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EARLY VIEW

RESEARCH PAPER

Effect of Stinging Nettle (*Urtica doica*) Essential Oil, Orange (*Citrus sinensis*) Essential Oil and Their Combinations on Chicken Wings Contaminated with *Salmonella* Typhimurium

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Abstract

The ingestion of broiler chicken has been associated with foodborne diseases arising from microbial growth. Marination with decontamination agents represents a widely adopted intervention to mitigate microbial proliferation. In this study, after the volatile components of nettle essential oil (NEO) and orange essential oil (CEO) were determined by gas chromatography, these essential oils and their combinations (100% NEO, 75% NEO - 25% CEO, 50% NEO - 50% CEO), 25% NEO - 75% CEO, 100% CEO) effect on *Salmonella t*yphimurium was determined in vitro. While the volatile component found in high amounts in NEO was found to be 21.83% limonene, it was found that 92.55% limonene was the main component in CEO. According to the disk diffusion results, the highest inhibition zone was detected in CEO, while the lowest inhibition zone was detected in NEO ($P<0.05$). In chicken wings NEO, CEO and combinations were found to reduce the number of *S*. Typhimurium compared to the control group ($P<0.05$). It was determined that as the amount of CEO in marination liquids increased, the effectiveness of marination liquids on *S.* Typhimurium increased ($P<0.05$). The use of NEO, CEO and combinations in broiler chicken wings is effective in suppressing *S.* Typhimurium contaminations.

Introduction

Microbial contamination is a major concern for the food processing industry, food manufacturers and consumers (Nannapaneni *et al*. 2008). The presence of pathogenic microorganisms in the production of poultry products constitutes a global human health problem (Byun *et al*. 2021). Chicken meat, with its high protein content, is an environment where microorganisms that cause food infections and intoxications can develop. Microbial contaminations due to environmental factors may occur in production farms and slaughterhouses. Slaughter equipment, plucking water and tools for cleaning the internal organs increase the risk of cross-contamination, additionally retail outlets are another source of cross-contamination (Byun *et al*. 2021).

Some pathogenic microorganisms originating from poultry have been associated with human diseases *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes* and *Campylobacter* are the most common pathogenic microorganisms causing food intoxications (Thanissery and Smith 2014). *Salmonella* spp. in poultry and poultry products. its presence poses a high risk to public health. Because *Salmonella* spp. can cause food poisoning and death in people with weak immune systems. *Salmonella* spp. causes salmonellosis in the human body (Byun *et al*. 2021). The number of food poisonings caused by *Salmonella* without typhoid subgroup exceeds 78 million per year (WHO 2015). More than 500,000 cases of invasive salmonellosis are

estimated worldwide each year, with the highest incidence observed in children under 5 years of age (Rama *et al*., 2022). In poultry slaughterhouses, the evisceration stage has been shown to be the most important source of *Salmonella* contamination. In addition, it has been stated that *Salmonella* maintains its vitality in processing equipment and infects clean carcasses because of insufficient/careless sanitation processes in these equipments (Thanissery and Smith 2014). Plants are considered as natural antimicrobial sources today due to their potential therapeutic effects and low/no side effects. Nettle (*Urtica dioica L.*) is a plant widely used in traditional medicine (Mahmoudi *et al.,* 2014). It has been stated in studies that nettle has many pharmacological effects such as anti-inflammatory, antirheumatic, antioxidant, antimicrobial, antiulcer and analgesic (Mahmoudi *et al*., 2014). The antimicrobial effect of stinging nettle is due to the tannins and terpenes found in its leaves and effective on microorganisms (Cesur and Soyer 2021).

In addition to its direct use in human nutrition, the incorporation of orange peels and essential oils into food products has been shown to improve the nutritional properties of biscuits by enhancing shelf life and physical quality without compromising sensory attributes (Rani *et al*., 2020). Abd El-Khalek and Zahran (2013) demonstrated that the combination of orange and mandarin peel powders with NaCl or γ-irradiation extended the shelf life by an additional 21 days. Furthermore, the inclusion of orange in food products has been reported to reduce total bacterial counts as well as yeast and mold populations, while exhibiting antioxidant properties by inhibiting lipid peroxidation and scavenging free radicals (Ademosun, 2022). In the late 19th century, the antimicrobial properties of essential oils were first discovered. Although essential oils are mainly used for aroma and fragrance, they have also been studied for their antimicrobial properties over time (Federman *et al.,* 2016). Essential oils have great potential to provide new drug precursors with proven mechanisms of action (Salehzadeh *et al.,* 2014)

In this study, the essential oil profiles of nettle and orange were analyzed, and their antimicrobial activities against Salmonella Typhimurium were evaluated both in vitro, using culture media, and in vivo, on broiler chicken wings. In the in vivo experiments, a marination-based decontamination approach was employed to assess the effectiveness of these essential oils in reducing microbial contamination, thereby providing a practical model for their potential application in poultry products.

