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Inhibitory effects of cinnamon oil, acetic acid, and lactic acid on *Escherichia coli* ATCC 23631

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ABSTRACT

In this study, the antimicrobial activities of cinnamon oil (Cinnamomum cassia), acetic acid, and lactic acid, alone or in combination, against Escherichia coli ATCC 23631 were evaluated by the agar disk diffusion, broth dilution, and UV absorption determination methods. In the agar disk diffusion assays, using the antibiotics ampicillin and nalidixic acid as the positive control, all three agents tested exhibited an effective inhibition against E. coli with inhibition zones ranging from 3.00 to 15.33 mm, compared to those of ampicillin and nalidixic acid ranging from 0.00 to 12.00 mm. In the broth dilution assays, the MBC (minimum bactericidal concentration) values of cinnamon oil and organic acid were determined from 300 to 5,000 ppm in which cinnamon oil giving the best result. In both assays, acetic acid and lactic acid showed similar antimicrobial activities, which are lower than those of cinnamon oil or their combinations. The assays also suggested that the combinations of cinnamon oil and lactic acid showed a higher synergic effect than those of cinnamon oil and acetic acid. Although the antimicrobial activities of the combinations of cinnamon oil with the organic acids were not higher than using the pure oil, the use of these combinations would reduce the essential oil amount needed to inhibit E. coli.

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1 INTRODUCTION

Pathogens can cause disease at low concentration as well as can rapidly multiply in food, in which *Escherichia coli* is one of the pathogens that are present in the human intestine and cause urinary tract infection (Rahman and Kang, 2009; Maziero and de Oliveira, 2010). Therefore, control of *E. coli* is necessary to limit the foodborne disease. Over the past several decades, the use of preservatives to enhance food preservation has been considered a viable solution. However, the disadvantages would be faced such as concern about residues in food, environmental impact, and human health impact (Škrinjar and Nemet, 2009), and their continuous application to control *E. coli* in poultry meat is not encouraged. Moreover, the foodborne disease is an important global issue, and need for more effective preservation strategies in which looking and researching for nature antimicrobials have received increasing attention (Marino *et al.*, 2001). Essential oils from plants have demonstrated as the antimicrobial agents that inhibit both Gram-positive, Gram-negative bacteria, and mold (Karabagias *et al.*, 2011; Lieu *et al.*, 2018; Dong and Thuy, 2019). Cinnamon oil from Vietnam origin has an efficient activity against bacteria, yeast, and mold (Lieu *et al.*, 2018). Similarly, organic acids are the natural constituents of plant and animal tissues that have antimicrobial activity. Many organic acids and essential oils are considered to be GRAS (Generally Recognized as Safe) (Friedly *et al.*, 2009; Del Nobile *et al.* 2012). In previous studies, essential oil showed better antimicrobial efficiency than organic acids. However, the antimicrobial activity of different essential oils is not the same, depending on the chemical composition of essential oils and the tested microorganisms (Ozogul *et al.*, 2015; Lieu *et al.*, 2018). Besides, the impact of organoleptic properties should be considered, because the use of essential oils in food preservation would have positive or negative effects that exceed the acceptant threshold (Škrinjar and Nemet, 2009). Using a high concentration would affect the surface of the food directly. Therefore, the use of essential oil at a low concentration that still ensures the antimicrobial activity is necessary. The concentration of essential oil using for food preservation is usually higher than *in vitro* tests for ensuring the antimicrobial activity, leading to an effect on the sensory properties (Gutierrez *et al.*, 2008). Therefore, recent studies focus on the combination of essential oils and other natural antimicrobial agents. This combination would enhance the antimicrobial effect as well as reduce the amount of essential oils. In a previous study showed that cinnamon oil in combination with xanthan gum as an emulsifying agent significantly enhanced its antimicrobial activity (Lieu *et al.*, 2018). In this study, the combined effect of cinnamon oil (from Vietnamese origin) and organic acids (lactic acid and acetic acid) on *E. coli* was evaluated. The agar diffusion method, broth dilution method, and ultraviolet (UV) absorption of the culture supernatant were used to determine the minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC) and cell membrane integrity.

