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Evaluation of the Bactericidal Effects of *Agrimonia eupatoria* and *Nepeta crispa* Extracts on Indicator Bacteria

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ABSTRACT

Background: The increasing prevalence of drug-resistant pathogens has drawn researchers' attention to studies on the antimicrobial activity of various substances. This study investigates the antimicrobial effects of the *Agrimonia eupatoria* and *Nepeta Crispa* extracts, which are used as herbal medicines.

Methods: The study investigated the effects of extract concentrations of 10, 20, and 40 mg/mL on three distinct bacterial strains, encompassing both types of Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Shigella*, and *Clostridium perfringens*. The evaluation employed the well diffusion method, supplemented by the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Results: The results showed that the *Nepeta Crispa* extract exhibited no antimicrobial effects at various concentrations, whereas the *Agrimonia eupatoria* extract was effective against all three types of bacteria, with the diameter of the inhibition zones increasing with higher concentrations. At a concentration of 40 mg/mL, the diameter of inhibition against the three types of bacteria was approximately 8 mm. The Minimum Inhibitory Concentration for *Escherichia coli* and *Shigella* was 2.5 mg/mL, and for *Clostridium perfringens*, it was 1.25 mg/mL. The Minimum Bactericidal Concentration for *Escherichia coli*, *Shigella*, and *Clostridium perfringens* was found to be 2.5 mg/mL, 5 mg/mL, and 2.5 mg/mL, respectively.

Conclusion: The extracts of some plants, such as *Agrimonia eupatoria* have antibacterial properties, but their usage of them needs more investigation.

1. Introduction

Bacterial resistance to antibiotics is a global challenge (Saffari et al., 2021). Over the years, antibiotics based on minerals and organic substances have been developed and used to combat infectious diseases. For example, antibacterial resistance in enteric bacteria is rapidly increasing in developing countries. Antibiotic-resistant bacteria are a serious threat to public health and a major factor in healthcare-related mortality and morbidity (Nazemi et al., 2005). These microorganisms possess the genetic ability to transmit and acquire resistance to drugs (Al Laham & Al Fadel, 2014). Consequently, the mortality rates among patients suffering from immune disorders, particularly those infected with new multi-resistant bacterial strains, are rising within hospital settings (Al Laham & Al Fadel, 2014). To address this pressing issue, three primary strategies have been adopted. First, there is an emphasis on the control and optimal utilization of antibiotics, alongside the rational prescription of these medications, particularly within healthcare institutions. Second, extensive research initiatives are being pursued to enhance understanding of the genetic mechanisms underlying antibiotic resistance. To better understand the genetic mechanisms of resistance. Finally, efforts are underway to develop novel pharmaceuticals and antibiotics