Materials and Methods

Material

S. Typhimurium (ATCC 14028) strain used in the experimental contamination of chicken wings was obtained from the Department of Food Hygiene and Technology of Burdur Mehmet Akif Ersoy University. Essential oils (Nettle essential oil and Orange essential oil) were obtained from Oz Aromatherapy (Turkey). Chicken wings (Şenpiliç, Turkey) were obtained from local markets. Also, in the research; Buffered Peptone Water (TPW- Biokar, France), Xylose Lysine Deoxycholate Agar (XLD Agar- Merck, Germany) and Tryptic Soy Broth (TSB-Merck, Germany) were used. PS (Physiological Saline) was prepared in the laboratory to be 0.9%

Methods

GS-MC - Analysis of volatile compounds

Working temperature: The furnace initial temperature is 60°C. After staying at 60 °C for 2 minutes, the furnace temperature was increased to 220 °C with an increase of 2°C/min and held at this temperature for 20 minutes. The contents of the essential oils used in the study were determined on the Agilent 5975C Agilent 7890A GC device by dissolving 10 µL of sample in 1 mL of hexane. The detector and injector temperature was 250 °C and 240 °C, while the column was CPWAX 52CB (50*0.25 (0.2)) (Baydar *et al*., 2013).

Determination of antimicrobial activity by disk diffusion method

S. Typhimurium ATCC 14028 strains inoculated on tryptic soy agar (Merck, Germany) medium by scratch plate method was incubated for 18 h at optimum temperature (37°C) and its density was adjusted to 0.5 McFarland with the help of physiological saline. 100 µL of the prepared inoculum was taken and swabbed onto the surface of petri dishes containing Mueller Hinton Agar (Merck, Germany). 6 mm sterile disks (Whatman™ 2017-006) impregnated with 25 µL of essential oil were placed in the petri dishes containing bacteria and incubated at 37 °C for 24 hours. The diameters of the inhibition zones formed at the end of the incubation were measured with a millimetric ruler (CLSI 2021).

Establishment of working groups

The preparation of the experimental groups was carried out aseptically. In the study, groups without and containing *S.* Typhimurium were evaluated as negative control group (K1) and positive control group (K2), respectively. A total of 7 trial groups were formed, including nettle and orange essential oils and their combinations. (Table 1).

Inoculum preparation

To prepare the contamination solution (inoculum), first 30 µl of *S*. Typhimurium (ATCC 14028) strain was added to 10 ml of TSB and incubated at 37 °C for 18 h. At the end of the incubation period, the tubes were centrifuged at 5000 rpm for 5 min (Eppendolf, Centrifuge 5810 R) and the pellet and supernatant were separated. Pellets were dissolved in 1 ml of sterile 0.1% Peptone Water (PW) and then centrifuged was repeated. After

the sec centrifugation, the supernatants were removed, and the pellets were dissolved in 1 ml sterile PW again and the inoculum was prepared (Dikici *et al*., 2013).

Preparation of marination solution

Each marination solution was prepared (v/v) with 0.9% PS (98%), essential oil/combinations (1%) and Tween 20 (1%) (Merck, Germany) in total amount of 200 ml (21°C).

Inoculation of pathogenic bacteria in chicken wings

The total inoculum volume of 200 ml was adjusted to a bacterial concentration of 0.5 McFarland (1.2×10^8 cfu/ml). Contamination was achieved by turning the chicken wings 3 times with the help of a spatula.

Marination procedure

The chicken wings used in the study were obtained from a local market. Care was taken to ensure that the chicken wings were not pre-processed or marinated, and were stored at 4 °C until processing. Two wings were used for each group. The wings in the first control group were washed with 25 ml of 0.1% TPW and planted without being contaminated and marinated. The wings in the sec control group were inoculated and placed on the grids with the help of a sterile spatula and left for 15 min to allow the bacteria to adhere. After washing with 25 ml of 0.1% BPW, serial dilutions were created and cultured using the spreading method (Yılmaz *et al*., 2024). The wings in the marination groups were inoculated in the same way, put in the marinating solution and left for 20 min, and then all of them were planted according to the specified procedure. All groups were studied in triplicate.