2 MATERIALS AND METHODS

2.1 Materials

Escherichia coli ATCC 23631 was obtained from the strain collection of Faculty of Food Science and Technology, Ho Chi Minh City University of Food Industry. *E. coli* was grown in NB (Nutrient Broth, Merck) medium at 37°C for 24 hours. The freshly grown microbial cell at approximately 6 Log CFU/mL by the spectrometer was used for the evaluation of the antimicrobial activity.

The essential oil in this study was cinnamon oil from Yen Bai of Vietnam. Cinnamon bark was hand-collected and immediately used to obtain essential oil by steam distillation. Cinnamon oil was stored in glass vials in the absence of light until analysis.

2.2 Evaluation of antifungal activity

2.2.1 Determination of MIC using diffusion agar method

The diffusion agar method was carried out according to the previous description (Dong and Thuy, 2019). The fresh biomass of *E. coli* (at final concentration 6 Log CFU/mL) was spread over the surface of TSA (Tryptic Soy Agar, Merck) medium. Cinnamon oil, acetic acid, and lactic acid at different concentrations (2, 4, 8, 16, 32, and 64 μL/mL) using alone or in combination (ratio of 1:1) were dripped (15 μL) on the medium surface. Tween 20 (without antimicrobial agents) was used as the negative control. The plates were incubated at 37°C for 24 hours. After 24 hours of incubation, the diameters of the inhibition zone were measured. The MIC values were determined as the lowest concentration of oil preventing visible growth of *E. coli*. Standard discs (6 mm) of ampicillin (10 μg/disc) and nalidixic acid (30 μg/disc) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity.

2.2.2 Determination of MBC using broth dilution method

The MBC value of cinnamon oil and organic acids was carried out according to the previous description (Lieu *et al.*, 2018). A range of concentration of cinnamon oil (100-1,000 μL/L in Tween 20 (0.3% v/v) as an emulsifying agent) and organic acids (1,000-20,000 μL/L) alone or in combination were prepared in NB medium. In the case of combination, the organic acid concentrations of 1/2 and 1/4 of their MBC values were combined with cinnamon oil.

Each flask was inoculated with 6 log CFU/mL of *E. coli*. Flasks containing only tween 20 (without antimicrobial agents) were used as control. The flasks were incubated at 37°C for 24 hours. One mL of culture was taken from each flask (where growth was not observed) for serial dilution to make the inoculum of 6 log CFU/mL and inoculated on NA (Nutrient Agar) medium plates at 37ºC in 48 hours for the determination of *E. coli* viability.

2.2.3 UV absorption determination

The experiments were carried out according to the previous description (Dong and Thuy, 2019). In brief, the biomass of *E. coli* was diluted to the test concentration by optical density (at final concentration 6 log CFU/mL, approximately) and separated into several flasks. The organic acids used alone or in combination at concentrations of 1/2, 1/4 of their MBC values with cinnamon oil were added to each flask, except for the control and incubated at 37ºC. During the incubation time, samples of 15 mL were removed from the flask at 0, 4, 8, 12, and 16 hours of incubation. The samples were immediately filtered using 0.22 µm syringe filters to remove bacterial cells and recorded optical density (OD) by a spectrophotometer at 260 nm. The effect of antimicrobial agents on the leakage of cytoplasmic contents was evaluated by the change of OD_{260nm} calculated according to the following equation:

$$\delta_{OD} = OD_t - OD_0$$

δ_{OD}: delta values of UV absorption

OD_t: OD value at t time

OD_0: Initial OD value

2.3 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SigmaPlot 11.0 followed by the Student-Newman-Keuls to compare means, with a significance level of 5% when a significant difference between treatments was noted. All tests were performed in triplicate and the data were expressed as means ± standard deviation.

