Archives of Razi Institute, Vol. 78, No. 1 (2023) 205-211

DOI: 10.22092/ARI.2022.358487.2228

Original Article

Antibacterial and Antioxidant Activity of *Ziziphora clinopodioid* Lam. (*Lamiaceae*) Essential Oil

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Received 10 April 2022; Accepted 7 May 2022
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Abstract

The genus *Ziziphora* belongs to medicinal plants. It is often used as a stomach tonic, carminative, antimicrobial, and expectorant; the extracted essential oils can be used as a second line of defence against pathogens. This study aimed to determine the antioxidant activity of essential oils of *Z. clinopodioides* as well as antibacterial activity against foodborne pathogens *(Bacillus* sp., *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas* sp.). The antibacterial activity of *Z. clinopodioides* essential oil was determined using the microdilution (M.D.) method in the nutritional broth medium and the agar disk diffusion assay. The result demonstrated that essential oil exhibit solid antibacterial properties against both gram-positive and gram-negative bacteria. Sequentially regarding MIC and MBC values, *Escherichia coli* was a higher level of resistance to the essential oil compared to *Bacillus* sp. Our findings suggested that the essential oil of *Z. clinopodioides* could be used as an antibacterial agent. The total antioxidant capacity of *Z. clinopodioides* leaves was assessed as ascorbic acid equivalents per gram of the leaves essential oil extract. The total antioxidant capacity was determined using ascorbic acid ($y=0.1185x + 49.508$, $R^2=0.3877$). While the result of *Z. clinopodioides* was ($y=0.1372x + 40.032$, $R^2=0.4503$).

Keywords: *Ziziphora clinopodioides*, Essential oil, Antibacterial, Antioxidant activity

1. Introduction

Medicinal herbs are the "backbone" of traditional medicine. Medicinal herbs were commonly used by people in developing countries (1). According to the history of human culture and civilization in Egypt, Assyria, China, and Indie's valley, the elders and wise men used medicinal herbs to heal various ailments. Ancient literature, mythological legends, folklore, medical treatizes, epic ballads, and thousand-year-old manuscripts all include information on these medicinal plants (2, 3). Many believe "green medicine" is safer and more reliable than expensive synthetic drugs, which may have adverse side effects (4). As a result of the indiscriminate use of commercial antimicrobial drugs, which are commonly used to treat infectious diseases, human pathogenic bacteria have developed diverse medication resistance (5). In the last several years, screening essential oils of medicinal plants has been a popular and scientific interest worldwide (6). Essential oils are “natural preservatives” and can be used to control pathogens (7). Essential oils have been utilized as bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, and antimicrobial agents. In addition, it is employed in the pharmaceutical, agricultural, and food industries (8). Essential oils found in medicinal plants have antibacterial properties and can be used to combat pathogenic microbes (9), *Z. clinopodioides*is belong to the Laminaceae family and is widely distributed in Iran, Turkey, and Iraq, specifically Kurdistan; its common Persian name is

"Kakuti-e-kuhi", and it is also known as a "Field Mint' (10). In traditional medicine, *Z. clinopodioides*is used as an expectorant, stomach tonic, carminative, and antistress action of the central nervous system depression (11, 12).

The main compounds of the essential oil are phenolic chemicals; higher concentrations of phenolic chemicals are thought to have stronger antibacterial actions against microorganisms, disrupt bacterial cell membranes and inhibit their proliferation, such as Carvacrol (65.22%), thymol (19.51%), p-cymene (4.86%) and ɣ-terpinene (4.63%). Geographical conditions, climate, seasonal oscillations, and the stage of plant development can all influence the chemical composition of plant essential oils and spices (13, 14).

Several investigations have shown that thymol and Carvacrol have antifungal properties against fungal infections. Thymol is more effective than Carvacrol. Thymol's low toxicity and pleasant odour and taste indicate that it can be employed as a preservative to avoid bacterial deterioration. Some researchers believe that thymol's antibacterial action involves changes in membrane permeability and intracellular content escape. In addition, Carvacrol is effective at causing spores to germinate and mycelium to proliferate. Individually and in combination, carvacrol and p-cymene work as a unique technique for regulating Escherichia coli. Carvacrol or p- cymene reduced the numbers of *E. coli* O157:H7 (18), p- cymene alone does not affect very well, but when combined with each, Carvacrol and thymol have a synergistic effect as an antimicrobial (15, 16). The acidic nature of Carvacrol and thymol's hydroxyl group, as well as their role in hydrogen bond formation, is the most fundamental explanation for their high antibacterial actions (13).

