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Antimicrobial activity of noni fruit essential oil on *Escherichia coli* O157:H7 and *Salmonella* Enteritidis [1]

JIAN YANG[2], SHAYNA JO AFAISEN, RAMA GADI
Western Pacific Tropical Research Center, College of Natural and Applied Sciences
University of Guam, Mangilao, Guam 96923
jyang@triton.uog.edu

Abstract— Noni (*Morinda citrifolia*, L.) is a medicinal plant in the Pacific Islands. The noni fruit juice and powders are used as botanical dietary supplements for health benefits. In this study, we extracted noni essential oil (EO) from ripe fruit by hydro-distillation, determined its antimicrobial efficacy against *Escherichia coli* O157:H7 and *Salmonella* Enteritidis, and elucidated major antimicrobial components of noni EO. Ripe noni fruit produced EO at a yield of 5% (w/w). When directly plated on tryptic soy agar (TSA), noni EO exhibited a surface minimum inhibitory concentration ($MIC_{surface}$) at 0.3 µl/cm^2 against *E. coli* O157:H7 and *S.* Enteritidis. With the dilution method using tryptic soy broth (TSB), noni EO exhibited a minimum bactericidal concentration (MBC) at 4.0 µl/ml against *E. coli* O157:H7 and *S.* Enteritidis. The survival curve shows that noni EO at 4.0 µl/ml inactivated more than 8 logs of *E. coli* O157:H7 and *S.* Enteritidis in TSB. With the gas chromatography/mass spectrometry (GC/MS) analysis, the major volatile compounds in noni EO were identified as caprylic acid (82%), 2-heptanone (8.6%), α-pinene (4.3%), camphene (2.3%), and methyl ester (2.3%). The results suggest that caprylic acid, a medium chain fatty acid, was the main component for the antimicrobial activity of noni EO.

Introduction

Noni (*Morinda citrifolia*) fruit juice and powders are botanical dietary supplements, possessing various biological properties, such as stimulating immune systems, scavenging radicals, and anti-cancer, anti-inflammatory, antimicrobial, and antiviral activities (Chan-Blanco et al., Dussossoy et al. 2011, Yang et al. 2007). Ripe noni fruit has butyric-acid-like rancid smell attributed to short-chain fatty acids (Chan-Blanco et al. 2006, Potterate 2007). Nearly one hundred volatile compounds are identified from noni fruits, including acids, aldehydes, ketones, alcohols, esters, terpenes, sulfur compounds, lactones, and others (Farine et al. 1996, Pino et al. 2010, Wei et al. 2011).

Volatiles are major components of plant essential oils (EOs) that possess bactericidal, virucidal, and fungicidal activities (Burt 2004, Fisher & Phillips 2008). The mechanisms of antimicrobial activity of EOs involved in acting on multiple targets of cells, including degrading cell walls, damaging cytoplasmic membranes, damaging membrane proteins, leaking cell contents, coagulating cytoplasm, and depleting the proton motive force (Burt 2004). Most of herb and spice EOs exhibit antimicrobial activity at 0.05-0.1% (Tajkarimi et al. 2010). The composition of EOs affects their antimicrobial efficacy (Burt 2004, Fisher & Philips 2008).

Foodborne pathogens *Escherichia coli* O157:H7 and *Salmonella.* Enteritidis cause significant foodborne illness outbreaks through contamination of raw meat, vegetables, fruits, milk, eggs, and

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[2] Corresponding Author

egg products (Foley et al. 2008, Hanning et al. 2009, Park et al. 1999). Although noni fruit EO exhibits antimicrobial activity against *Bacillus subtilis*, *Enterococcus facecalis*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* (Brophy et al. 2008), the efficacy of noni EO on *E. coli* O157:H7 and *S.* Enteritidis is not known. The objectives of this study were to determine the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of noni fruit EO against *E. coli* O157:H7 and *S.* Enteritidis and to identify the major components of noni EO with antimicrobial activity.

Materials and Methods

Materials

The bacteria strains *E. coli* O157:H7 (ATCC 35150, 43889, 43890) and *S.* Enteritidis (ATCC 19585, 13311, 14028) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The strains were maintained at -20°C in Brain Heart Infusion broth containing 25% glycerol for less than 1 year before use.