3 RESULTS AND DISCUSSION

3.1 Antimicrobial effects of cinnamon oil, acetic acid, and lactic acid against *E. coli*

The antimicrobial activity of cinnamon oil, acetic acid, and lactic acid separately and combined is shown in Figures 1, 2 and 3. In the experiments with individual chemicals, the results showed that the antimicrobial diameters ranged from 3.0 mm to 15.33 mm, compared with 0-12 mm of the antibiotics ampicillin and nalidixic acid. At the same test concentration, cinnamon oil gives a larger antimicrobial zone diameter than those of the samples tested with an organic acid (p <0.05) (Figures 1 and 3). It had a MIC value of 4 µL/mL while the MIC value of acetic acid and lactic acid was 8 µL/mL (Figure 1). The combined effect of pairs of agents also showed differences (Figure 3). The combination of cinnamon oil and organic acid showed a synergistic effect while this interaction was not recorded in the combination of the two organic acids. The combination of cinnamon oil and organic acid only contained 50% of essential oils and organic acids, but it still achieved the desired antimicrobial effect (Figures 1 and 3). The results also showed that lactic acid used alone or combined with cinnamon oil gave higher antimicrobial activity than acetic acid, but this difference was not significant (p <0.05) (Figures 1 and 3).

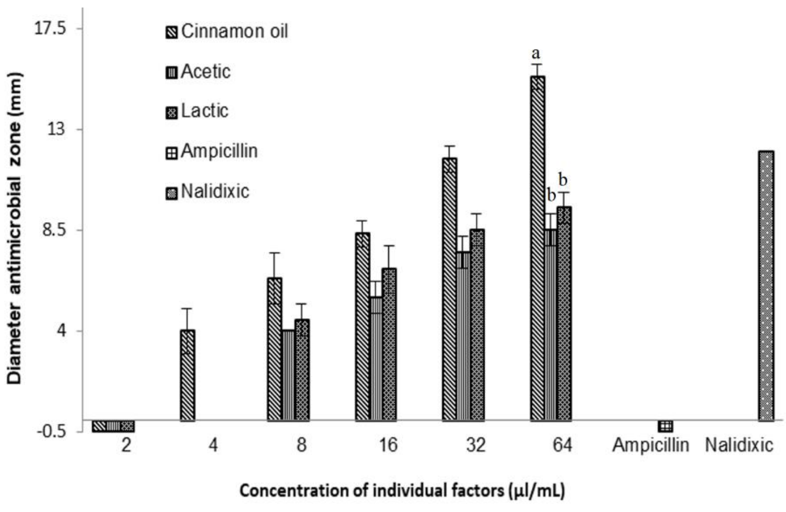

Fig. 1: Effect of individual chemical on *E. coli.* [ab] Means in the same column followed by different superscripts are significantly different (p < 0.05)

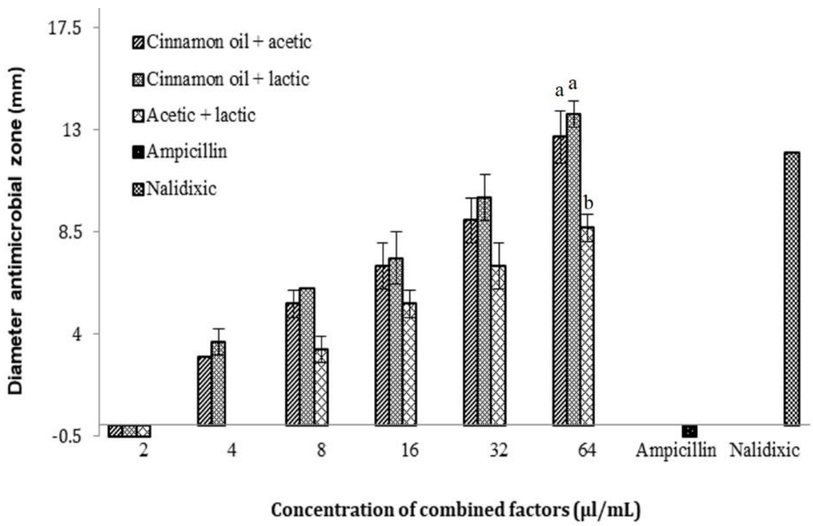

Fig. 2: Effect of combined chemical (1:1) on *E. coli.* [ab] Means in the same column followed by different superscripts are significantly different ($p < 0.05$)