This study aimed to determine the antioxidant activity of essential oils of *Z. clinopodioides* as well as antibacterial activity against foodborne pathogens (*Bacillus* sp., *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas* sp.).

2. Materials and Methods

2.1. Plant Samples

Z. clinopodioides aerial parts were taken locally in August 2018 during its flowering stage from Penjwin (Kurdistan region, north of Iraq (Figure 1).

Figure 1. A) *Z. clinopodioides;* **B)** Essential oil droplets of *Z. clinopodioides* on the aerial part (leaf) were observed under dissecting microscope

2.2. Essential Oil Plant Extraction

The Clevenger apparatus was used in essential oil extraction from the plant, which exists in models (Figure 2). The leaves of the areal plants (leaves) were dried at room temperature before being ground in a grinder machine. Leaves were added to 100 mL of water as a solvent. The apparatus contains a varying size flask that holds boiled water and the plant to be extracted. The oil floats on top of the water, which is slowly returned to the heated flask via the diagonal conduit; after 6 hours of extraction, the essential oil is collected in the burette (2).

Figure 2. Clevenger apparatus for essential oil

2.3. Bacterial Strains

Four different types of bacterial species were used: *Bacillus* sp., *E. coli* (ATCC:8739), *and Pseudomonas* sp. (ATCC: 9027). Moreover, *Staphylococcus aureus* (ATCC: 6538) was cultivated on nutrient agar and incubated at 37 °C for 24 hours.

2.4. Antimicrobial Activity Analysis

The disc diffusion method was used to test the antibacterial activity of the essential oil from *Z. clinopodioides* using 0.5 McFarland standard bacterial strains suspension spread on Muller-Hinton agar (MHA) medium. Wells were made on agar and treated with 30 µl of essential oil; another was filled with 30 µl of D.W. as a negative control and a reference antibiotic (tetracycline 10 µg/disc). Positive control was utilized. The diameter of the clear zone surrounding the disc was measured and expressed in millimetres as its antibacterial activity after 24 hours of incubation at 37°C (17).

2.5. Establishment of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The bacterium's inoculum (*Escherichia coli* and *Bacillus* sp.) was prepared from suspensions, and turbidity was set at 0.5 McFarland standard. The MIC of *Z. clinopodioides* essential oil against bacterial strains was calculated using a 96-well micro-well dilution method plate. The stock solution was prepared by mixing 20 µl of *Z. clinopodiodes* essential oil with 20 µl of %20 tweens. The 12-well plates were made by pouring 90 µl of nutrient broth and 5 µl of inoculum into each well. Then 100 µl of their serial dilutions were put into ten wells in a row. As a negative control, a well containing each had a final volume of 135 µl. The contents of each well were stirred for 20 seconds on a plate shaker at 300 rpm. A total of 135 µl of nutritional broth and inoculum solely without oil was employed. The final capacity of each well was 135 µl. On a plate shaker set to 300 rpm, the contents of each well were swirled for 20 seconds and then incubated at 37°C for 24 h. The absorbance at 600 nm was used to measure microbial growth. The essential oil's MIC was determined to be the lowest concentration that inhibited growth. In triplicate experiments, the MICs for each test microorganism were determined. To confirm MICs and to establish MBCs, 20 µl of the solution was extracted and inoculated on Muller-Hinton agar (MHA) plates. The agar plates were incubated at 37°C for 24 hours before being checked for growth. MBC was the lowest concentration at which organism growth was observed (18). All procedures were carried out in triplicate.

