

Research Article

Evaluation of the Bioactivity of Honey in Combination with Alcoholic Extract of Black Cardamom and *Zataria multiflora*

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Received 1 December 2022; Revised 10 February 2023; Accepted 19 May 2023; Published 14 June 2023

Academic Editor: Nadica Maltar Strmečki

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Considering the high rates of global antimicrobial resistance, researchers are looking for new ways to deal with the resistance. Therefore, this study is aimed at analyzing the bioactive compounds and antimicrobial effects of honey and alcoholic extracts of *Zataria multiflora* and Black cardamom. The mixture of honey (30%) and alcoholic extracts of *Zataria multiflora* (35%) and Black cardamom (35%) as named F6 revealed the antibacterial activity against three tested bacteria (MIC = 1000 µg/ml). Thymol, resorcinol, and phenol, 2-methyl-5-(1-methylethyl, 3,7-octadiene-2, 6-diol, 2, 6-dimethyl) were identified as potential antimicrobial agents using GC-MS. The F6 toxicity to RBCs and HEK293 human cells was 11.13 and 13.84 times lower for Triton X-100 toxicity (95%), respectively. We concluded that F6 has the best antibacterial activity against *E. coli* and less toxicity against tested eukaryotic cells due to the presence of chemicals such as hydrogen peroxide, thymol, and resorcinol.

1. Introduction

Infectious diseases, especially those caused by antibiotic-resistant bacteria, are one of the most important causes of death in patients and a problem for the medical community in the world [1]. Nosocomial infections caused by especially resistant bacteria end in the deaths of more than 700,000 people and impose, so these deaths can reach 10 million people per year in 2050, a high cost on health care worldwide [1, 2]. Common bacteria in nosocomial infections include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *E. faecium*, and *Acinetobacter* spp. [3–5]. Many commercial antibiotics are used all over the world to control human infectious diseases. Long-term use of these antibiotics has led to the emergence of multidrug-resistant bacteria and has created significant clinical problems in the treatment of infectious diseases [6]. In 2017, the World Health Organization (WHO) published a list of antibiotic-resistant bacteria, and the purpose of publishing this list was to explore and develop new antibiotic

therapies [7]. The lack of new effective antibiotics is a significant economic challenge; it has led to decreased antibiotics in the market [8]. The spread of antibiotic resistance in a short period has made investment in the production of new antibiotics difficult. These conditions, while requiring governments to intervene in this area to resolve the economic challenges, on the other hand, make it necessary to provide new solutions to tackle resistant bacteria. One of these methods can be the use of natural substances and plant extracts [9]. Today, it is believed that the use of whole plant extract instead of active ingredients isolated from it, due to the synergistic effect and the masking effect of toxicity between the substances in the plant, is preferred in many cases, and a better therapeutic effect is obtained [10]. There are about 250,000–500,000 plant species on Earth. Some of these plants are part of the food chain of animals and humans. Also, many of them are used for therapeutic purposes due to their secondary metabolites [11]. Various secondary metabolites such as carotenoids, terpenoids, flavonoids, alkaloids, tannins, saponins, enzymes, minerals,

and vitamins are found in plants that have antimicrobial, antiviral, and antifungal properties [12].

Zataria multiflora is one of the dicotyledonous plants of the mint family that grows in different parts of Iran. The most important active ingredients of *Zataria multiflora* are thymol, carvacrol, and parasimol [13, 14]. This plant also contains tannins, flavonoids, saponins, and bitter substances. Among the *Zataria multiflora* compounds, thymol composition and phenolic compounds are the most characteristic active chemical composition of *Zataria multiflora*, which is present in different amounts in different parts of this plant, including leaves, flowers, and roots [15].

Black cardamom, also known as *Amomum subulatum*, is a small perennial herbaceous plant of the *Zingiberaceae* family, has a strong aroma, and is used as a spice and condiment in foods [16]. This plant is found at altitudes of 600 to 2,000 in the humid evergreen tropical forests of the Eastern Himalayas, India, and Nepal. The fruit is triangular, reddish brown to dark pink, and the seeds of the fruit contain 2-3% of essential oils that have medicinal properties [16]. Black cardamom essential oil contains 1 and 8 cineole (35-35%) and alpha-pinene (5%) whose biological activities such as analgesic, anti-inflammatory, antioxidant, and antidiabetic have been proven in various studies [16]. This plant has antimicrobial properties against *Streptococcus mutans*, *S. aureus*, *E. coli*, *P. aeruginosa*, *Candida albicans*, and *Lactobacillus acidophilus* [17].

Honey is a useful food product and a valuable antioxidant that has been known as the highest and most powerful food for centuries and has also been used as a medicine in the treatment of most diseases in all areas due to its healing properties [18]. The compound has been used because honey is a bioactive compound derived from bees and plants, compounds, and may be associated with antimicrobial activity that can kill or inhibit the growth of some pathogenic microorganisms [19]. The main antibacterial agent in honey is hydrogen peroxide, which is produced through the activity of glucose oxidase. Glucose oxidase is secreted by the bee's pharyngeal glands, which converts glucose in honey to glucuronic acid and hydrogen peroxide [20]. Small amounts of diastase, invertase, protease, catalase, and phosphatase enzymes are involved in the antimicrobial activity of honey [21]. As a result, the antimicrobial activity of honey varies according to its plant source, bee diversity, and geographical origin [22].