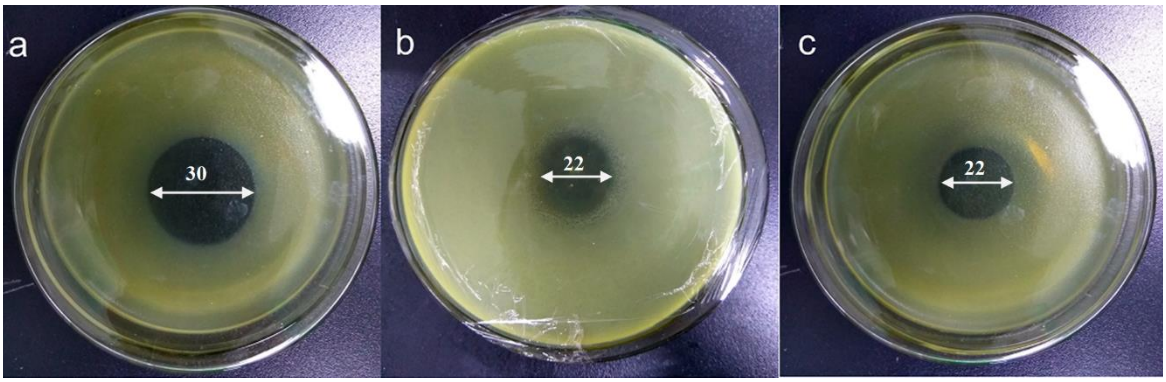

Fig. 3: The diameter (mm) of the antimicrobial zone of antimicrobial agents (256 µl/mL), cinnamon oil (a), lactic acid (b), and acetic acid (c)

Antibacterial agents of natural origin are currently receiving a lot of interest. Some organic acids (acetic, lactic, benzoic, and sorbic acid) have a long history of use in the food industry as preservatives (Skřivanová *et al.*, 2006). The mechanism of antibacterial action of cinnamon oil and organic acids has been reported in previous studies. Acetic and lactic acids (1% to 2% v/v) inhibited the growth of *E. coli* O157: H7, *Salmonella typhimurium* and *Listeria monocytogenes* in the apple from 0.52 to 2.78 log CFU/apple in case of acetic acid treatment, and 1.69 to 3.42 log CFU/apple in case of lactic acid treatment compared to control sample (Park *et al.*, 2011). Organic acids have an antimicrobial effect by affecting the integrity of cell membranes or large molecules of cells or disrupting the transport of nutrients and energy exchange (Del Nobile *et al.*, 2012). Jay *et al.* (2005) indicated that the antimicrobial activity of organic acids is due to a reduction in pH, a decrease in the internal pH of bacterial cells by ionizing acid molecules and disrupting the transport of substrates by changing the permeability of the cell membrane (Jay *et al.*, 2005). Similarly, plant-based essential oils also show a high antimicrobial effect. Chao *et al.* (2000) examined the antibacterial activity of 45 essential oils on eight bacteria (four Gram positive and four Gram negative), two fungi, and one yeast in which cinnamon bark oil (*Cinnamomum zeylanicum*) showed an inhibitory effect against all the test microorganisms and phage (Chao *et al.*, 2000). Similarly, cinnamon oil from Vietnamese origin, which contained cinnamaldehyde (77%), coumarin (10.7%), and 2-methoxy cinnamic aldehyde (2.7%) as the main compounds, has a broad-spectrum activity against bacteria, yeast, and mold (Lieu *et al.*, 2018). The hydrophobicity is an important property of essential oils and their components, which helps them separate bacterial and mitochondrial cell membranes, affecting the structure and permeability of membranes (Sheeladevi and Ramanathan, 2012) as well as deforms the structure of microbial cells (Dong and Thuy, 2019). Cui *et al.* (2016) suggested that the direct damage effect on bacterial cell membranes is the main mechanism of action of cinnamon oil.