2.6. Determination of Antioxidant Activity by DPPH Assay

The DPPH free radical scavenging activity (RSA) of *Z. clinopodioides* leaves essential oil was determined by Elshikh (18) with some modifications. The *Z. clinopodioides* leaves essential oil extract was prepared in various concentrations (0, 10, 15, 50, 200, 500) µg/ml diluted with methanol. 0.004 mg DPPH was dissolved in 100 mL methanol. A spectrophotometer was used to compare the absorbance at 517 nm to the control after a 30-minute incubation at room temperature. For the standard, ascorbic acid was used in triplicate.

The following formula was used to compute the percentage of free radical DPPH antioxidant activity:

Antioxidant activity (Inhibition) %=[($A_{control}$–A_{sample}) /$A_{control}$] X 100 Where: $A_{control}$ is the absorbance of the control reaction, and A_{sample} is an absorbance in the presence of extract.

3. Results and Discussion

3.1. Antibacterial Activity of Essential Oil

The antibacterial activity of *Z. clinopodioides* essential oil is shown in table 1. The essential oil of *Z. clinopodioides* was found to be effective against all of the microorganisms tested (Figures 3, 4, 5 and 6) when both *Bacillus* and *Escherichia coli* showed inhibition zones (all replicates) for each strain using well diffusion method procedure.

Table 1. The essential oil action *Z. clinopodioides* against four tested bacterial species

Bacteria	*Bacillus* sp	*Escherichia coli*	*S. aureus*	*Pseudomonas*
Essential oil	5.48±0.36	2.90±0.38	4.88±0.75	5.8±0.14
SEM	0.2088	0.2204	0.434294	0.255767

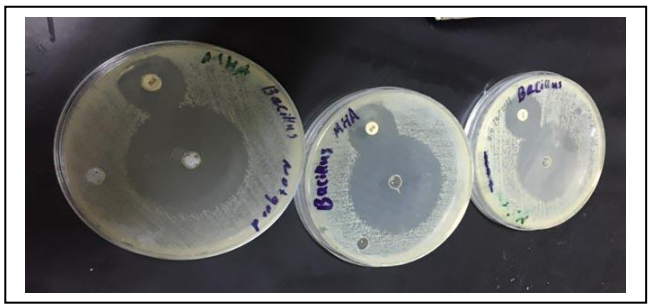

Figure 3. The inhibition zone of *Bacillus* sp. in (triplicates)

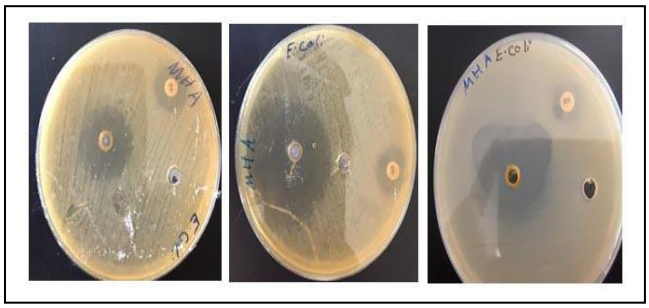

Figure 4. The inhibition zone of *Eacherichia coli* in triplicates

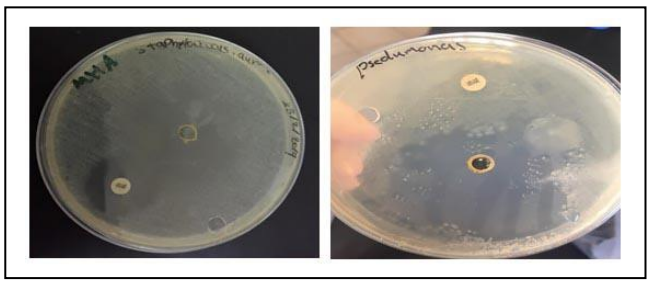

Figure 5. The inhibition zone of *Staphylococcus aureus* and *Pseudomonas* sp

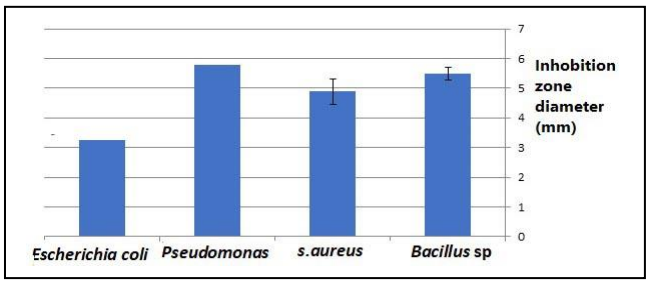

Figure 6. Inhibition zone diameter (mm) of essential oil action against four different microorganisms (mm)