Although some studies carried out on the antimicrobial effects of medicinal plants and honey, they have not studied the effects of the unique plant or honey in combination with each other. This study is aimed at evaluating the antimicrobial effects of the combination of honey, alcoholic extracts of *Zataria multiflora*, and Black cardamom on *S. aureus*, *E. coli*, and *P. aeruginosa*, and also to investigate the possible cytotoxicity effects of the mentioned combination on renal epithelial cells and human erythrocytes.

2. Material and Method

2.1. Source of Honey. In this study, honey was purchased from the most natural jars of honey in Kurdistan, Iran, so

10 grams was dissolved in 10 ml of sterile distilled water. It was then passed through a syringe filter (Sartorius Co., Germany) and dispersed in microtubes for 1 ml, and stored at -80°C until laboratory time.

2.2. Plant Material and Processing. Two plant species (Black cardamom and *Zataria multiflora*) to the extent of 100 grams were collected from the Atark Medicinal Plants Distribution Center (Tehran, Iran), and they were examined by the toxicology specialist of Tabriz University for the non-toxicity of the plants. The plants were transferred to Urmia University of Medical Sciences. Then, two types of plants were powdered by an electric mill (Pars Khazar, Iran), and 100 grams of silica gel was poured into the bottom of the desiccator to completely dry the plants. So, the plants were placed inside this device for 24 hours. In order to get alcoholic extraction, 10 g of each complete drying plant was weighed with a scale. Each of the weighted herbal powders was transferred to a separate Erlenmeyer flask, and 100 ml of 96% ethanol was added to each Erlenmeyer flask. The Erlenmeyer was placed in a shaking incubator at 37-40°C at a speed of 200 rpm for 48 hours until the active ingredient of the plants was completely extracted into the solvent. After this step, the contents of the Erlenmeyer flask were filtered using 0.4 µm Whatman's filter paper (Whatman, UK). The filtered samples were poured into test tubes and centrifuged at 3,000 rpm for 10 minutes. The samples were concentrated in a distillation apparatus (Heidolph, Germany) (and collected in a falcon tube and stored in a freezer at -80°C until the test was performed. Alcoholic extracts and honey were then lyophilized to dry by freezing. After complete drying of the alcoholic extracts of plants and honey, these materials were stored in a freezer at -20°C until the tests were performed [23].

3. In Vitro Antimicrobial Assays

Required microorganisms including *S. aureus* (ATCC-25923), *E. coli* (ATCC-25922), and *P. aeruginosa* (ATCC-27853) were prepared from the microbiology laboratory of Urmia Medical School. The antimicrobial properties were investigated in two stages.

Step 1: the antimicrobial properties of alcoholic plant extracts and honey were investigated individually using the broth microdilution method for the detection of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Initially, 2 mg/ml of honey and alcoholic plant extracts were prepared and poured into the first well of the 96-well microtiter plate. The rest of the well volume was filled with Müller-Hinton broth until the final volume of the first well was poured to 200 µl. Serial dilution was carried out to obtain the doses (1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/ml) in each microtiter plate. Finally, 100 µl of the bacterial suspension (1.5×10^6 CFU/ml) was added to each well. One column was considered negative control (culture media without bacteria) and another one was treated with culture media and bacteria without antibiotics as a positive control. The microtiter plates were incubated at 37°C for 24 hours. The lack of

visible turbidity of any concentration is determined by the MIC score.

MBC of each substance was determined from the broth dilution of MIC tests by subculturing to agar plates that do not contain the test agent [24].

Step 2: at this stage, the antimicrobial activity of the various combined concentrations of honey and alcoholic extracts from plants was evaluated under the following mixing scheme (Table 1). In each microtiter plate, one column with antibiotic and one column with no bacterial suspension were considered to be positive and negative control, respectively. Antibiotics consisted of vancomycin, gentamicin, and cefixime for *S. aureus*, *P. aeruginosa*, and *E. coli*, respectively.

As a result of the antimicrobial evaluation of all compounds, the results of their MIC and MBC were determined. Any compound with an improved antimicrobial effect was selected for GC-MS analysis and cellular toxicity.

4. Gas Chromatograph-Mass Spectrometry (GC-MS) Analysis

A gas chromatograph-mass spectrometer (GC-MS) (Agilent 7890B/5977A Series Gas Chromatograph/Mass, USA), equipped with a split/splitless injection system and electron bombardment ionization model, and NIST and Wiley mass libraries were used to identify the chemical compounds and active ingredients of Black cardamom and *Zataria multiflora*. In this analysis, an HP5-MS column with a length of 60 m, an inner diameter of 0.25 mm, and a film thickness of 0.25 μm was used. The analysis state in the HP5-MS was regulated as follows condition: Injection site temperature, interface temperature, and ionization site temperature were set at 250, 270, and 250°C, respectively. The column temperature program started with an initial temperature of 50°C and was kept at this temperature for 5 minutes. Then, the temperature of the column reached 180°C with a slope of 15°C/min and remained constant at this temperature for 2 minutes. Finally, the temperature reached 290°C with a slope of 10°C/min and remained constant at this temperature for 10 minutes. The split ratio was set to 110, and the injection volume was half a microliter [25].