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that can effectively combat resistant bacterial strains (Al Laham & Al Fadel, 2014). Plants have long been used as medicines to maintain human health and as complementary therapies. According to the World Health Organization (WHO), medicinal plants are a vital source for the development of various pharmaceuticals. Approximately 80% of people in developed countries use traditional medicines derived from these medicinal plants, underscoring the necessity for rigorous studies on the safety and efficacy of such treatments (Eloff, 1998). Despite their widespread use, many herbal medicines may not adequately fulfill their intended purposes, and there exists insufficient scientific evidence to substantiate their effectiveness. This gap highlights the critical need for comprehensive research to validate the therapeutic claims associated with herbal remedies and to ensure their safe application in health care practices. Since years ago, the use of herbal extracts as therapeutic treatments and as antibiotics has become very common and many studies have been conducted on them (Ikram & Inamul-Haq, 1984; Izzo et al., 1995; Kubo et al., 1993; Martinez et al., 1996). A variety of plants have been identified for their medicinal potential, including *Sudanese plants* (Almagboul et al., 1985)*, Cynodon dactylon essential oil* (Artizzu et al., 1996), *C. macrostachys* (Dubale et al., 2023), *Seriphidium oliverianum* stem extracts (Abbas et al., 2021), *Sisymbrium officinale* (Khalid et al., 2022), *Cistus salviifolius,* and *Punica granatum* (Álvarez-Martínez et al., 2021). Plants produce secondary metabolites in different environmental conditions that have various applications for human health. Among these metabolites, plant antibacterial agents such as phenolic compounds, which are found in essential oils and tannins, are well documented (Janssen et al., 1987; Santos Filho et al., 1990). Antibiotics show their antibacterial properties through various mechanisms, with the most important of which include inhibition of protein synthesis, disruption of metabolic pathways involved in cell wall synthesis, interference with DNA and RNA synthesis, and lysis of the bacterial membrane. On the other hand, mechanisms of antibiotics resistance have been identified, including antibiotic modification by enzymes , inactivation, and the expression of efflux pumps (Kubo et al., 1993). In the realm of plant-based research, various phytochemicals have been recognized as effective antibacterial agents. Recent studies have increasingly focused on the antibacterial potential of plant extracts and essential oils, highlighting compounds such as terpenoids, polyphenols, and alkaloids (Kubo et al., 1993). Polyphenols, in particular, represent a large and diverse group of antibacterial agents, categorized into numerous subgroups. Given the ongoing development of bacterial resistance to these agents, extensive research continues to explore novel antibacterial compounds derived from plants. A significant number of plants have been identified and reported as antibacterial agents, including *Acacia nilotica, Bauhinia kockina korth, Cistus salvifolius* and *Cytinus hypocistis* (Kubo et al., 1993). Studies have indicated that these plants have shown antimicrobial effects with MICs of 125, 80.7, 62.5, and 1560 µg/mL, respectively (Nascimento et al., 2000).

The medicinal plant of *Agrimonia eupatoria L.* (AE) is found throughout Europe, Asia, Africa, and North America. In Iran, it is named Ghafath and is prevalent in various regions, including the northern provinces of Mazandaran and Gilan, as well as in Hamadan and Kermanshah in the west, and central areas such as Merazi Province. AE typically thrives in pastures, along forest edges, and in foothills regions, favoring environments with moderate temperature, light, and humidity. AE is a stable, herbaceous, erect plant with a height of about 30-60 cm, with branched and running roots. In traditional medicine, almost all parts of AE are recognized for their medicinal applications, including the treatment of liver, kidney, lung, and eye infections, as well as diabetes management and cardiovascular disease prevention. The secondary metabolites of this plant include polyphenols and flavonoids such as procyanidin, quercetin, catechin, and kaempferol, tannins, along with triterpenoids (Saffari et al., 2021; Khazaei & Mirazi, 2018).

Nepeta crispa (NC), commonly referred to as Mofrah in Iran, is a perennial herb native to the Hamadan region and belongs to the *Lamiaceae* family. This plant has garnered attention in traditional medicine for its various therapeutic properties, including its ability to strengthen the stomach, provide calming effects, and alleviate flatulence. Additionally, it is considered beneficial in the treatment of nervous and respiratory disorders. As a prominent genus within the *Lamiaceae* family, Nepeta comprises numerous aromatic species known for their medicinal attributes. Globally, there are over 250 species, with 67 identified in Iran. Literature has documented the diverse properties of *Nepeta crispa*, emphasizing its significance in herbal medicine (Badrehadad & Piri, 2014; Sayyari et al., 2022). In this study, extracts of AE and NC plants were studied and their antimicrobial effects were studied. Given that these two plants have not been studied for their antibacterial properties, if they have significant effects, they can be used as new sources for the preparation of antibiotics.

