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Comparison and Determination of Minimum Inhibitory Concentration of Essential Oils Against *Escherichia coli* O157:H7

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The minimum inhibitory concentration (MIC) of essential oils of garlic, cinnamon, clove, oregano and sage against *E. coli* O157:H7 was determined by broth and agar dilution method. Antimicrobial activity of essential oils was also compared using disk diffusion method. The MIC of essential oils of oregano, cinnamon, clove, sage and garlic against *E. coli* O157:H7 was 300-ppm, 350-ppm, 750-ppm, 1750-ppm and 3000-ppm, respectively. Oregano essential oil was the most inhibitory and garlic was the least inhibitory against *E. coli* O157:H7 (oregano > cinnamon > clove ≫ sage ≫ garlic).

Keywords: Essential oil, *E. coli* O157:H7, MIC.

1. Introduction

Many of the spices and herbs used today have been valued for their preservative effects and medicinal powers in addition to their flavor and fragrance qualities. Scientific investigations concerning the inhibition of microorganisms by spices, herbs and their essential oils and various components have been reported [1]. Most of the foodborne bacterial pathogens examined were sensitive to extracts from plants such as garlic, onion, mustard, and clove [1-2]. The extent of sensitivity varied with the strain and environmental conditions imposed [2].

The antimicrobial compounds in spices and herbs are mostly in their essential oil fraction [3]. Water extract of cloves reduced the decarboxylase activity of a crude extract of *Enterobacter aerogenes* by about 40%. Cinnamon, sage, nutmeg, and allspice were also very effective inhibiting the decarboxylase activity of *Enterobacter aerogenes*[4]. Farag *et al.* [5] tested sage, rosemary, caraway, cumin, clove, and thyme for their inhibitory effects against Gram-positive bacteria *(Staphylococcus aureus, Micrococcus* spp., *Sarcina* spp., and *Bacillus subtilis)*, and Gram-negative bacteria *(Pseudomonas fluorescens, Escherichia coli*, and *Serratia marcescens)*. The Gram-positive bacteria were more sensitive to the antimicrobial compounds in spices [1]. Phenol compounds containing a hydroxyl group have been recognized as antimicrobial components in spices [2]. Eugenol in clove, carvacrol and thymol in oregano, cinnamaldehyde in cinnamon, and thujone, borneol and cineole in sage, and allicin in garlic have been identified as major essential oil components [1, 2, 6]. Sivropoulou et al. [7] found that carvacrol and thymol content of *Origanum* essential oils exhibited high level of antimicrobial activity against test organisms, *E. coli, Pseudomonas aeruginosa, Salmonella* Typhimurium, *S. aureus, Rhizobium leguminosarum* and *B. subtilis*. Ryu and Holt [8] showed that cinnamon oil and cinnamaldehyde were each effective in inhibiting the growth of *Penicillium expansum* in apple juice.

There are several methods to evaluate antimicrobial activity of essential oils. Serial dilution of spices, essential oils or their components in a laboratory broth medium is a commonly used method to determine MIC. In this method, essential oils are serially diluted in the broth medium. After proper inoculation and incubation period, the growth of a particular microbial culture is evaluated. The MIC is defined as the lowest concentration of essential oil that inhibits the growth of the microorganism. Agar dilution method is similar to broth dilution method. In this method, instead of using a broth medium, an agar medium is used [1, 6, 9].

The disk diffusion method is another method to determine antimicrobial activity of essential oils. A paper disk deposited with an essential oil is placed on agar medium inoculated with a test microorganism. After a suitable incubation period, the zone of inhibition is measured. The essential oils with strong antimicrobial activity exhibit a

larger inhibition zone. Essential oil vapor technique also uses a zone of inhibition to compare efficacy of essential oils. In this technique a small cup or a paper disk with essential oil is placed on petri plate cover and inoculated agar placed on top. After an appropriate incubation period the zone of inhibition is measured. Double plate method, agar cup method [1] and a gradient agar method [10] are other methods developed to measure antimicrobial activity of spices, their essential oils or major components.

Diffusion, optical density, impedance, and mycelial weight methods are also used to determine antimicrobial efficacy or minimum inhibitory concentration of spices or their essential oils and major components. Although each method gives a general idea about microbial activity of spices, essential oils and major components, it has its own limitations.