5. In Vitro Hemolysis Assay

The toxicity of honey and alcoholic extracts of *Zataria multiflora*, Black cardamom, and also their combinations against human red blood cell was evaluated using a hemolysis assay. At first, 5 ml of fresh human blood was taken and promptly mixed with 0.5 ml of sodium dihydrate citrate (as an anticoagulant) in a tube. The tube was centrifuged (1000 rpm/10 min), and the plasma (as supernatant) was removed. To complete the elimination of plasma remnants, the sediment of red blood cells (RBCs) was washed twice with phosphate-buffered saline (PBS). In the next step, a concentration of 2% RBCs was prepared in PBS sterile buffer. Serial concentrations of the F6 compound (Table 1) were also prepared in a 96 microplate, and then 100 μl of human RBCs (2%) was added into the wells. The microplate was incubated at 37°C for 24 hours. The next day, the microplate was centrifuged in a condition of

TABLE 1: The various mixture concentrations of honey and alcoholic extracts from plants.

	Combination ratio		
	Honey	Black cardamom	<i>Zataria multiflora</i>
1	—	50%	50%
2	—	75%	25%
3	—	25%	75%
4	50%	50%	—
5	50%	—	50%
6	75%	25%	—
7 (F1)	75%	—	25%
8 (F2)	25%	75%	—
9 (F3)	25%	—	75%
10 (F4)	50%	25%	25%
11 (F5)	70%	15%	15%
12 (F6)	30%	35%	35%

1,000 rpm for 5 minutes. The optical density of the wells was measured at 540 nm using a microplate spectrophotometer (Agilent, USA). The hemolysis percentage was calculated based on the following formula [26].

$$[(\text{hemolysis } (\%)) = 100 \times \left[\frac{(\text{OD sample} - \text{OD blank})}{(\text{OD triton} - \text{OD blank})} \right]]. \quad (1)$$

Triton X-100 (0.1%) was used as a positive control and PBS was used as a negative control.

6. In Vitro Cytotoxicity Assay

The cytotoxicity of the F6 compound (Table 1) was evaluated against the HEK293 cell line (the renal epithelial cell line) using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) method. Initially, 3×10^4 cells were seeded in 96 microplates containing 90% DMEM (Dulbecco's Modified Eagle Medium) culture medium supplemented with 10% fetal bovine serum and 1% Pen Strep. The cells were incubated for 24 hours under 5% CO₂, 95% humidity, and a temperature of 37°C. Serial concentrations of the F6 compound ranging from 1000 to 7.812 $\mu\text{g/ml}$ were added to each well and incubated at 37°C for 24 hours. The next day, 20 μl of MTT solution with a concentration of 5 $\mu\text{g/ml}$ was added to each well and incubated at 37°C for 4 hours. The supernatant of wells was gently removed, and 100 μl of dimethyl sulfoxide (DMSO) was added to each well and shaken for 15 minutes to release insoluble purple formazan from the cells. Finally, the amount of optical density (OD) of the samples was measured at 570 nm using a microplate spectrophotometer. The following formula was used to calculate the percentage of cell toxicity [26].

$$\text{Dead}(\%) = \left[\frac{1 - (\text{OD test})}{(\text{OD control}) \times 100} \right]. \quad (2)$$

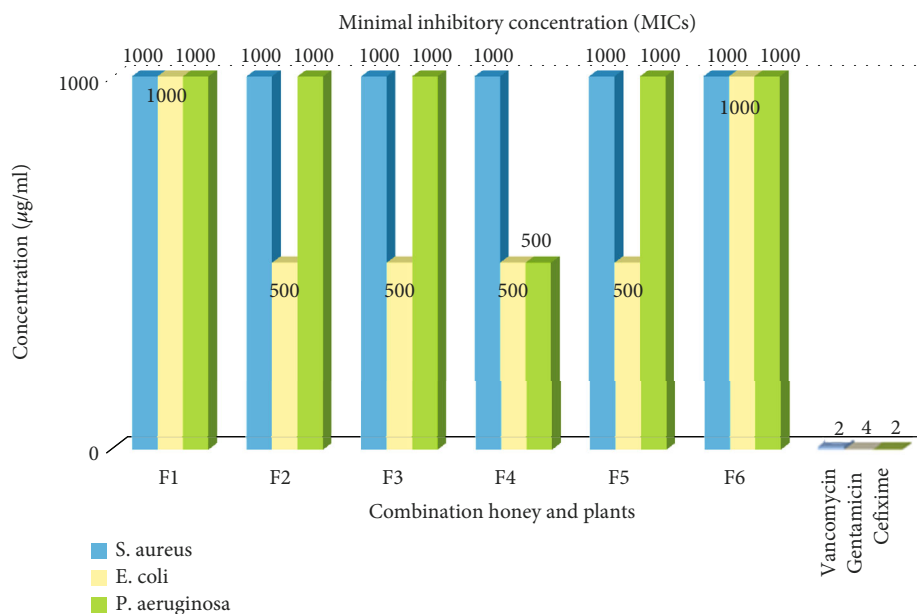


FIGURE 1: Minimum inhibitory concentration (MIC) of honey and alcoholic extracts of *Zataria multiflora* and Black cardamom and comparing it with antibiotics against ATCC strains of *S. aureus*, *E. coli*, and *P. aeruginosa*.