3.2. Calculation of the MIC and MBC Values

The MIC value of *Z. clinopodioides* essential oil for all test bacteria is displayed in table 2; figures 7 and 8. The essential oil inhibited *B. subtilis* growth in the fifth dilution (MIC=0.031 g/mL). And *Escherichia coli* (MIC=0.25 µg/mL) in the second dilution. Gram-positive bacteria were more sensitive during the MIC test than Gram-negative *E. coli*. In MBC, the essential oil killed 99.9% or more of *E. coli* in the first dilution (MBC=0.5 g/mL); while the essential oil did not kill *Bacillus* at any concentration, this indicates that the MIC was not equivalent to the MBC for *E. coli* and *Bacillus* sp. MBC was unable to obtain, indicating that the oil has a bactericidal effect.

Z. clinopodioides are widely employed in traditional medicine. Essential oils have long been used as flavouring ingredients in food and beverages, and they must act as natural agents for food preservation due to the presence of antimicrobial components (16). Furthermore, for removing pathogenic germs, researchers have been interested in biologically active chemicals extracted from plant species because of their resistance to antibiotics (3). The essential oil's antibacterial activity could be linked to the presence of some compounds such as pulegone content, piperitenone and 1-8 cineole. These components have been shown to have substantial antibacterial properties (19). Pulegone is also a prominent component of *Ziziphora* species (*Z. hispanica*, *Z. brevicalyx* and *Z. tenuior*) (20), pulegone essential oil compound was (34.4%) in *Z. clinopodioides* Lam. *Z.hispanica* (78.6%), Ziziphorabrevicalyx ((75.0–88.0), and *Z. tenuior* (71.2–85.3) (21).

Table 2. Average and standard deviation of MIC and MBC for both *Bacillus* sp. and *Escherichia coli* in each dilution

M. O	1	2	3	4	5	6
Bacillus	0.044±0.0066	0.057±0.0065	0.077±0.012	0.086±0.006	0.151±0.077	0.1356±0.012
E. coli	0.077±0.0025	0.0853±0.005	0.0976±0.0025	0.133±0.017	0.143±0.081	0.149±0.0075

Figure 7. MIC and MBC for *Bacillus* sp. (triplicate)

Figure 8. MIC and MBC for *Escherichia coli*i (triplicate)

3.3. DPPH Radical-Scavenging Antioxidant Assays of *Z. clinopodioides* Leaves Essential Oil

The inhibitory activity of *Z. clinopodioides* leaves essential oil was determined using five different concentrations (Figures 9 and 10). The results demonstrated that radical scavenging activity increased with increased concentrations; the inhibition percentage of *Z. clinopodioides* leaves was 95.77% in 500 µg/mL, compared with standard ascorbic acid was 97.33 % (Figure 9).

The total antioxidant capacity of *Z. clinopodioides* leaves was assessed as ascorbic acid equivalents per gram of the leaves essential oil extract. The total antioxidant capacity was determined using ascorbic acid ($y=0.1185x + 49.508$, $R^2=0.3877$). While the result of *Z. clinopodioides* was ($y=0.1372x + 40.032$, $R^2=0.4503$) (Figure 10). According to our results, the DPPH radical scavenging activity of the extract from *Z. clinopodioides* increased with increasing concentration (22). This may be due to the finding that certain chemical compounds have antioxidant properties (23, 24).

IC50 values were used to show the antioxidant properties of various concentrations of *Z. clinopodioides*; all data were compared with the IC50 value of standard ascorbic acid. The IC50 of *Z. clinopodioides* obtained in our study was 72.653 µg/ml, while it was 4.151 µg/ml in ascorbic acid.