To prepare inocula, strains of *E. coli* O157:H7 and *S.* Enteritidis culture were respectively thawed and transferred to 10 ml of sterile tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA). After incubation overnight at 37°C, the TSB culture was streaked on a tryptic soy agar (TSA) (Difco) plate and then incubated overnight at 37 °C; the plates were stored at 4°C for less than 1 month before use. A single colony from TSA plate was inoculated into 10 ml TSB and incubated at 37 °C for 24 h. Then, the cocktail, which was prepared by combining three stains of *E. coli* O157: H7 or *S.* Enteritidis in equal volume, were centrifuged at 3,600 rpm for 25 min with a IEC Centra CL2 centrifuge (Thermo Electronic Cooperation, Milford, MA, USA). The pellets were resuspended in peptone water to obtain a concentration at 1.0×10^3 and 1.0×10^8 cfu /ml as inoculum for the MIC and MBC analyses, respectively.

Hydro-Distillation of Essential Oil

Unripened, white hard noni fruits collected from wild noni plants on Guam were cleaned, air-dried, and ripened at 24 °C for 2-3 days. Noni EO was extracted from ripened noni fruit with hydro-distillation. Briefly, mashed noni fruit (300 g) and distilled water (100 ml) were placed in a 500 ml round-bottom boiling flask. During extraction at 100 °C for 4 h, the steam and the noni volatiles were condensed through a cold-water condenser and collected in a flask. The noni EO oil floating on top of the hydrosols was transferred by pipette into a brown vial and stored at 4°C for further use. The yield of noni EO was expressed as the percentage of the EO weight to the initial fruit weight (w/w %).

MIC Determination

Noni EO was diluted with 10% dimethyl sulfoxide (DMSO) and spread evenly on sorbitol-MacConkey agar (SMAC) (Difco) and xylose lysine deoxycholate (XLD) (Difco) plates to test the MIC values of *E. coli* O157:H7 and *S.* Enteritidis, respectively. The noni EO concentrations on the plate surface were at 0.1, 0.2, 0.3, and 0.4 μl/cm^2. A 0.1 ml of *E. coli* O157:H7 and *S.* Enteritidis at a level of 1.0×10^3 CFU/ml were inoculated on the SMAC plates and XLD plates, respectively. The plates were then incubated at 37°C for 24 h (*E. coli* O157:H7) and 48 h (*S.* Enteritidis). The MIC value was determined as the concentration at which no pathogen colonies were observed on the plates. Two replicates were conducted for this experiment.

MBC Determination

A broth dilution method was used to determine the MBC values of noni EO against *E. coli* O157:H7 and *S.* Enteritidis. Briefly, *E. coli* O157:H7 and *S.* Enteritidis suspensions (1 ml) were inoculated in TSB (9 ml) to obtain a concentration of 10^8 CFU/ml; then noni EO dissolved in 10% DMSO was added to obtain EO concentrations of 0, 1, 2, 4, and 8 µl/ml. After pathogens with EO were treated at 37 °C for 16 h, the samples (0.1 ml) of *E. coli* O157:H7 and *S.* Enteritidis were plated on the SMAC and the XLD agar, respectively. After incubation at 37 °C for 24 h (*E. coli* O157:H7) and 48 h (*S.* Enteritidis), the MBC value was determined as the lowest concentration of noni EO with no growth of the pathogens on the selective-media plates. Two replications of this experiment were conducted.

Pathogen Survival Analysis

To ascertain the survival of *E. coli* O157:H7 and *S.* Enteritidis (for MBC determination, above), 1 ml of pathogen sample was serially diluted (1:10) with 9 ml of sterile peptone water (1%). Portions (0.1 ml) from appropriate dilutions were spread plated with a spiral plater (Model D, Spiral Biotech, Bethesda, MD) on the SMAC plates for *E. coli* O157:H7 and on the XLD plates for *S.* Enteritidis. After incubation at 37°C for 24 (*E. coli* O157:H) and 48 h (*S.* Enteritidis), the colonies of surviving cells were counted and expressed as colony forming units (log cfu/ml). The survival curves of *E. coli* 015:H7 and *S.* Enteritidis were plotted as surviving cells vs the noni EO concentrations.