2. Materials and Methods

Preparation of Plant Extracts Dried samples of the AE and NC plants were obtained from the Agricultural Research Center of Hamadan Province. In the laboratory, the plants were washed with double-distilled water. The plants were placed separately on a clean cloth in a clean environment to dry completely. After a few days, once the plants were thoroughly dried, they were ground into a powder. Solutions of 1, 2, and 4 g of powder in 100 mL of distilled water (1%, 2%, and 4%) were made. The solutions were incubated in a water bath at 45 °C for 30 min, then removed and allowed to reach room temperature. Afterward, they were filtered using the Whatman 42 filter paper.

2.1 Cultivation of Microbial Strains

Various bacterial strains, including *Escherichia coli* (PTCC 1399, ATCC 255922), *Clostridium perfringens* (PTCC 1765), and *Shigella dysenteriae* (PTCC 1188) were obtained from

the Industrial Research Center of Iran in lyophilized form. *Escherichia coli* is the best biological drinking water indicator for public health protection. *Clostridium perfringens* is one of the most common causes of food poisoning and can be found on raw meat and poultry, in the intestines of animals, and in the environment, *Shigella spp*. are very significant in food safety and are the most common cause of acute, bloody diarrhea (dysentery) and are responsible for a significant proportion of morbidity and mortality associated with diarrheal disease. After performing the rehydration steps, each bacterium was cultured on an agar medium for use in experiments. Following the rehydration procedures, each bacterial strain was cultured on an agar medium to facilitate subsequent experimental applications. This methodological approach is essential for assessing the pathogenicity and antimicrobial susceptibility of these organisms.

2.2 Preparation of Bacterial Suspension (0.5 McFarland)

To prepare a 0.5 McFarland turbidity standard, a 24-hour culture of each bacterium was grown on Mueller-Hinton agar. After inoculation into a tube containing physiological saline, a microbial suspension equivalent to 0.5 McFarland was prepared. The absorbance of the prepared solution was measured at a wavelength of 620 nm using a spectrophotometer. It was 0.08-0.1 in the prepared solutions according to the method (Krishnan et al., 2015).

2.3 Evaluation of Antimicrobial Effects

In the plates containing Mueller-Hinton agar, 100 µL of the 0.5 McFarland solution was spread evenly across the entire plate using a sterile swab. Subsequently, wells with a diameter of 6 mm were created on the agar surface. Extracts of AE and NC plants, at specified concentrations, were added to the wells. Tetracycline solution and distilled water were used as positive and negative controls, respectively. The plates were incubated at 37 °C for 24 h. All procedures were repeated three times, and the average diameters of the inhibition zones were recorded.

2.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Broth microdilution and disk diffusion susceptibility testing were performed as described by the CLSI (CLSI, 2012a, CLSI, 2012b) (Tato et al., 2014). The MIC test was performed in a sterile 96-well microplate using the microdilution method. A specified amount of Mueller-Hinton broth was prepared according to the protocol. Initially, 100 µL of Mueller-Hinton broth was added to each well. Then, 100 µL of the extracts from the AE and NC plants (40 mg/mL) were added to the first well of each row. Following this, 100 µL were transferred from the first well to the second well, and this dilution process continued through the last well, from which 100 µL were removed. Finally, 100 µL of a microbial suspension equivalent to 0.5 McFarland (10^6 bacteria per mL) was added to all wells. This process was performed for all three bacterial types-*Escherichia coli*, *Shigella*, and *Clostridium perfringens*-and both types of extracts, with three consecutive replicates. Tetracycline solution and distilled water served as positive and negative controls, respectively. The 96-well microplates were incubated at 37 °C for 24 h. The results were read using an Elyza reader at a wavelength between 630-578 nm. After determining the MIC values, 100 µL from each well corresponding to the MIC point and the clear wells were transferred to Mueller-Hinton agar for further cultivation and spreading. These plates were then incubated at 37 °C for 24 h. Subsequently, the results for the MBC were observed and recorded.