In this assay, Triton X-100 and peptide-free cell suspension were used as the positive and negative control, respectively.

7. Results

7.1. In Vitro Antimicrobial Assays. Honey and alcohol extracts from *Zataria multiflora* and Black cardamom showed antimicrobial effects above 1000 µg/ml when tested individually. According to Figure 1, the F4 compound has a better MIC value, but the MBC value of this compound was higher than 2000 µg/ml. On the other hand, the F6 compound [honey (30%) and alcoholic extracts of *Zataria multiflora* (35%) and Black cardamom (35%)] had better antimicrobial activity than other compounds against *E. coli*, *S. aureus*, and *P. aeruginosa* with MICs = 1000 µg/ml. In addition, this combination had equal MIC and MBC (1000 µg/ml) against *E. coli* (Figures 1 and 2). While the substances with lower MIC scores are more efficient antimicrobial agents, however, the closer the MIC is to the MBC, the more bactericidal the compound.

7.2. Processing, Phytochemical, Analysis, and GC-MS Analysis. Phytochemical analysis of 12 compounds of alcoholic extracts of *Zataria multiflora* (35%) and Black cardamom (35%) by GC-MS revealed that phytochemicals including carbohydrates, amino acids, steroids, and saponins contain more bioactive compounds (Figures 3–5). The molecular weight, structural aspects, and molecular formula of these compounds were demonstrated in Figures 5 and 6. Based on our findings, thymol at 100% and resorcinol at 64.64% were the most bioactive compounds. Other identified compounds include phenol, 2-methyl-5-(1-methyl ethyl, 3,7-octadiene-2,6-diol, 2,6-dimethyl) (Figures 2, 6, and 7).

7.3. In Vitro Hemolysis Assay. The compound F6 had about 11.13 times less hemolytic activity than Triton X-100 (positive control) against humans (RBCs). The compound F6 at a concentration of 1000 µg/ml lysed about 8.53% of human erythrocytes, but Triton X-100 lysed 100% of human erythrocytes. The hemolysis effects of compound F6 were evaluated in hours 1 and 24, and the obtained results were the same (Figure 8).

7.4. In Vitro Cytotoxicity Assays. The compound F6 showed significantly less cytotoxic activity than Triton X-100 against human embryonic kidney epithelial cells (HEK293). The cytotoxic activity of compound F6 was 13.84 times lower than the positive control Triton X-100. The compound F6 at a concentration of 1000 µg/ml lysed 6.86% of kidney epithelial cells, while Triton X-100 lysed 95% of kidney epithelial cells (Figure 9).

8. Discussion

Increasing antibiotic resistance is causing a serious crisis around the world. Due to the reduced effectiveness and high toxicity of antimicrobial drugs, researchers need to look for natural and herbal substances to solve this problem. In order to provide an alternative method to deal with antibiotic-resistant bacteria, the present study was conducted to investigate the antimicrobial effects and identify active compounds and cytotoxicity of the combination of honey and alcoholic extracts of two plants including *Zataria multiflora* and Black cardamom. In this study, the combinations F1 to F6 showed better antimicrobial activity with a concentration of 500-1000 µg/ml against tested bacteria, including *S. aureus* (ATCC-25923), *E. coli* (ATCC-25922), and *P. aeruginosa* (ATCC-27853) which, were the commonest nosocomial infectious agents. In comparison to the different types