Analysis of Volatile Compounds

The analysis of gas chromatography/mass spectrometry (GC/MS) on noni EO was performed on a combined Varian 3900 GC and Saturn 2100T Ion trap Mass Spec system (Varian Analytical Instruments, Walnut creek, CA, USA). The gas chromatograph was equipped with a DB-5 CP-Sil 8 CBMS analytical capillary column (30 m × 0.25 mm I.D., coated with 5% phenyl 95% dimethylpolysiloxane, 0.25 µm film thickness). The oven temperature was programmed from 50 to 250 °C at 5 °C/min. Helium was used as the carrier gas at 1.0 ml/min. The injection port and detector was set at 250 °C. One µl of noni EO diluted with hexane was injected. Mass spectra were scanned in the mass range of 40-350 *m/z.* The volatiles of noni EO were identified by comparison of their retention indices and mass spectra with values in the NIST Mass Library. The percent content was calculated by peak area normalization.

Results and Discussion

Yield of Noni EO

The yield of noni ripe fruit EO obtained by hydro-distillation was 5.0%, higher than that of most other plant or fruit EOs. Normally, the yields of plant EOs is less than 5%; for example, the EO yield of thyme is 2% (Arraiza et al. 2009), orange peel 1.6% (Allaf et al. 2013), and coriander 0.32% (Msaada et al 2009). The high content of EO in ripe noni fruit could be one of reasons for the strong unpleasant butyric acid-like smell of the fruit.

The $MIC_{surface}$ Value

In the treatment of noni EO against *E. coli* O157:H7 on the surface of the selective SMAC agar, the tiny colorless colonies (or bacteria lawn) were observed at EO concentrations of 0.1 and 0.2 µl/cm^2 (Fig. 1). The growth of E. coli O157:H7 also turned the red color of the SMAC agar to

colorless, because E. coli O157:H7 used peptone in the medium, which raised the pH, and changed the color of the "neutral-red" indicator in the agar (Fig. 1). No colonies of *E. coli* O157:H7 were observed on the plates treated with noni EO at 0.3 and 0.4 μl/cm^2, indicating inhibition of *E. coli* O157:H7 growth.

In the treatment of noni EO against *S.* Enteritidis on the surface of selective XLD agar, back-centered colonies of *S.* Enteritidis at the edges of plates were observed at noni EO concentrations of 0.1 and 0.2 μl/cm^2 (Fig. 1). No colonies of *S.* Enteritidis were observed on the plates treated with noni EO at 0.3 and 0.4 μl/cm^2, indicating inhibition of *S.* Enteritidis growth. The results suggest that noni EO completely inhibited the growth of *E. coli* O157:H7 and *S.* Enteritidis at the concentration of 0.3 μl/cm^2 on the surface of the selective media in this study. Therefore, 0.3 μl/cm^2 is minimum minimum inhibition concentration ($MIC_{surface}$) of noni EO for both *E. coli* O157:H7 and *S.* Enteritidis.

The MIC values of plant EOs varies greatly with composition of EOs, microbial strains, and testing methods (Burt 2004). Ousealah et al. (2007) observed MIC values of 18 plant EOs against *E. coli* O157:H7 ranging from 0.025 to 0.8% (v/v). Bajpai et al. (2012) reported the MIC values of 20 plant EOs against *S.* Enteritidis from 0.5 to 4000 μg or μl/ml and the MIC values of 3 herb EOs at 20-160 μg/ml. Hulánková & Bořilová (2011) noted the MICs of oregano EO against *E. coli* O157:H7 and *S.* Enteritidis are 0.51 and 0.50 μl/ml, respectively. In comparison with these reported MIC values for other plant EOs, noni EO was an effective agent for inhibiting the growth of *E. coli* O157:H7 and *S.* Enteritidis.