Escherichia coli O157:H7 is a Gram-negative, facultative anaerobic rod bacterium. *E. coli* O157:H7 is an enterohemorrhagic (EHEC) strain of *E. coli* with somatic (O) and flagella (H) antigens. *E. coli* O157:H7 produces large quantities of one or more related toxins (verotoxins or shigatoxins) that cause severe diseases [11, 12]. *E. coli* O157:H7 was first identified as human pathogen in 1982, after two outbreaks of hemorrhagic colitis associated with the consumption of undercooked hamburgers [11, 12]. Foods of animal origin are principally the major vehicle of transmission of *E. coli* O157:H7 infections. Raw or undercooked ground beef has been most often associated with *E. coli* O157:H7 infections compared to other foods.

Zaika [1] and Shelef [6] reviewed the methods used to determine the antimicrobial activity of spices and herbs, and their essential oils and active components. The objectives of this study were to determine the minimum inhibitory concentration of essential oil of oregano, cinnamon, clove, sage and garlic using broth and agar dilution methods against *E. coli* O157:H7, and to compare antimicrobial activity of the essential oils against this pathogen using disk diffusion method.

2. Materials and Methods

2.1. Culture preparation

Five strains of *E. coli* O157:H7 (ATCC 35150, 43889, 43894, 43895 and 51657) were obtained from the American Type of Culture Collection (Rockville, MD). Cultures were grown in Brain Heart Infusion (BHI) broth at 37°C for 24 hr. Cultures were serially diluted with 9 ml of 0.1% peptone water. An equal volume of each culture (2 ml) was transferred into a sterile test tube to make a cocktail culture of ca. 6.0 log cfu/ml.

2.2. Preparation of essential oil solutions

Essential oil of garlic, cinnamon, clove, sage and oregano was obtained from the Essential Oil Company (Portland, OR). A stock solution of 1,000, 5,000, 10,000 and 20,000 ppm of each essential oil was prepared as follows: an appropriate amount of each essential oil was dissolved in a sterile distilled water and ethyl alcohol mixture (1:1) (Fisher Scientific, Fair Lawn, NJ). The mixture then was completed to the appropriate volume with sterile distilled water. The final concentration of alcohol in the essential oil stock solutions was 10%(vl/vl). Preliminary studies showed that alcohol content of stock solutions did not exhibit any antimicrobial activity against *E. coli* O157:H7 cultures in this particular method.

2.3. Preparation of broth medium and inoculation

Preliminary studies were performed to determine the approximate concentration of each essential oil with inhibitory activity against *E. coli* O157:H7 in 1,000 ppm range. BHI broth was prepared. Appropriate amount of each essential oil from the stock solutions was transferred into a sterile test tube and completed to 10 ml with BHI broth. Essential oil-BHI broth mixture was mixed for 15 seconds using a vortex. The cocktail solution of *E. coli* O157:H7 was inoculated into the broth medium using a 1 μl calibrated inoculating loop (Difco Laboratories, Detroit, MI). The essential oil-BHI broth tubes inoculated with *E. coli* O157:H7 were incubated at 32°C for 24-48 hr. The minimum inhibitory concentration of each essential oil was recorded as the lowest concentration of each essential oil in the broth tubes with no growth (i.e. no turbidity) of inoculated *E. coli* O157:H7.

2.4. Preparation of agar slants and inoculation

Preliminary studies were performed to determine the approximate concentration of each essential oil with inhibitory activity against *E. coli* O157:H7 in 1,000 ppm range. Tryptic Soy Agar

(TSA) was prepared and tempered at 48°C. Appropriate amount from the stock solutions of each essential was transferred into a sterile test tube and completed to 10 ml volume with tempered TSA. Essential oil-TSA agar mixture was mixed for 15 seconds using a vortex and solidified to make slants. Using a 1 μl calibrated inoculating loop (Difco Laboratories, Detroit, MI), the cocktail solution of *E. coli* O157:H7 was inoculated onto the surface of slants. The slants were incubated at 32°C for 24-48 hr. The minimum inhibitory concentration of each essential oil was recorded as the lowest concentration of each essential oil in the agar slants with no growth of inoculated *E. coli* O157:H7.