3. Results and Discussion

The antimicrobial effects of AE and NC extracts using the well diffusion method are presented in Tables 1 and 2 and Figure 1. The results of the antimicrobial activity tests for AE at concentrations of 10, 20, and 40 mg/mL showed that the inhibition zones increased with increasing concentrations. At the concentration of 10 mg/mL, the bactericidal effect of the extracts was minimal, while at 40 mg/mL, the diameters of the inhibition zones were increased and approximately equal across all three types of bacteria. Tetracycline was used as a positive control, yielding an inhibition zone diameter of approximately 21 mm, while distilled water served as a negative control. The results indicated that the NC extract exhibited no antimicrobial effects at any of the tested concentrations.

The results of the MIC and MBC tests for the bacteria, after serial dilution of the extracts from AE and NC extracts at concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.312 mg/mL, are shown in Tables 3 and 4 and Figure 2. As indicated, NC extract showed no antimicrobial activity at the specified concentrations, while AE extract exhibited a MIC of 0.5 mg/mL against *Shigella* and *Escherichia coli*, and a MIC of 0.25 mg/mL against *Clostridium perfringens*. Additionally, the MBC was found to be 0.25 mg/mL against *E. coli* and *C. perfringens*, and 0.5 mg/mL against *Shigella*, demonstrating a stronger effect on *Clostridium perfringens*.

Table 1. Diameter of inhibition zones (mm) induced by AE extract

Concentration (mg/mL)	*Escherichia coli*	*Shigella dysenteriae*	*Clostridium perfringens*
10	6/5	6/5	6/5
20	7	6/5	6/5
40	8	8	8
Tetracycline antibiotics	21	21	21
Distilled water	ND	ND	ND

ND: Not determined

Table 2. Diameter of inhibition zones (mm) induced by NC extract

Concentration (mg/mL)	*Escherichia coli*	*Shigella dysenteriae*	*Clostridium perfringens*
10	ND	ND	ND
20	ND	ND	ND
40	ND	ND	ND
Tetracycline antibiotics	21	21	21
Distilled water	ND	ND	ND

ND: Not determined

Figure 1 visually represents the antibacterial effects of both plant extracts, highlighting the effectiveness of AE extract in creating inhibition zones, while NC extract shows no inhibition across all tested concentrations. The comparison provides a clear illustration of the disparity in antimicrobial activity between the two extracts.

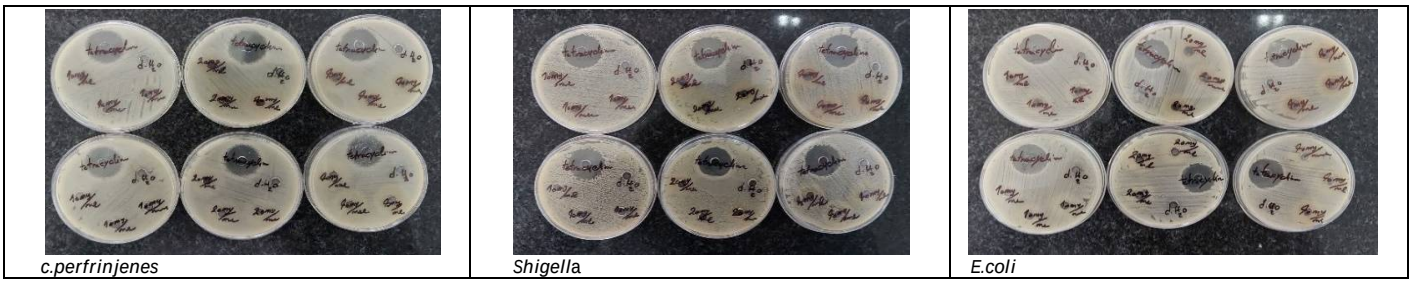

Figure 1. Inhibition zones induced by AE and NC extracts (Top Row: Diameter of inhibition zones (mm) in response to AE extract against *Escherichia coli*, *Shigella dysenteriae*, and *Clostridium perfringens* at various concentrations (10, 20, and 40 mg/mL)). Bottom Row: Diameter of inhibition zones (mm) in response to NC extract against the same bacterial strains at the same concentrations

The results demonstrate the varying levels of antibacterial activity of *Agrimonia* extract against the selected bacterial strains, with specific MIC and MBC values, while *Nepeta* extract did not exhibit any measurable activity.