THE MBC VALUE

After the treatment of noni EO against *E. coli* O157:H7 and *S.* Enteritidis in TSB, pathogens were cultured on the selective SMAC and XLD plates. Colonies of of both bacteria declined greatly with an increase of noni EO from 0 to 2.0 μl/ml (Fig. 2). At the noni EO concentration of 4.0 μl/ml^2, no colonies of *E. coli* O157:H7 on the SMAC plates and of *S.* Enteritidis on XLD plates were observed, indicating that noni EO eliminated both pathogens. Therefore, the concentration of noni EO at 4.0 μl/ml was the minimum bactericidal concentration (MBC) against both *E. coli* O157:H7 and *S.* Enteritidis in TSB.

Bajpai et al (2012) reported that the MBC values of 4 herb EOs (*Thymus vulgaris*, *Ocimum basilicum*, *Origanum vulgare*, and *Salvia officinalis*) are at 0.078-80 μl/ml against *S.* Enteritidis. Smith-Palmer et al (1998) observed the MBC values of 6 plant EOs (basil, bay, clove, cinnamon, spearmint, and thyme) are at 0.1-0.5% against *E. coli* O157:H7 and at 0.075-0.5% against *S.* Enteritidis. Compared with these reported MBC values for other plant EOs, noni EO was an effective bactericide agent against *E. coli* O157:H7 and *S.* Enteritidis.

PATHOGEN SURVIVAL

In treatment at a noni EO concentration of 0-1 μl/ml^2, *E. coli* O157:H7 exhibited a lag phase with less than 0.2 log reduction, while *S.* Enteritidis exhibited 1.0 log reduction without a lag phase (Fig. 3). At an EO concentration of 2 μl/ml^2, although both *E. coli* O157:H7 and *S.* Enteritidis reduced 2 logs, the log-reduction rate of *E. coli* O157:H7 from 0 to 2 μl/ml was slower than that of *S.* Enteritidis. At noni EO concentrations of 2 to 4 μl/ml^2, both *E. coli* O157:H7 and *S.* Enteritidis growth were reduced 7 logs with the same log-reduction rate. Overall, noni EO at 4 μl/ml in TBS

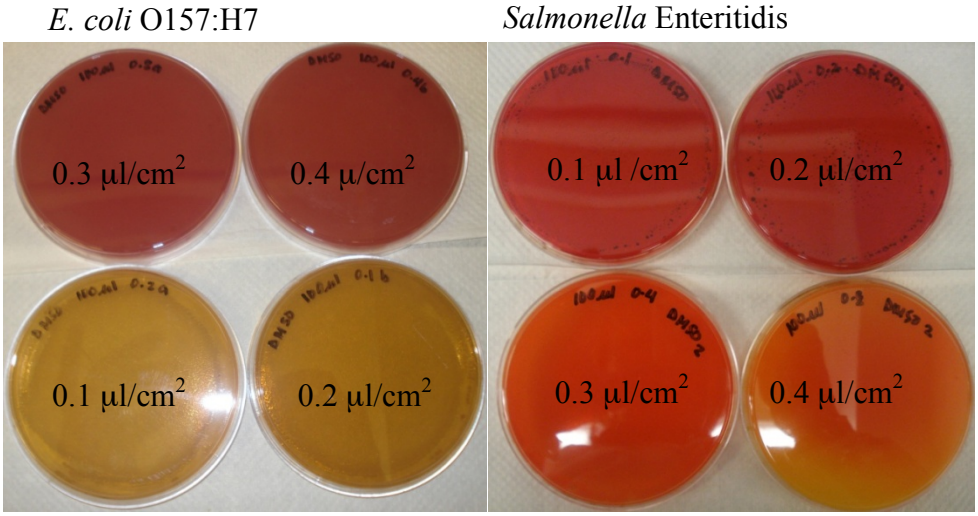

Figure 1. The effect of noni EO on *E. coli* O157:H7 and *S.* Enteritidis using the direct spreading-plate method on the MIC value of noni EO towards both pathogens.