3.2 Effect of cinnamon oil and organic acids on the growth of *E. coli* in liquid medium

The effects of cinnamon oil and organic acids on the growth of *E. coli* in liquid medium are presented in Table 1. The results showed that in the experiments with individual chemicals, cinnamon oil had the highest bactericidal effect with an MBC value of 500 ppm, that 10 times lower than those of the organic acids. Although the MBC values of acetic acid and lactic acid were not different, when combined with cinnamon oil, lactic acid showed higher antimicrobial activity than acetic acid.

Table 1: MBC values of cinnamon oil, the organic acids, and their combinations. [a-d] Means in the same column followed by different superscripts are significantly different ($p < 0.05$)

Ingredient	MBC (ppm)
Cinnamon oil + Tween 20 0.3%	500[a]
Acid acetic	5,000[b]
Acid lactic	5,000[b]
Cinnamon oil + acetic acid 1,250 ppm	450[c]
Cinnamon oil + acetic acid 2,500 ppm	350[d]
Cinnamon oil + lactic acid 1,250 ppm	350[d]
Cinnamon oil + lactic acid 2,500 ppm	300[e]

Antimicrobial efficacy of cinnamon oil and organic acids on agar medium and the liquid medium was not homogeneous in assay concentration (Figure 1, 2 and Table 1). This is because the agar environment causes the diffusion of essential oils to be limited by the antibacterial components of the essential oils that are less soluble in this environment (Aleksic and Knezevic, 2014). Previous studies have shown that the agar diffusion method requires a higher concentration of antimicrobial agents than the liquid medium to achieve the desired antimicrobial effect (Boubaker *et al.*, 2016; Dong and Thuy, 2019). This makes the assay by agar diffusion method (Figure 1) requires a higher concentration than the liquid medium (Table 1).

3.3 Effect on bacterial cell membrane integrity of cinnamon oil, the organic acids, and their combinations

The UV absorption values of the *E. coli* suspension are shown in Figure 4. The δ_{OD} values of the samples without *E. coli* were stable during the survey period (data not showed), while significant changes were recorded in samples containing *E. coli* bacteria. In the sample containing cinnamon oil, the value of δ_{OD} began to change after 4 hours while this change was recorded in lactic acid samples after 8 hours, this change was also recorded in the combination of the sample of cinnamon oil and organic acids. The results also showed that the δ_{OD} value of acetic acid samples did not differ during the survey period, while the lactic acid sample recorded a higher δ_{OD} value during the survey period. Similarly, the δ_{OD} values of lactic acid samples combined with cinnamon oil were higher than acetic acid samples (Figure 4).

The UV absorption at a wavelength of 260 nm is often used to assess the effect of essential oils on cells that have been reported in previous studies. Combined with the SEM observation, it shows that the increase in OD value corresponds to the material leakage inside the cell and the alteration of the microbial cell structure (Cui *et al.*, 2016; Dong and Thuy, 2019). However, similar studies of organic acids have not been fully published. Figure 4 shows that the effect of cinnamon oil and lactic acid increases the value of δ_{OD}, whereas the obtained results in acetic acid samples were not clear. This suggests that lactic acid may have caused a leak of intracellular protoplasm, resulting in a more effective lactic acid synergistic effect with cinnamon oil than acetic acid (Figure 4).