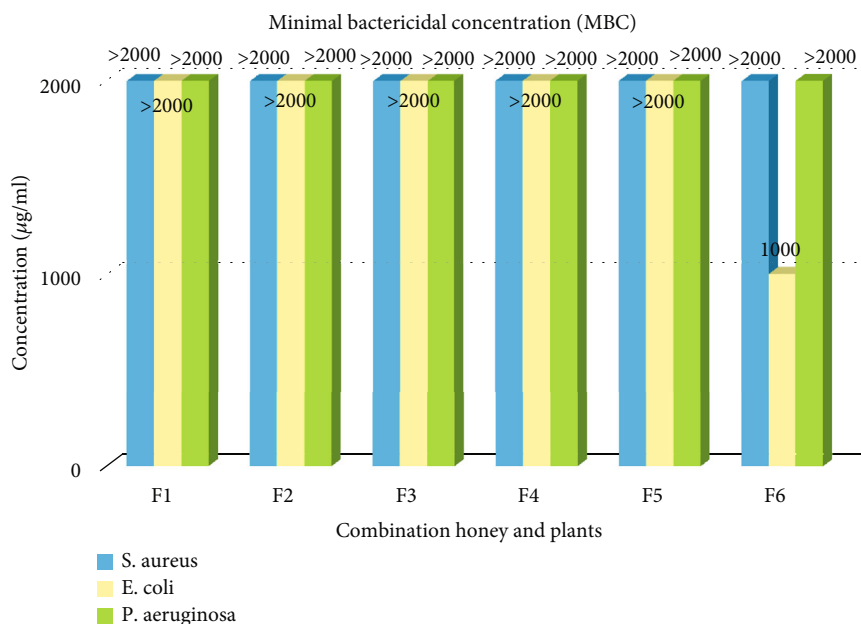


FIGURE 2: Minimum bactericidal concentration (MBC) of honey and alcoholic extracts of *Zataria multiflora* and Black cardamom and comparing it with antibiotics against ATCC strains of *S. aureus*, *E. coli*, and *P. aeruginosa*.

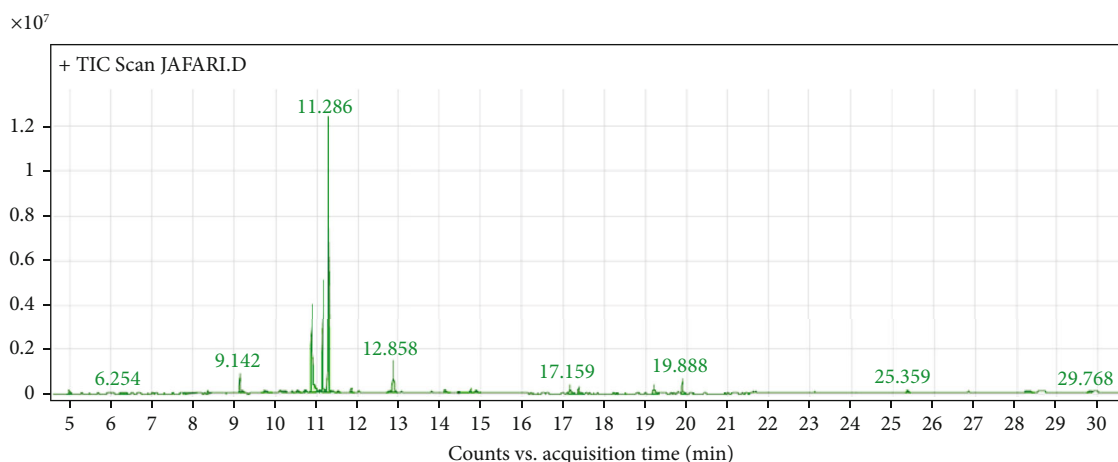


FIGURE 3: GC-MS analysis of compound F6. As shown in the graph, thymol is the main component of alcoholic extracts of *Zataria multiflora* and Black cardamom.

of compounds tested, the compound F6 was found with higher antimicrobial activity against *E. coli*, therefore, the chemical analysis of that seemed essential. According to the identified main antimicrobial substance in honey called hydrogen peroxide [27], in the chemical analysis stage of this research, the mentioned two alcoholic extracts were entered into the copper GC-MS; the compounds found in the F6 were the same as thymol and resorcinol, which have been reported as good antibacterial substances [23]. In the last stage, the cytotoxicity of compound F6 against human blood cells (RBCs) (11.13 times) and human kidney epithelial cells (HEK293) (13.84 times) was less compared with Triton X-100 toxicity (95%). In a study conducted by Zeedan et al., in 2016, honey and ethanolic extracts of ginger, clove, and black cumin revealed the highest antimicrobial effects on *E.*

coli (57.14%), which is consistent with the results of our study [28]. In the study conducted by Ghalamfarsa et al. in 2018, the antimicrobial activity of several kinds of honey in comparison with ciprofloxacin was examined [29]. Similar to our finding, they indicated that honey has antibacterial activity, and its combination with antibiotic promotes such abilities. In the study of Sultan et al. in 2020, *Artemisia absinthium* L. plant showed a moderate antimicrobial effect against *S. aureus* and *E. coli*. The chemical analysis of this plant proved the existence of compounds like epiyangambin, flavone, octadecanoic acid, 2,3-dihydroxypropyl ester, palmitic acid β -monoglyceride, α -D-mannofuranoside, camphor, and terpineol. In addition, the plant revealed cytotoxicity against MCF-7 cells at a concentration of $80.96 \pm 3.94 \mu\text{g/ml}$ as the IC₅₀ value, but it could not