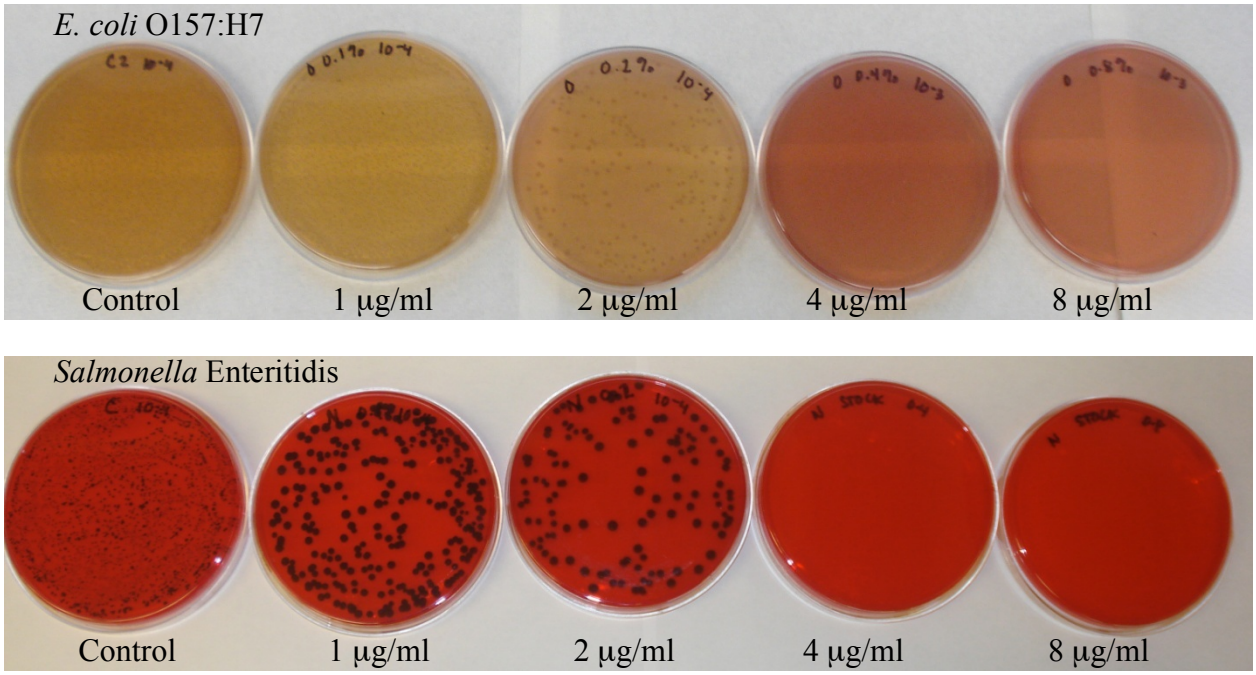

Figure 2. The effect of noni EO on *E. coli* O157:H7 and *S.* Enteritidis using the broth dilution method in TBS to determine the MBC value of noni EO against both pathogens.

caused more than 8-log growth reductions for both *E. coli* O157:H7 and *S.* Enteritidis; the result confirms their MBC values (4 μl/ml) in TSB (Fig. 2, 3).

E. coli O157:H7 and *S.* Enteritidis are both gram-negative organisms. Based on 17 plant EOs, Friedman et al. (2004) observed that *Salmonella enterica* in apple juice is more susceptible to EOs than *E. coli* O157:H7. Hulánková & Bořilová (2011) noted no significant difference in MIC values of oregano EO against *E. coli* O157:H7 and *S.* Enteritidis. In our study, at low concentrations (< 2 $\mu l/ml^2$), *E. coli* O157:H7 exhibited a higher resistance to noni EO than *S.* Enteritidis. However, at high concentration (>2 $\mu l/ml^2$), both pathogens exhibited the same susceptibility to noni EO.

GC/MS Analysis

The result of the GC-MS analysis shows that the EO of noni ripe fruit was mainly composed of caprylic acid (octanoic acid) (82%), 2-heptanone (8.6%), α-pinene (4.3%), camphene (2.3%), and methyl ester (2.3%) (Fig. 4). Caprylic acid and 2-heptanone are major volatile components accounting for 90.6% of noni EO. Caprylic acid (58-90%) and hexanoic acid (3.6-19%) are considered to be major volatiles in noni fruit (Brophy et al. 2008; Farine et al. 1996). Caprylic acid (53.7%) and hexanoic acid (10.3%) are also noted as major components of noni EO in another study (Piaru et al. 2012). Hexanoic acid was not detected in our noni EO samples, perhaps due to differences in sample preparation and noni fruit variety.