Figure 2 illustrates the comparative analysis of MIC and MBC values for both AE and NC extracts. The top rows highlight the lack of significant antibacterial activity for *Nepeta* extract, while the bottom rows showcase the effective concentrations of *Agrimonia* extract that inhibit and kill the target bacteria. This visual representation aids in understanding the differential effects of the two extracts on the assessed bacterial strains.

The prolonged use of antibiotics has led to the development of drug resistance, prompting researchers to explore plants for new medicinal compounds that serve as rich sources of various phytochemicals, including polyphenols. Polyphenols are a group of highly hydroxylated phenolic compounds found in various plant extracts, known for their antibacterial activities against a wide range of pathogenic bacteria (Al Laham & Al Fadel, 2014).

Table 3. MIC and MBC results induced by AE extracts

Bacterial Strain	Minimum Inhibitory Concentration (MIC) (mg/mL)	Minimum Bactericidal Concentration (MBC) (mg/mL)
Escherichia coli	2/5	0.25
Shigella dysenteriae	2/5	0.5
Clostridium perfringens	1/25	0.25

Table 4. MIC and MBC results induced by NC extracts

Bacterial Strain	Minimum Inhibitory Concentration (MIC) (mg/mL)	Minimum Bactericidal Concentration (MBC) (mg/mL)
Escherichia coli	ND	ND
Shigella dysenteriae	ND	ND
Clostridium perfringens	ND	ND

This study investigated the antimicrobial effects of extracts from the medicinal plants of AE and NC on the Gram-positive bacterium *Clostridium perfringens* and the Gram-negative bacteria *Shigella* and *Escherichia coli*, which are significant contributors to water and food contamination. The results indicated that the NC extract showed no antibacterial activity at the tested concentrations across all assays, including the determination of inhibition zones and MIC and MBC. Conversely, the AE extract demonstrated a slight increase in inhibition zone diameters with increasing concentrations, indicating its effectiveness against all three bacterial types. However, at the initial concentration of 10 mg/mL, the inhibition zone was minimal, measuring less than one mm. The evaluation of MIC revealed that the Gram-positive bacterium *Clostridium perfringens* exhibited the highest sensitivity among the tested bacteria.

Some plant metabolites can exhibit antimicrobial activity. This is particularly relevant for Gram-positive bacteria, which possess a single layer of peptidoglycan in their cell membrane, making them more sensitive to antimicrobial agents. In contrast, the cell membrane of Gram-negative bacteria is characterized by a more complex structure, featuring a layer of lipopolysaccharides and phospholipids, resulting in lower permeability and greater resistance to hydrophobic antimicrobial compounds (Barzegar & Alizadeh Behbahani, 2023). Overall, while the AE extract shows potential antimicrobial properties, and NC extract did not demonstrate any significant antibacterial effects, further research is needed to explore the mechanisms of action of these extracts and their potential applications in combating antibiotic-resistant bacterial strains.

The effects of the extracts investigated in this study were compared with results from studies on other plants. The findings were consistent with some studies while differing from others. In some studies, the lack of antibacterial properties of some plant extracts is reported. For instance, Nazemi et al. (2005) reported that the aqueous extract of *heracoleum persicum* exhibited no antimicrobial effects on the tested strains. In a study conducted by Salmaniann et al. (2023), the aqueous extract of *Citrullus colocynthis* was investigated for its effects on *Escherichia coli* at two concentrations of 0.5 and 1 mg/mL. The results indicated that there was no antimicrobial effect at the lower concentration, while significant bacterial growth inhibition was observed only at the higher concentration. Pajohi-Alamoti et al. (2015)