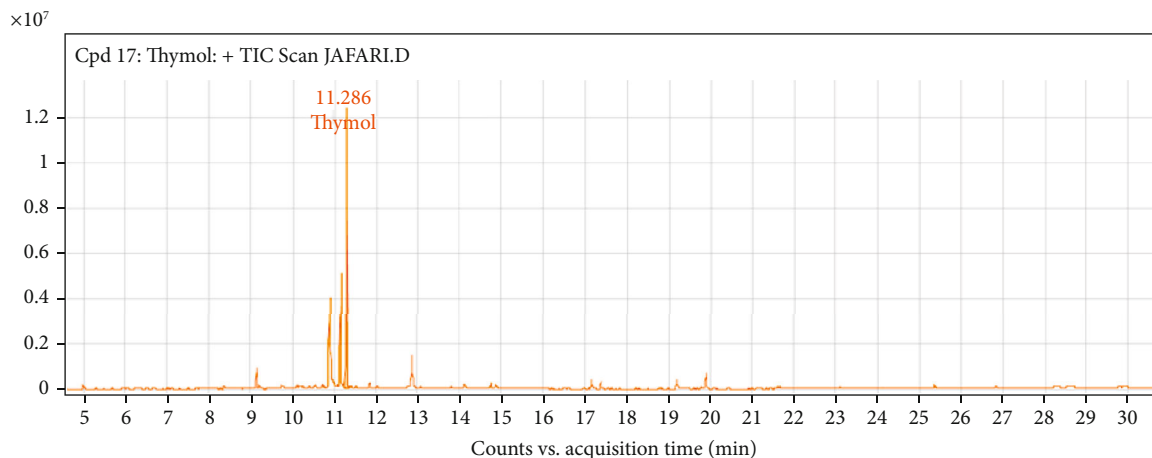


FIGURE 4: GC-MS analysis of compound thymol. It shows that thymol is the main component of alcoholic extracts of *Zataria multiflora* and Black cardamom.

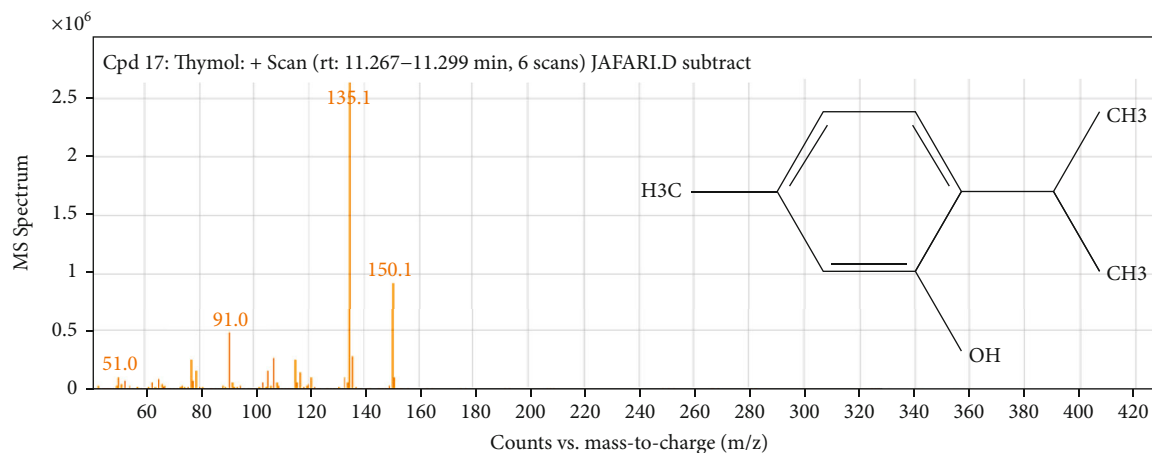


FIGURE 5: Chemical thymol obtained from GC-MS device.

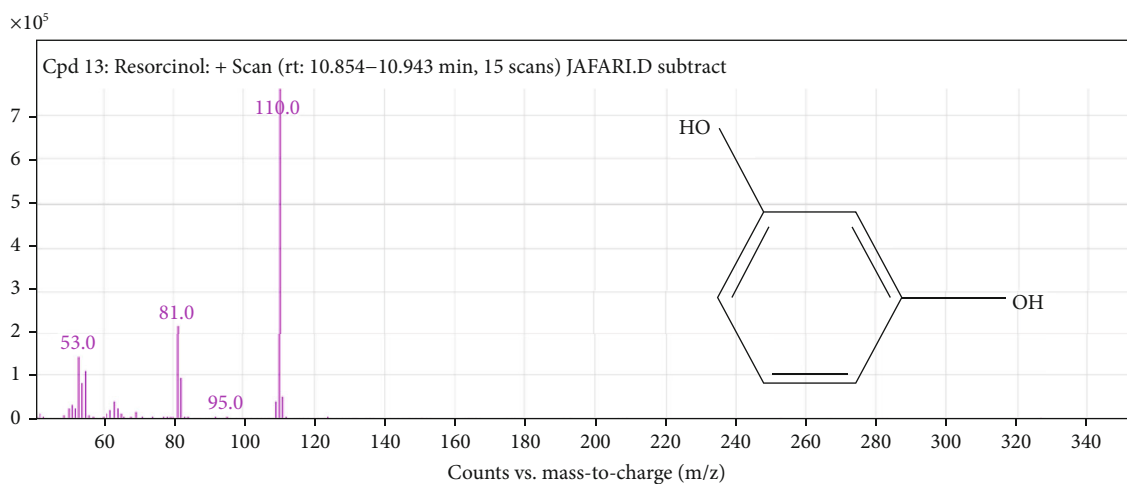


FIGURE 6: Chemical resorcinol obtained from GC-MS device.