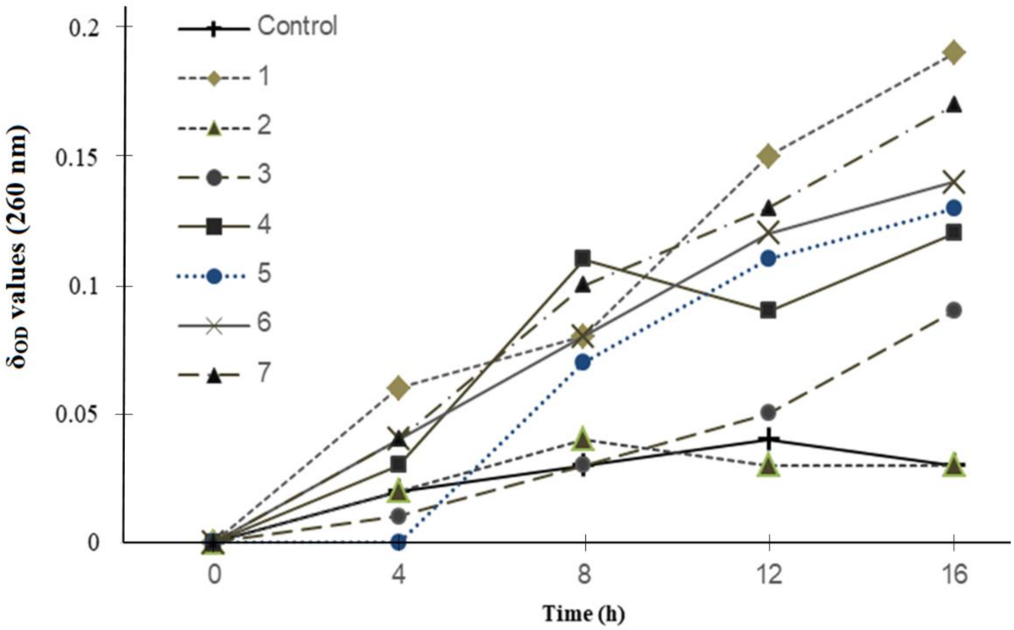

Fig. 4: Variation of the δ_{OD} absorption values

1- cinnamon oil (500 ppm) + Tween 20 0.3%; 2- acetic acid (5,000 ppm); 3- lactic acid (5,000 ppm); 4- cinnamon oil (450 ppm) + Tween 20 0.3% + acetic acid (1,250 ppm); 5- cinnamon oil (350 ppm) + Tween 20 0.3% + acetic acid (2,500 ppm); 6- cinnamon oil (350 ppm) + Tween 20 0.3% + lactic acid 1,250 ppm; 7- cassia oil (300) + Tween 20 0.3% + lactic acid 2,500 ppm.

Essential oils showed more effective antibacterial activity than organic acid (Ghellai and Beral, 2015). In addition, the antimicrobial activity of essential oils is not due to a single substance but by the action of many of the ingredients in the essential oils, which slows down the microbial adaptation to the antibacterial agent in the essential oil and ensures the stability of the application of essential oils in food preservation (Chao *et al.*, 2000). However, essential oils and organic acids at concentrations that achieve antimicrobial efficacy can cause a negative effect on the sensory property of the products as well as economic problems. Therefore, combining essential oils with organic acids will significantly reduce the amount of essential oils and organic acids to use. Though the antimicrobial activity of lactic acid and acetic acid was not significantly different ($p > 0.05$), the combination of lactic acid and cinnamon oil was significantly improved ($p < 0.05$) compared to the combination of cinnamon oil and acetic acid (Figure 1, 2 and Table 1). The synergistic effect may be due to organic acid altering cell membrane integrity (Del Nobile *et al.*, 2012), affecting membrane permeability (Jay *et al.*, 2005), creating conditions for the cinnamon oil to easily affect cell membranes and invade and cause bacterial cell death. This synergistic effect helps to reduce the amount of cinnamon oil and still ensures antimicrobial efficacy (Figure 2 and 1).

4 CONCLUSIONS

In this study, cinnamon oil, acetic acid, and lactic acid have been shown to effectively inhibit *E. coli*. Cinnamon oil provides better activity against *E. coli* than acetic acid and lactic acid when being assayed in high concentrations. The obtained antibacterial data showed that at a concentration of 4 μL/mL, only cinnamon oil gave an antimicrobial zone. Besides, cinnamon oil has an MBC value that was 10 times lower than the organic acids. The combination of cinnamon oil and organic acids significantly reduced the amount of cinnamon oil and organic acids to use. Besides, cassia oil combined with lactic acid has a higher UV absorption than the pair of cinnamon oil and acetic acid. The result indicated that the combination of cinnamon oil and organic acids is highly effective as an antimicrobial agent. In addition, because of its economic efficiency, it is a promising candidate for application in food preservation.

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