studied the effects of sumac fruit extract against *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli*. Their findings showed that as the concentration of the extract increased, the antibacterial activity also improved, effectively inhibiting bacterial growth. Das et al. (2012) studied the antimicrobial effects of fresh wheatgrass aqueous extract on the growth of four bacterial strains (two Gram-positive and two Gram-negative) and one fungus, including foodborne pathogens. The study revealed that the extract was effective against all tested organisms, with Gram-positive strains exhibiting greater sensitivity than Gram-negative strains. Among the Gram-positive organisms, *Bacillus cereus* was identified as the most sensitive, followed by *Staphylococcus aureus*, while *Escherichia coli* (a Gram-negative bacterium) showed the lowest sensitivity. Moazami et al. (2012) examined the antimicrobial effect of saffron petal aqueous extract on *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, and *Bacillus cereus*. The study found *Salmonella typhimurium* to be the most sensitive bacterium, while *Staphylococcus aureus* and *Escherichia coli* were identified as the most resistant.

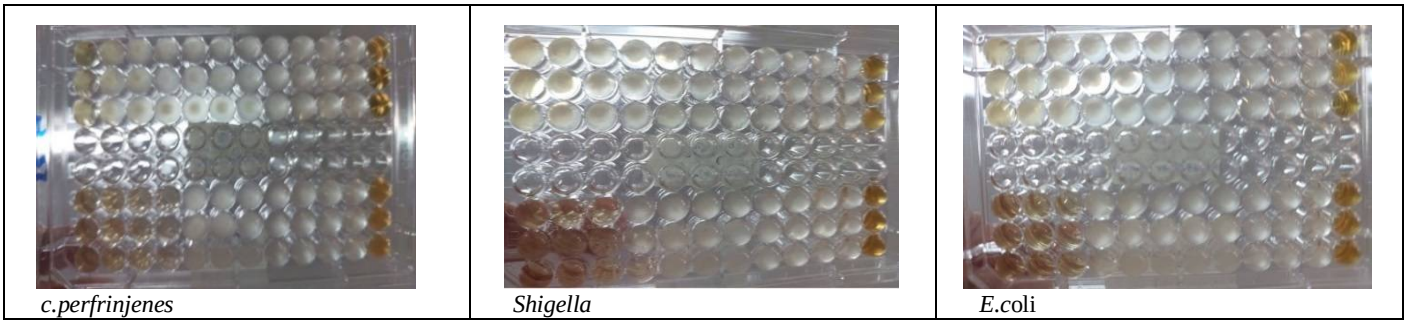

Figure 2. Results of MIC and MBC of AE and NC extracts. (Top Three Rows: Data corresponding to *Nepeta* extract, showing the results for MIC and MBC against *Escherichia coli*, *Shigella dysenteriae*, and *Clostridium perfringens*. Bottom Three Rows: Data corresponding to AE extract, displaying the MIC and MBC results for the same bacterial strains)

4. Conclusion

The results of the present study indicated that the extract of the *NC* plant, at the specified concentrations and using the outlined method, exhibited no antimicrobial effects. In contrast, the extract of *the AE* plant demonstrated increased antimicrobial activity with higher concentrations and effectively inhibited the growth of all three types of bacteria, especially the Gram-positive bacterium *Clostridium perfringens*.

Authors' Contributions

Zohre Farahmandkia: Conceptualization; Formal analysis; Writing-original draft. **Somayeh Zolghadr, Sara Fathi:** Methodology; Data curation; Investigation; Formal analysis. **Mohammad Reza Mehrasbi:** Methodology; Validation; Supervision. All authors reviewed and approved the final manuscript.

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Conflicts of Interest

The authors declare they have no conflict of interest.

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Ethical considerations

This study was approved by the Ethics Committee of Zanjan University of Medical Science. (IR.ZUMS.BLC.1401.048).

Using artificial intelligence

Artificial intelligence was not used in this research.

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