prevent the proliferation of HCT116 ATCC-treated human colon cancer cells [30]. The main point of this research was the simultaneous study of the combination of honey

with extracts of *Zataria multiflora* and Black cardamom plants by having active chemical compounds, such as hydrogen peroxide, thymol, and resorcinol. This has resulted in

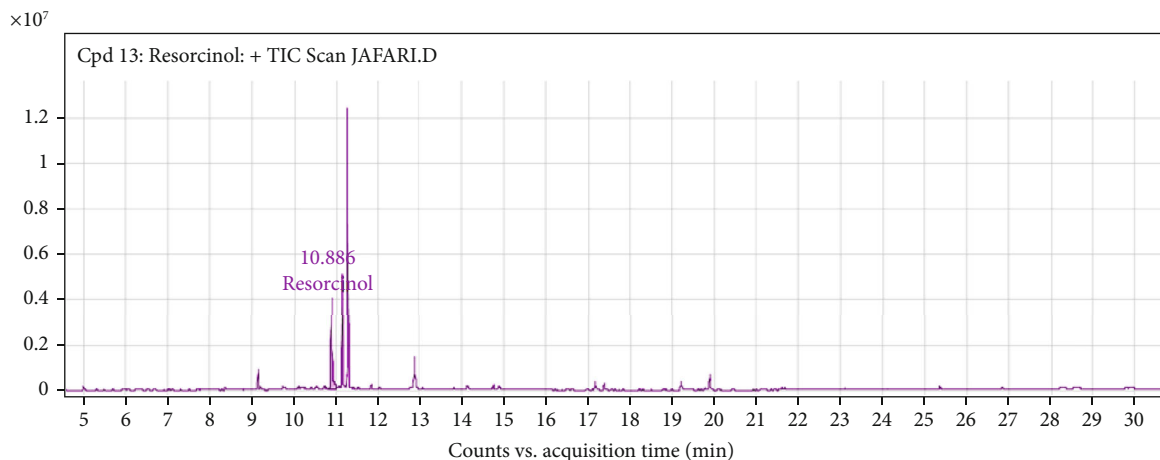


FIGURE 7: GC-MS analysis of the compound of F6. As the graph shows resorcinol is the main component next to thymol of alcoholic extracts of *Zataria multiflora* and Black cardamom.

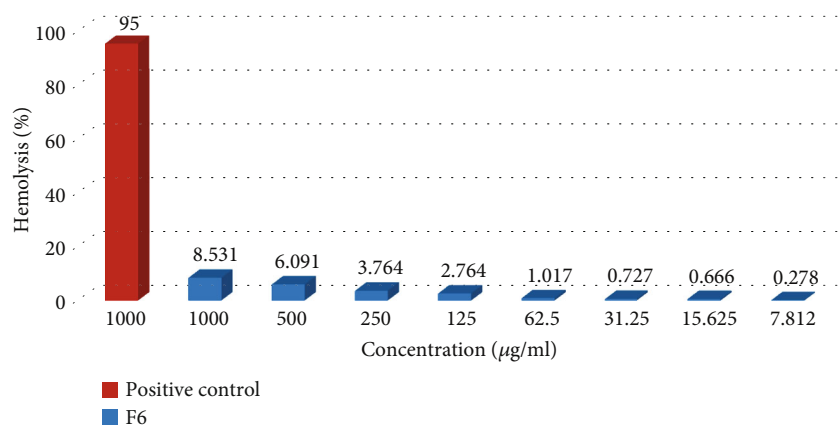


FIGURE 8: Hemolytic activity of compound 6 in different concentrations (1,000 to 7.812 µg/ml) (Data were the results of three repetitions).

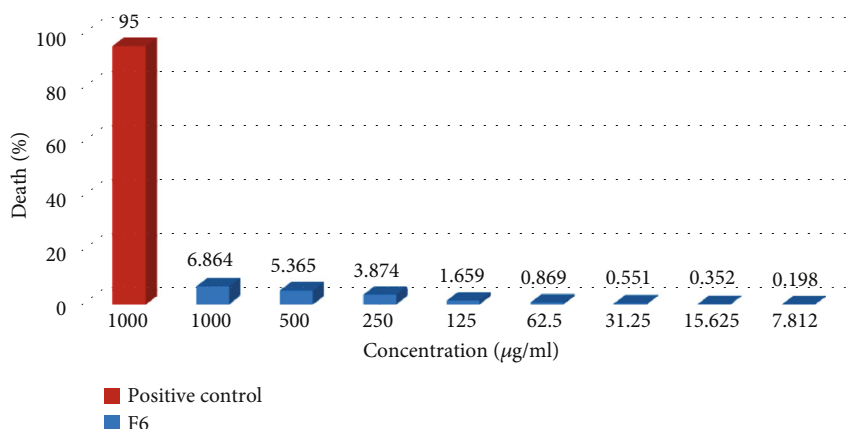


FIGURE 9: Toxicity activity of the compound F6 in different concentrations (1000 to 7.812 µg/ml) (Data were the results of three repetitions).