Caprylic acid is a medium chain fatty acid (containing 8 carbons) with strong unpleasant rancid smell. The high level of caprylic acid in noni EO suggests that caprylic acid is a major compound contributing to the butyric acid-like smell of ripe noni fruit. Caprylic acid is also an antimicrobial agent against fungus, bacteria, and virus (Chang et al. 2010, Kim & Rhee 2016). The MIC values of caprylic acid against *E. coli* O157:H7 and *S.* Enteritidis are 3.6 and 4.33 μl/ml, respectively (Hulánková & Bořilová 2011). Caprylic acid has been used to inactivate foodborne pathogens in alfalfa seeds and minced beef (Chang et al. 2010, Hulánková et al. 2013). α-Pinene also exhibits antimicrobial activity against *Salmonella* Typhimurium with a MIC value of 8-16 μl/ml (Bajpai et al. 2012). However, the quantity of α-pinene is much lower than that of caprylic acid in noni EO; also the MIC value of α-pinene is higher than that of caprylic acid. Therefore, caprylic acid could be the main agent of antimicrobial activity in noni EO.

Antimicrobial Mechanisms of Noni EO

Although the specific effects of noni EO on bacteria are not fully understood, antimicrobial activity is generally attributed to various phytochemicals in EOs that target bacterial cell membranes and cellular biochemical pathways (Lv et al. 2011, Seow et al. 2014). Due to their hydrophobicity, the EO molecules react with lipids in bacterial cell membranes, resulting in increasing the cell-membrane permeability, disrupting the membranes, and breaking cell homeostasis (Seow et al. 2014). Such changes in cell membranes not only cause the leakage of intracellular components, but also facilitate the movement of antimicrobial compounds into the cytoplasm, inducing cell death (Seow et al. 2014, Kim & Rhee 2016). Caprylic acid, the major compound in noni EO, has a pKa of 4.86 and is primarily in the dissociated form at a pH of 7.1-7.4 in TBS broth and SMAC and XLD agar. The mechanism of antimicrobial activity of caprylic acid against *E. coli* O157:H7 and *S.* Enteritidis in this study could be attributed to the following: (1) the dissociated form of caprylic acid first disintegrates cell membranes, changing cell-membrane permeability and disrupting the cell membranes; and (2) the changes in cell membrane faciliate the entrance of noni-EO antimicrobial compounds (including caprylic acid) into cells, which acidifies the pH of cell plasma, impairs microbial enzyme systems, and destroys cellular biochemical pathways, eventually inactivating *E. coli* O157:H7 and *S.* Enteritidis (Chang et al. 2010, Seow et al. 2014, Kim & Rhee 2016).