2.5. Disk diffusion method

The TSA agar plates were spread plated with 100 μl of the cocktail solution of *E. coli* O157:H7 and air dried for 30 min. A sterile paper disk (6.4 mm diameter) (Difco Laboratories, Detroit, MI) was placed on TSA agar. 15 μl of each essential oil was deposited on the paper disks. The plates were immediately inverted and incubated at 32°C for 24-48 hr. Zone of inhibition (mm) was measured using a ruler. Experiments were repeated three times.

2.6. Statistical Analysis

Analysis of variance (ANOVA) was performed using a general linear model procedure. A randomized complete block design with repeated measurements was applied in this study. The significance of differences of the MIC values of three replicates were determined at the 95% confidence limit (p=0.05). Differences among treatments were examined for levels of significance by the least significant differences (LSD) test (SAS Inst., Inc., Cary, NC).

3. Results and Discussion

The minimum inhibitory concentration (MIC) of essential oil of garlic, cinnamon, clove, oregano and sage against *E. coli* O157:H7 was determined using both broth and agar medium dilution method. Inhibitory activity of essential oils against the pathogen was also compared using the disk diffusion method. The MIC value of each essential oil was examined at 50 ppm proximity.

In the broth dilution method, proper amounts of essential oil of each spice was added into BHI broth and inoculated with *E. coli* O157:H7 cocktail. The MIC was recorded as the lowest concentration of essential oil in BHI broth without turbidity (no growth of *E. coli* O157:H7). After incubation at 32°C for 24-48 hr, the MIC value of oregano was 150-200 ppm, cinnamon was 250-300 ppm, clove was 250-300 ppm, sage was 3500-3750 ppm and garlic was greater than 5000 ppm (Table 1).

Table 1
Minimum inhibitory concentration of oregano, cinnamon, clove, sage and garlic essential oils against *Escherichia coli* O157:H7[1].

	[2]Min. inh. conc. (ppm)	
Essential oils	In broth	In agar
Oregano	$150-200^{A}$	300^{A}
Cinnamon	$250-300^{A}$	350^{B}
Clove	$250-300^{A}$	750^{C}
Sage	$3500-3750^{B}$	1750^{D}
Garlic	$>5000^{C}$	3000^{E}

[1] The MIC values (ppm) with different letters in the same column are significantly different ($p < 0.05$)
[2] Min. inh. conc.: Minimum inhibitory concentration

In the agar dilution method, molten TSA agar after mixing with the proper amount of essential oils was solidified as slants and inoculated with *E. coli* O157:H7. The MIC values were recorded as the lowest concentration of essential oil with no growth of *E. coli* O157:H7 on the slants. In the agar dilution method, oregano had the lowest MIC value of 300 ppm. The MIC value of cinnamon was 350 ppm, clove was 750 ppm, sage was 1750 ppm and garlic was 3000 ppm (Table 1).

Antimicrobial activity of essential oil of garlic, cinnamon, clove, oregano and sage against *E. coli* O157:H7 was also compared using the disk diffusion method. Sterile paper disks were placed on TSA plates seeded with *E. coli* O157:H7. Inhibition zone was expressed as mm diameter of the zone after subtracting 6.4 mm of disk diameter. Cinnamon showed the highest inhibitory activity with 21.3 mm inhibition zone. Oregano had 16.0 mm, cloves had 10.0 mm and sage had 5.5 mm inhibition zone. Garlic showed the weakest inhibition with 3.0 mm inhibition zone (Table 2).

Three different methods showed that essential oil of oregano and cinnamon had the highest inhibitory activity against *E. coli* O157:H7 followed by clove and sage. Garlic essential oil showed very

Table 2
Antimicrobial efficacy of oregano, cinnamon, clove, sage and garlic essential oils against *Escherichia coli* O157: H7 determined by disk diffusion method[1].

Essential oils	Inhibition zone by disk diffusion method (mm)
Oregano	21.3^{A}
Cinnamon	16.0^{B}
Clove	10.0^{C}
Sage	5.5^{D}
Garlic	3.0^{E}

[1] The inhibition zone values (mm) with different letters within a column are significantly different ($p < 0.05$)

weak inhibitory activity against *E. coli* O157:H7.