Microbiological analysis

All groups were immediately evaluated for microbiological analysis after treatments. Samples for counting were placed in sterile bags one by one. It was rinsed with 25 mL of 0.1% buffered peptone water for 1 min, serial dilutions were prepared by taking 1 ml of this liquid and seeding was done by surface spreading method. XLD agar was used for Salmonella enumeration and incubated at 37 °C for 24 h after sowing. The number of colonies was expressed as cfu/ml in the wing washed with 25 ml of peptone water.

Statistical analysis

The study was carried out in 3 parallels and Minitab® 19.1.1 (64-bit) (USA) package program was used to evaluate the data. ANOVA test was applied to the data) first, and Duncan multiple comparison test was applied to the parameters found statistically significant ($P<0.05$) in the ANOVA test (n=3). Data are given in the study as mean ± standard deviation.

Results

Gas chromatography/mass spectroscopy (GC-MS)

A total of 18 volatile compounds were found in nettle essential oil. The highest amounts of these were limonene (21.827%), pentane (21.042%), 3-methyl and 2-heptenal (12.786%) (Table 2). A total of 54 compounds were found in orange essential oil, the highest amount of which was limonene (92.546%). GC-MS analysis results of orange essential oil are given in Table 2.

Determination of antimicrobial activity by disk diffusion method

Inhibition zones of essential oils on the growth of *S.* Typhimurium are given in Table 3. According to the data obtained, the inhibition zones of essential oils and their combinations on *S.* Typhimurium were found to be important ($P<0.05$). Similar to our microbiological analysis results, the highest inhibition diameter was in the M5 group, and the lowest inhibition diameter was M1 ($P<0.05$). Generally, it was found that as the amount of orange essential oil increases, the inhibition zone diameter increases.

Microbiological analysis

The decontamination effects of essential oils and their combinations on *S.* Typhimurium are shown in Table 4. As a result of our study, the effect of both essential oils on *S.* Typhimurium was statistically significant ($P<0.05$). It was determined that the M1 group was less effective than the other groups with a decrease of approximately 0.99 log cfu/ml compared to the control group in *S*. Typhimurium inhibition. The highest decrease in the number of *S*. Typhimurium was seen in the M3, M4 and M5 groups ($p<0.05$). When the groups in which both oils were used alone (M5-M1) were compared, the antimicrobial effect of orange essential oil was found to be higher than the antimicrobial effect of nettle essential oil ($p<0.05$). It was determined that all groups (M2, M3, M4, M5) in which orange essential oil was added had more antimicrobial effect than the M1 group. The highest effect was determined in the M3 group, where both oils were used at a 50%-50% ratio, with 1.81 cfu/mL. With this effect, it can be said that the oils synergistically increase each other's antimicrobial effect. Figure 1. *S.* Typhimurium count results

Discussion

Essential oils obtained from plants are natural sources used to increase food safety and extend shelf life in poultry meat (Marzlan *et al.,* 2021). In line with this information, the inhibitory effects of nettle essential oil and orange essential oil on *Salmonella* Typhimurium in chicken wings were investigated.

As a result of the GS-MC analysis, 54 volatile compounds were found in orange essential oil, and limonene was the component found at the highest rate with 92.5%. Dosoky and Setzer (2018) investigated the biological activities of *Citrus spp*. essential oils and reported that the main component in the GS-MC analysis of sweet orange and bitter orange oils was d-limonene, and the ratios were 83.9% and 89.7%, respectively (Dosoky and Setzer 2018). Bourgou *et al*. (2012) investigated the composition of citrus essential oils during the ripening process and identified forty volatile compounds in bitter orange essential oil. The highest component in orange essential oil was limonene with 81.52-86.43%throughout the ripening process (Bourgou *et al*., 2012). Small variations may be due to differences in the ripening stage or oil extraction method.

