strains isolated from clinical specimens in Menoufia hospitals.

Gastrointestinal infections pose a substantial risk to hospitalized patients, with a notable prevalence in Egyptian healthcare facilities, underscoring the importance of thorough investigation. E. coli remains a key culprit in causing enteric diseases within Menoufia hospitals, manifesting symptoms ranging from diarrhea akin to cholera to severe dysentery. Moreover, E. coli infections can extend to the urinary tract, leading to conditions such as cystitis or pyelonephritis, and may result in other extraintestinal infections like septicemia and meningitis. Hospital-acquired infections attributable to E. coli contribute to numerous fatalities annually (Asili et al 2015).

There is a growing interest in exploring the antibacterial properties of herbal extracts, particularly due to their efficacy against multidrug-resistant bacterial strains. In 2015, the Nobel Prize in Physiology/Medicine was awarded for the discovery that artemisinin, a sesquiterpene lactone endoperoxide, is an effective treatment for malaria. Artemisinin, a semi-synthetic compound present in varying concentrations in all Artemisia plants, serves as the primary active ingredient in Artemisia annua L. (A. annua L.), commonly known as sweet wormwood. Belonging to the Asteraceae family, A. annua L. grows wild in Asia, notably in China, Japan, and Korea, and has been introduced and cultivated in several other countries including Poland, Brazil, Spain, France, Italy, Romania, the United States, and Austria. Chinese herbalists have utilized A. annua L. since ancient times for treating various health conditions. Present research endeavors are concentrated on elucidating the mechanisms of action of A. annua L. and the antimalarial effects of artemisinin. Moreover, recent studies have investigated the potential therapeutic effects of A. annua L. on inflammatory and cancerous conditions, as well as infections caused by viruses, bacteria, and parasites (Mathlouthi et al., 2021).

The concentration of phenolic and flavonoid compounds in Artemisia correlates positively with its antioxidant capacity. Artemisia exhibits numerous health benefits, including antioxidant, neuroprotective, hepatoprotective, anti-inflammatory, renoprotective, gastroprotective, digestive, and antibacterial properties. The antibacterial activity of Artemisia is particularly noteworthy in light of the increasing resistance to antibacterial medications among pathogens.

Our study found that the ethanolic extract of A. annua exhibited higher antibacterial activity compared to methanolic and chloroform extracts. This aligns with previous research indicating varying levels of antibacterial activity across different Artemisia species and extracts. For instance, studies by Erel et al. (2012) and Sengul et al (2011) demonstrated moderate antibacterial effects of A. absinthium L. methanolic and aqueous extracts against E. coli and S. aureus. These findings suggest that the geographical origin of the plant and the type of extract used can influence the antimicrobial activity of wormwood extracts.

Furthermore, recent studies have highlighted the role of specific compounds, such as chlorogenic acid found in certain Artemisia species, in disrupting bacterial cell membranes and inducing cell death. Additionally, the presence of terpenoids, phenolics, and volatile oils in Artemisia extracts contributes to their antimicrobial potency. This was observed in studies by Mamatova et al (2019), Poiata et al (2009), Suresh et al. (2011), Solís et al. (2004), and Gallucci

et al. (2009), which demonstrated significant antimicrobial activity of various Artemisia extracts against clinically important pathogenic bacteria.

Overall, our findings, along with those from previous studies, underscore the potential of Artemisia extracts as effective antimicrobial agents against a range of pathogenic bacteria, including *Klebsiella spp. ESBL, Klebsiella spp. CRE, and E. coli.*

In our study, both ethanol and methanol extracts exhibited notable inhibition against *E. coli.* The phytochemical analysis revealed that the ethanolic extract of *A. annua* was particularly rich in phenolic compounds such as phenolic acids and tannins, holding potential for medicinal applications. The active constituents found in these extracts offer promising alternatives to conventional antibiotic drugs for combating multidrug resistance.

5. Conclusion

In summary, this study emphasizes the pressing issue of antimicrobial resistance (AMR) in *E. coli,* which poses a serious public health concern, especially in healthcare settings like Menoufia hospitals. The escalation of multi-drug resistant (MDR) *E. coli* strains is primarily attributed to the In conclusion, this study highlights the urgent issue of antimicrobial resistance (AMR) in *E. coli*, a significant public health threat, especially in healthcare environments like Menoufia hospitals. The rise of multi-drug resistant (MDR) *E. coli* strains is largely due to the overuse and misuse of antibiotics, emphasizing the need for effective antibiotic stewardship and continuous surveillance. The research also points to the promising antimicrobial potential of *Artemisia annua* extracts against MDR *E. coli*, demonstrating substantial antibacterial activity and suggesting a natural alternative to conventional antibiotics and food preservatives. These findings strongly support further investigation into *Artemisia* extracts for therapeutic use against resistant infections, indicating that natural extracts could be crucial in developing new treatments and preventive measures against MDR bacteria, offering a sustainable and eco-friendly solution to antibiotic resistance.

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