Differences were observed among the three methods used to determine the antimicrobial efficacy and the MIC of essential oils against *E. coli* O157:H7. Each essential oil showed different MIC values in both broth and agar dilution methods. In broth and agar dilution methods oregano essential oil was the most inhibitory and garlic was the least inhibitory against *E. coli* O157:H7 (oregano > cinnamon > clove ≫ sage ≫ garlic). The disk diffusion method showed that cinnamon oil was the most inhibitory and garlic was the least inhibitory against *E. coli* O157:H7 (cinnamon oregano > clove > sage > garlic).

Results of these three methods indicated that antimicrobial activity of essential oils depends on environmental conditions. Antimicrobial activity of spices and essential oils may vary in laboratory medium, and in different food systems against microorganisms. Solubility or hydrophobicity, reactions between essential oil and compounds in the system, presence of volatile compounds in essential oils and diffusivity of essential oils might affect the antimicrobial activity of essential oils. Each method has its own advantages and disadvantages. Although the disk diffusion method does not indicate the MIC values, it might provide a fast screening method to compare antimicrobial activity of essential oils.

Antimicrobial properties of essential oils have been well documented in both laboratory and food systems. Although most of the essential oils have been classified as generally recognized as safe (GRAS) substances, organoleptic acceptance of these oils limits their use as additives or preservatives. Antimicrobial activity of essential oils depends on their major components [13]. The major components in essential oils may vary with the origin of spices and climate condition of the location [14, 15].

The test medium and method for testing the antimicrobial activity of spices, their essential oils and major constituents might influence the outcome of the study [1]. Kim et al. [16] used a paper disk method and liquid medium to determine antibacterial activity and the minimum inhibitory concentrations of 11 essential oil compounds against *E. coli*, *E. coli* O157:H7, *S.* Typhimurium, *Listeria monocytogenes*, and *Vibrio vulnificus*. *V. vulnificus* was the most susceptible organism to essential oil compounds. Carvacrol showed strong antibacterial activity against all bacterial strains tested and was highly bactericidal against *S.* Typhimurium and *V. vulnificus* in liquid medium. They found that the size of the zone inhibition in the paper disk method did not accurately reflect the antimicrobial activity of the essential oil compounds in liquid medium. Kivanc and Akgul [17] evaluated antibacterial activities of 22 essential oils against *Aerobacter aerogenes, B. subtilis, E. coli, Proteus vulgaris, P. aeruginosa, Staphylococcus albus*, and *S. aureus*. They found that *S. aureus* and *P. vulgaris* were the most sensitive organisms, while *P. aeruginosa* was the most resistant organism to essential oils. They also showed that the agar diffusion and serial dilution methods were not always comparable and recommended to use both methods to determine inhibitory activity of essential oils. Ross et al. [18] found that the minimum inhibitory concentration of garlic oil against 62 human enteric bacteria (Gram-negative, Gram-positive bacteria and pathogenic forms) was in the range of $0.02 - 5.5 mg/ml$. However they stated that test methodologies might produce underestimates of antimicrobial activity of essential oil of garlic. Hammer et al. [19] studied antimicrobial activity of 52 plant essential oils using broth and agar dilution methods. The MIC values obtained by broth dilution were higher than the MIC values obtained by agar dilution. Rapid spectrophotometric methods based on the measurement of the turbidity with indicator compounds have been used to determine antimicrobial activity of natural products (e.g. extracts and essential oils). The assays based on hydrolysis of fluorescein diacetate [20] and the reduction of resazurin [21] have been found to be more accurate and repro-

ducible compared to conventional broth and agar dilution methods. Skandamis et al. [22] compared the inhibitory activity of oregano essential oil against *S.* Typhimurium in liquid and solid matrix containing gelatin. The oregano essential oil inhibited the growth of *S.* Typhimurium more strongly in liquid medium than in the gelatin matrix.

4. Conclusions

The MIC of essential oils of garlic, cinnamon, clove, oregano and sage determined by broth and agar dilution methods showed that oregano oil was the most and garlic oil was the least inhibitory against *E. coli* O157:H7. However cinnamon inhibited *E. coli* O157:H7 more strongly in disk diffusion method than in broth and agar dilution methods. Although disk diffusion method is not used to determine MIC values, it might be useful for rapid screening of antimicrobial activity of a large number of samples.

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