As a result of the analysis of nettle essential oil, 18 components were detected and the main components were limonene (21.827%), pentane (21.042%), 3-methyl and 2-heptenal (12.786%). Gül *et al*. (2012) found 43 components in their study investigating the chemical composition of nettle essential oil and the main component was carvacrol (38.2%) (Gül *et al.,* 2012). Altuner et al. (2018) determined the main essential oil component as cis-9-hexadecenal with a rate of 33.81% according to GC-MS results of *U. dioica* oil (Altuner *et al* 2018). Dhouibi *et al.* (2018) identified 24 compounds in their study investigating the main bioactive compounds of nettle extract with GC-MS analysis and determined that the main component was 2-methyltetradecane dodecane with a rate of 24.80% (Dhouibi *et al.* 2018). When the findings obtained in the study and the above-mentioned studies are compared, differences are seen in the results of each. It is thought that the reason for these differences is the variability in the regions where the herb grows and also that using only the leaf or the whole plant in obtaining the oil may affect the results.

This study investigating the antimicrobial activity against *S.* Typhimurium, the inhibition zone diameter of orange essential oil was found to be 15.27 mm, while the inhibition zone diameter of nettle essential oil was found to be 10.16 mm. Ambrosio *et al*. (2017) investigated the antimicrobial activity of various essential oils on pathogenic and beneficial bacteria and determined the inhibition zone diameter of orange essential oil against *S.* Enteritidis as 16.2 mm (Ambrosio *et al.* 2017). Tao *et al.* (2009) investigated the chemical composition and antimicrobial activities of the essential oil obtained from the peel of Bing Tang sweet orange and found that the inhibition zone diameter of orange oil against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) was 23.37 and 17.21 mm. The results given above are parallel to the results of the study (Tao *et al*. 2009).

In another study, Naveed *et al*. (2021), showed that orange essential oil showed antimicrobial activity against *S. aureus*, *B. subtilis*, *E. coli* and *S.* Typhi by agar disc diffusion method and they also stated that 10 mg/mL orange essential oil forms a 21 mm inhibition zone on *S typhi.* O'Bryan *et al.* (2008) investigated the antimicrobial effects of orange essential oils against *Salmonella* spp. and showed that the inhibition zone diameter against *S.* Typhimurium was 7.3 mm. The results are not consistent with the data of this study. It is thought that this may be due to the different strains used or the different content of the orange oils used. Modarresi-Chahardehi *et al*. (2012) investigated the antimicrobial activity of various nettle extracts in their study. The researchers measured the inhibition zone diameter of the nettle extract obtained with chloroform and ethyl acetate as <9 mm against *Salmonella paratyphi B.* Saklani and Chandra (2012) determined the inhibition zone diameter of ethanolic root extract of nettle plant against *Escherichia coli* (MTCC 729), *Streptococcus pyogenes* and *Salmonella entericatyphim* as 17±1 mm, 16±1 mm and 14±1 mm, respectively. In the study conducted by Gülçin *et al*. (2004) on the antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica L.*), the inhibition zone diameter of the nettle extract against *Escherichia coli* and *Staphylococcus aureus* was determined as 8 mm (Gülçin *et al*. 2004). As a result of the data scan, no study was found that nettle oil has antimicrobial activity against *S.* Typhimurium. For this reason, comparisons were made with different species and strains, so it is thought that the reason for the different results of the studies given is this. Irokanulo *et al.* (2021) investigated the activity of *Citrus sinensis* (sweet orange), *Citrus lemon* (lemon) and *Citrus aurantifolia* (lime) extracts at two different concentrations (400 mg/ml and 200 mg/ml) against bacteria in fresh chicken meat contaminated with *E. coli*, *S.* Typhi and *S.* Enterica. In the results, it was reported that after 400 mg/ml and 200 mg/ml orange peel essential oil treatment of meat inoculated with *E. coli*, *S.* Typhi and *S.* Enterica, the number of viable organisms in the meat decreased on average by 3.3 log, 2 log and 1 log [15]. In addition, it was stated that the number of viable organisms of meat inoculated with *S.* Enterica decreased by 1.1 log at 400 mg/ml concentrations of sweet orange peel essential oil and 0.8 log at 200 mg/ml concentration (Irokanulo *et al.* 2021). The results of this study are like the results of the study conducted. In a study conducted by Thanissery and Smith (2014), it was reported that marination with thyme and orange essential oil reduced *S.* Enteritidis by 2.6 log and *E. coli* by 3.6 log in broiler breast fillets (Thanissery and Smith 2014). It is thought that the differences between the studies may be due to the application methods of antimicrobial agents, as Lu and Wu (2012) stated that natural antimicrobials provided lower reductions in *Salmonella* spp. with immersion and spray applications. Lin *et al*. (2010) stated that Tween-20/water emulsion of sweet orange essential oil showed antibacterial activity against *V. parahaemolyticus*, *S.* Typhimurium, *E. coli* and *S. aureus* on stainless steel and plastic cutting board surfaces. Based on the results of this study, it is thought that orange essential oil can be used for decontamination purposes against *S.* Typhimurium in foods or on surfaces. Nettle (*Urtica dioica L*.) is a