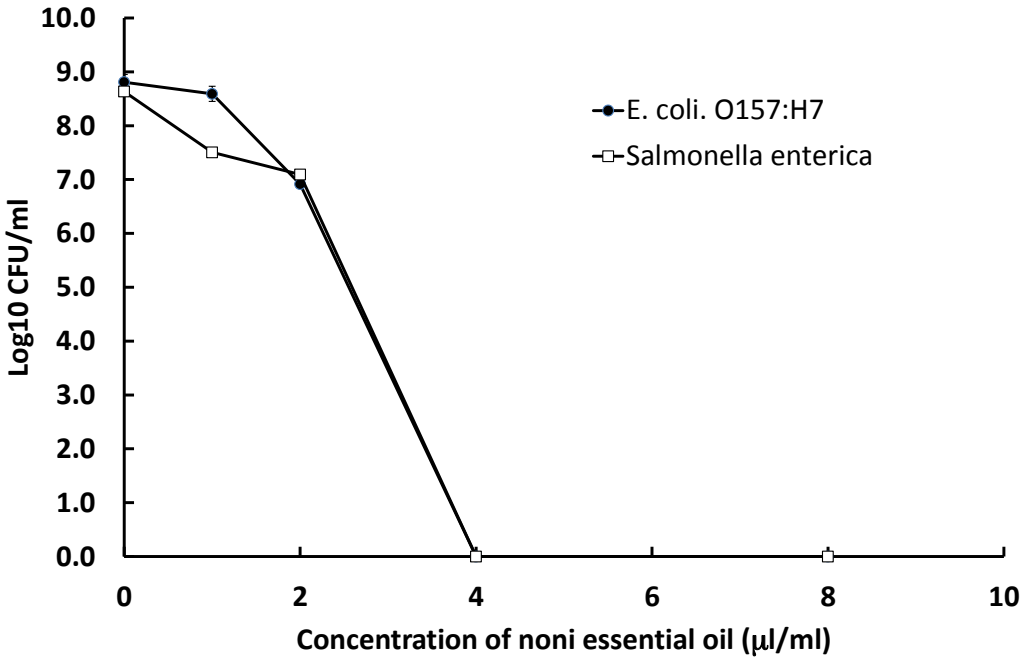

Figure 3. The survival of *E. coli* O157:H7 and *S.* Enteritidis as affected by noni EO in TBS after a treatment for 16 hours.

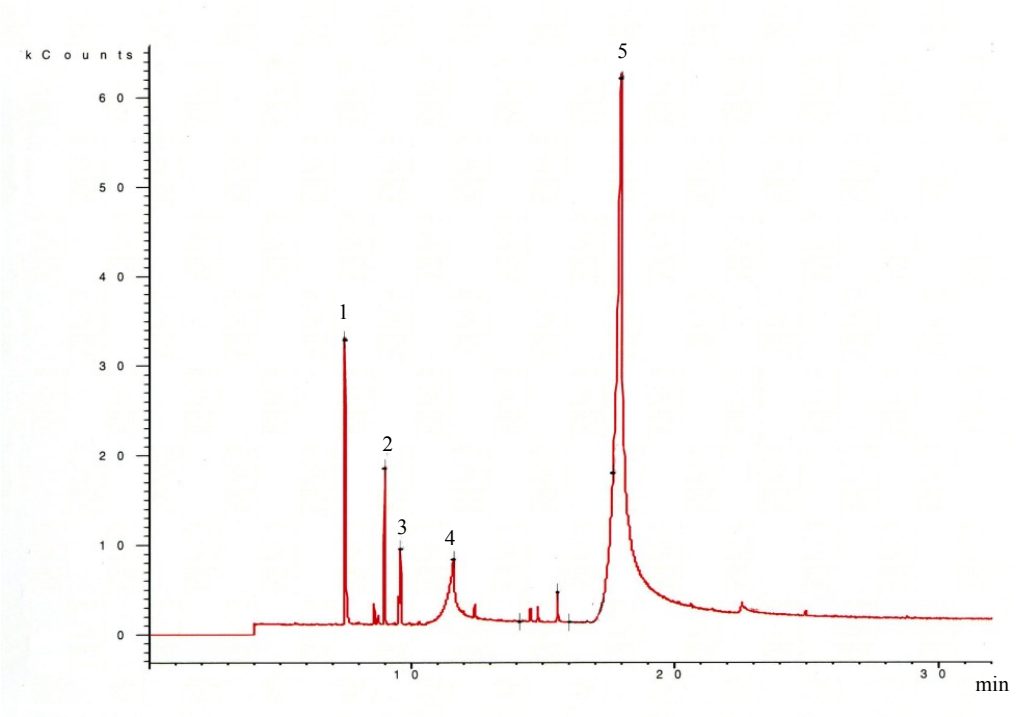

Figure 4. The GC chromatogram of noni EO: 1. α-pinene; 2. camphene; 3. Methyl ester; 4. 2-heptanone; 5. Caprylic acid.

Conclusion

Hydro-distillation of ripe noni fruit produced a yield of 5% EO. The noni EO exhibited $MIC_{surface}$ at 0.3 μl/cm^2 and MBC at 4 μl/ml against both *E. coli* O157:H7 and *S.* Enteritidis. Caprylic acid and 2-heptanone were the major components of noni EO accounting for 90.6%. Caprylic acid is likely the main agent of noni EO antimicrobial activity towards *E. coli* O157:H7 and *S.* Enteritidis. Ripe noni fruit EO has potential as an antimicrobial agent or ingredient to control foodborne pathogens.

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