IC50 value differed amongst the studied, IC50 value from 3.60 to 3.90 mg/ml (25), but the ability *of Z. clinopodioides* essential oils to scavenge the DPPH radical was assessed from 3.60 to 4.20 mg/ml (26), while it was 147.2±1.3 µg/mL and 111.4±1.2 µg/mL in *Z. tenuior* and *Z. clinopodioides*, respectively (27). *Z. clinopodioides*phy to chemical compounds could be the source of the most important bioactive chemicals. Some researchers reported that secondary metabolites in many *Z. clinopodioides* have anti-inflammatory, antibacterial, anticancer activities, and antioxidant effects (26, 27).

According to the results of this investigation, *Z. clinopodioides* essential oil has an excellent antibacterial impact on human pathogenic bacteria. Gram (+) bacteria are more vulnerable to essential oil than Gram (-) bacteria, according to numerous reports (28). This may be due to changes in the cell wall structure. Gram (-) bacteria's tolerance to essential oils has been attributed to a hydrophilic outer membrane that prevents hydrophobic essential oils from penetrating the target cell membrane (29).

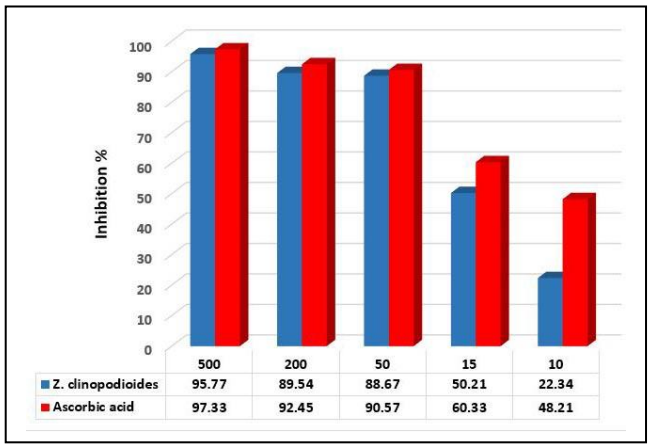

Figure 9. The percentage inhibition of *Z. clinopodioides* leaves essential oil by the antioxidant ascorbic acid

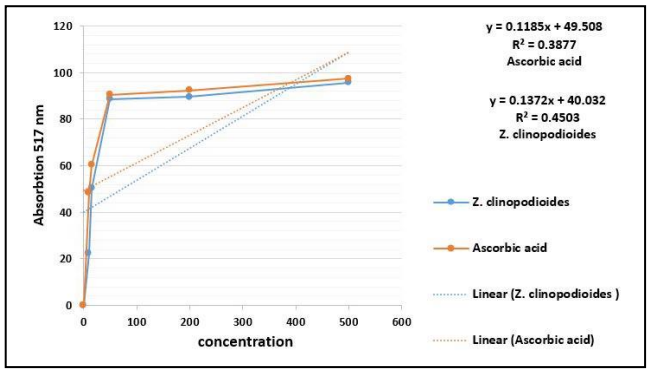

Figure 10. Calibration curve of percentage inhibition of the free radical DPPH by *Z. clinopodioides* leaves and ascorbic acid

The results of this study corroborate traditional usage of *Z. clinopodioides*, which appeared to be effective sources of antibacterial medicines, especially against *Bacillus* sp. and *Escherichia coli*. Based on these findings, it is feasible to conclude that essential oil extracted from *Z. clinopodioides*leaves has potent and wide antibacterial action against various human pathogenic microorganisms.

The presence of phytochemical compounds in the leaves of *Z. clinopodioides* makes it biologically active, and it may be responsible for disease control. *Z. clinopodioides*leaves has led to finding that the DPPH is well exhibited. The results of this investigation indicate that *Z. clinopodioides* leaf essential oil extract has excellent antioxidant properties.

Authors' Contribution

Study concept and design: K. O. Q.
Acquisition of data: S. A. A. M. A.
Analysis and interpretation of data: H. H. A.
Drafting of the manuscript: D. F. A.
Critical revision of the manuscript for important intellectual content: K. O. Q. and S. A. A. M. A.
Statistical analysis: H. H. A.
Administrative, technical, and material support: D. F. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

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