medicinal plant widely used in traditional medicine (Mahmoudi *et al*. 2014). In a study by Bagheri *et al*. (2021), it was stated that encapsulating nettle with chitosan and basil seed gum and adding it to beef patties increased the shelf life of the meatballs and preserved the physicochemical parameters of the meatballs (Bagheri *et al.* 2021). Mahjoorian *et al.* (2021) reported that edible films containing nettle essential oil showed inhibitory effects against *E. coli*, *L. monocytogenes*, and *S. aureus*, but no effect was observed on *P. aeruginosa*. In another study, aqueous extracts of nettle were reported to significantly reduce the motility of *S. mikawasima* and *S. virchow* serovars and disrupt biofilm formation on *Salmonella*-contaminated spinach (Cesur and Soyer 2021). Shabani *et al.* (2023) investigated the effect of *Urtica dioica L.* essential oil on *Escherichia coli* and *Listeria monocytogenes* in minced camel meat and found that it reduced the growth of both bacteria during 18 days of storage (Shabani *et al*. 2023). Although there is no study to fully compare the decontamination effect of nettle against *S.* Typhimurium in foods, considering the sources given above and the study conducted, it is thought that nettle can be used as a preservative in foods.

Chicken meat and meat products have an important place in human nutrition due to their high protein content and cheapness. The risk of cross-contamination increases during processing stages such as slaughter, removal or transportation of post-slaughter internal organs. Therefore, preservatives are used to ensure food safety. Since chemical preservatives have toxic effects and cause consumer concerns, the use of plant-derived preservatives is becoming increasingly popular. Our study revealed that orange and nettle essential oils and their combinations ensure food safety in chicken meat. It is believed that eliminating the scientific deficiencies in the suppression mechanisms of microbial growth of vegetable oils in chicken meat will expand the use of vegetable sources in ensuring food safety.

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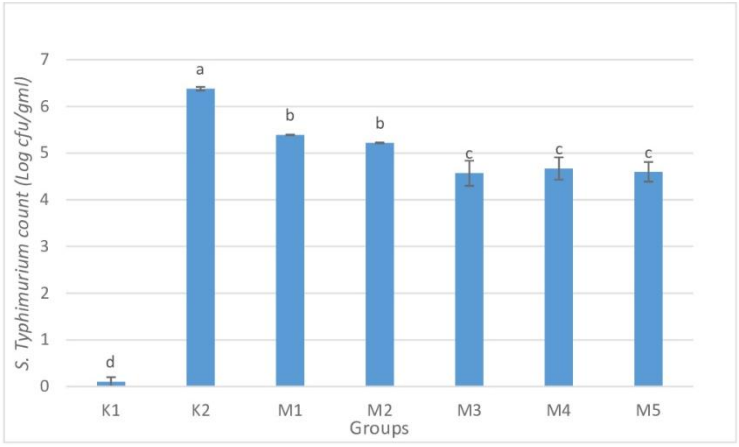

Figure 1. *S.* Typhimurium count results

Table 1. Experimental groups

Groups	*S.* Typhimurium	NEO*	CEO**
K1	-	-	-
K2	+	-	-
M1	+	100%	-
M2	+	75%	25%
M3	+	50%	50%
M4	+	25%	75%
M5	+	-	100%