better antimicrobial effects and less toxicity. According to various studies [27] and also our findings, honey indicates antimicrobial properties when used. Individually, these effects depend on some factors, including the origin of beed-fed plants, bee diversity, and geographical origin. Consider-

ing the increasing resistance of bacteria, the authors suggest more attention to using these natural substances in combination with chemical or herbal substances whose synergistic effects induce better inhibition and lethality among bacteria. The toxic effect of the F6 compound on human kidney

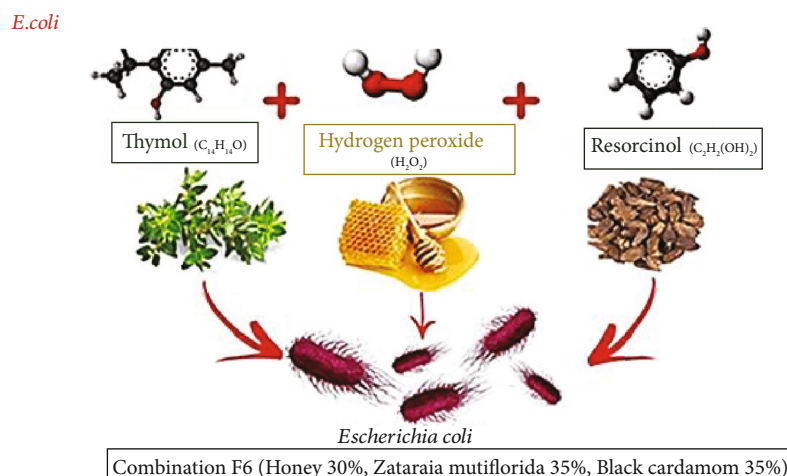


FIGURE 10: Combination F6 (Honey 30%, *Zataria multiflora* 35%, and Black Cardamom 35%).

epithelial cells (HEK293) and human blood cells (RBCs) at 1000 $\mu\text{g/ml}$ concentrations was 6.86% and 8.53%, respectively. The effect of cell toxicity due to this compound was much lower in comparison to Triton X-100. In contrast, Daneshmand et al. did not report any hemolytic effect of *Ziziphus jujube* on blood cells [31]. Roby et al. [32] studied the physicochemistry properties of two kinds of honey (*Trifolium hybridum* and *Citrus sinensis*). They indicated two different phenolic compounds (syringic acid and quercetin) in extracts of honeys. However, they concluded that features of honey examples generally depend on the floral origin of nectar foraged by bees [32]. In accordance, we also detected phenolic compounds as the main compounds of the combination F6, and these compounds with thymol and resorcinol have acceptable antimicrobial effects. In the other study, the antimicrobial effects of *Elettaria cardamomum* plant oil were evaluated on *S. aureus*, *Listeria monocytogenes*, and *E. coli*. The most important phenolic compound in this oil was sitosterol, and the plant had better antimicrobial effects on tested bacteria. Inconsistent with our study, this study confirmed again that phenolic compounds in natural substances can show better antimicrobial effects [33]. Based on the researches, thymol has structural isomers and a phenolic hydroxyl on the ring. The hydroxyl group increases its hydrophilic ability, disrupts membrane integrity, increases membrane permeability, and ultimately causes proton and potassium leakage. Accordingly, it leads to a loss of membrane potential in *E. coli* [34, 35]. Resorcinol shows the same antimicrobial mechanism concerning the phenolic group. Since the main antibacterial agent in honey is hydrogen peroxide, H₂O₂ has been shown to disrupt the structure and permeability of cell walls and cytoplasmic membranes, as well as induce ribosomal lesions and DNA rupture in bacteria [36]. Therefore, it seems that the antimicrobial properties of thymol and hydrogen peroxide compounds, as well as the synergism of these substances in the F6 compound cause an increase in the ability to inhibit or kill *E. coli*. In the study of Dušan et al. [37], the short-term presence of thymol had no destructive effect on Caco-2 cells [37] (Figure 10).

9. Conclusion

Our study showed that the combination F6 [honey (30%) and alcoholic extracts of *Zataria multiflora* (35%) and Black cardamom (35%)] had a higher antimicrobial activity against *E. coli*. The GC-MS analysis of compound F6 revealed that thymol and resorcinol are the major components of the alcoholic extracts of *Zataria multiflora* and Black cardamom with antibacterial activity. The compound F6 had lower hemolytic activity (11.13 times) compared to Triton X-100 against human red blood cells (RBCs). Furthermore, F6 significantly exhibited 13.84 times lower cytotoxic activity than Triton X-100 against HEK293 cells. It appears that compound F6 is a respectable candidate for further studies in animal models.

Data Availability

All study data can be made available to the public.

Additional Points

Novelty Impact Statement. To the best of our knowledge, this is the first study that evaluated the combination antimicrobial activities of the honey and alcoholic extracts of the mentioned plants and indicated that the combination is more effective than any of the individual substances.

Ethical Approval

This study has been approved by the Vice President of the Research and Ethics Committee of Urmia University of Medical Sciences with the code IR. UMSU. REC.1400.190 of ethics.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

This study was done with the financial support of the Urmia University of Medical Sciences Research Vice-Chancellor, so the authors express their gratitude to all colleagues.

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