* NEO: Nettle essential oil, ** CEO: *Citrus orange* oil

Table 2. Essential oil analysis results of NEO and CEO,

COMPONENT	NEO	CEO
Limonene	21.827	92.546
2.3.6.7-tetramethyl-10-(4-methylphenylsulfonyloxy)- 1.4.4. alpha.5.8.8a. beta.9. beta.9a. beta.10. beta. 10a.alpha. -	9.339	0.305
Pentane. 3-methy	21.042	-
2-Heptenal	12.786	-
Cyclopentanol. 2-(aminomethyl)-. trans	9.608	-
2.4-Decadienal	2.271	-
1-Octadecanamine	1.818	-
N-(3-Methylbutyl) Acetamıde	1.421	-
Methyl palmitate	1.079	-
Ethyl palmitate	0.792	-
Decanoic acid	0.733	-
1.1.3.3-Tetramethyl-1.3-Dısılaından	0.484	-
Stearic acid. methyl ester	0.439	-
Oleic Acid Methyl Ester	0.276	-
Ethyl stearate	0.246	-
Ethyl Oleate	0.206	-
Methyl dihydromalvalate	0.138	-
Ethyl linoleate	0.088	-
beta. -Myrcene	-	1.576
beta. -Terpinene	-	0.66
Alpha. -Pinene	-	0.612
Limonene oxide. cis	-	0.468
Sabinene	-	0.423
2-Cyclohexen-1-one. 2-methyl-5-(1-methylethenyl)	-	0.359
Trans- (+)-Carveol	-	0.287
Trans-Limonene Oxide	-	0.285
trans-p-Mentha-2.8-dienol	-	0.259
p-mentha-E-2.8(9)-dien-1-ol	-	0.233
Delta.3-Carene	-	0.205
cis-Carveol	-	0.194
1.3.6-Heptatriene. 2.5.6-trimethyl	-	0.191
Decanal	-	0.166
[1R-(1. alpha.7. beta.8a. alpha.)]	-	0.131
Alpha. Terpineol	-	0.104
Bomylene	-	0.101
2-Cyclohexen-1-ol. 2-methyl-5-(1-methylethenyl)-. cis	-	0.084
1.8-menthadien-4-ol	-	0.073
Cymol	-	0.057
Germacrene-D	-	0.052
Beta. -Pinene	-	0.05
Citronella	-	0.028
Cyclobutane. methylene-	-	0.027
Copaene	-	0.026
1-Silacyclo-2.4-hexadiene	-	0.026
1H-Azonine. octahydro-	-	0.025
1-Cyclohexene-1-carboxaldehyde. 4-(1-methylethenyl)-	-	0.025
Acetonitrile. bromo	-	0.024
2.4-Cyclopentadiene-1-ethanamine	-	0.024
Camphor	-	0.023
Limonene	-	0.021
beta. -Cubebene	-	0.021
4-(1-Methyletheny1) cyclohex-2-enone	-	0.021
2-Cyano-1-Hydroxyimidazole	-	0.021
Cyclohexene. l-methyl-4-(1-methylethenyl)	-	0.02
Phenylethanolamine	-	0.02
Citral	-	0.019
2-isopropylthio-5-trifluoracetyl-1.3-oxathiolyium-4-olat	-	0.018
1-Isopropylidene-3-Methyl-3-vinylcyclobutane	-	0.016
3-Isopropoxypropylamine	-	0.016
Acetic acid. nonyl ester	-	0.014
2-Cyano-5-Aminomethyl-Bicyclo [2.2.1] Heptane	-	0.014
3.8-Dioxatricyclo [5.l .0.02.4] octane. 4-ethenyl	-	0.014
delta. -Cadinene	-	0.013
Nerol	-	0.013
Lavandulyl acetate	-	0.012
2-methylene-7-oxabicyclo [4.1.0] heptane	-	0.012
Acetamide. N-ethyl	-	0.012
1-Decanamine	-	0.01
1.6-Hexanediamine	-	0.01

Table 3. Disk diffusion results of NEO and CEO to *S*. Typhimurium

Groups	Zone of Inhibition (mm)
M1	10.16±0,21^{c}
M2	10.09±0,13^{c}
M3	13.06±0,09^{b}
M4	13.27±0,17^{b}
M5	15.27±0,25^{a}

M1: 100% NEO, M2: 75% NEO 25% CEO, M3: 50% NEO 50% CEO, M4: 75% NEO 25% CEO, M5: 100%

* NEO: Nettle essential oil, ** CEO: *Citrus orange* oil

^{abc}: Means in the same column with different superscripts are statistically different ($p<0.05$)

Table 4. *S.* Typhimurium count result after treatment

Groups	K1	K2	M1	M2	M3	M4	M5
Count Results (log_{10} kob mL^{-1})	<1^{d}	6.38±0.04^{a}	5.39±0.10^{b}	5.22±0.01^{b}	4.57±0.27^{c}	4.67±0.24^{c}	4.60±0.21^{c}

^{abc}: Means in the same column with different superscripts are statistically different ($p<0.05$) (n=3)