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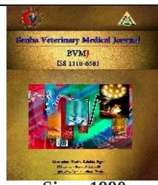

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Original Paper

Evaluation of the anti-vibrio effects of eucalyptus and neem essential oils in shrimp

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ABSTRACT

The preservation of seafood is critical for ensuring food safety, extending shelf life, and keeping quality. Therefore, the present study was planned to investigate the antibacterial effect of eucalyptus and neem essential oils (1.5% and 3.0%) on the sensory and *V. parahaemolyticus* contamination in shrimp mince during refrigeration storage. Results revealed significant reductions in *V. parahaemolyticus* counts (CFU/g) in the oil-treated groups accompanied by sensory characters' enhancement up to 10 days of storage compared with control positive samples which appeared spoilage signs after the 4th day of storage. Results of the in vitro anti-Vibrio assessment of the used oils showed wider zones of inhibition, measuring 10 mm and 12 mm for eucalyptus oil and 4 mm and 6 mm for neem oil, at concentrations of 1.5% and 3%, respectively proving the superiority of eucalyptus oil against *V. parahaemolyticus*. Furthermore, the effects of the used oils were concentration dependent; which was also approved by the *in vivo* assessment; where higher concentrations (3.0%) made higher reduction effects with reduction % of 37.5, 53.1, 21.9, and 43.8 for the eucalyptus (1.5% and 3.0%) and neem (1.5% and 3.0%), respectively. The obtained results encourage using of eucalyptus and neem oils as food additives in seafood production for safer and higher-quality products.

1. INTRODUCTION

Shrimp is recognized as a highly nutritious seafood option, offering a wealth of health benefits. Additionally, shrimp is an excellent source of essential minerals such as iodine, phosphorus, zinc, and selenium, which are crucial for various body functions including thyroid health and immune response. Moreover, shrimp is low in saturated fat and contains omega-3 fatty acids, which are beneficial for heart health. Furthermore, the antioxidant astaxanthin found in shrimp is also noteworthy; it has been studied for its potential to reduce inflammation and protect against oxidative stress, contributing to both cardiovascular and cognitive health (Dayal et al., 2013).

Despite its nutritional advantages, shrimp can pose health risks if not handled properly. One major concern is contamination with *Vibrio* bacteria, particularly *Vibrio parahaemolyticus*, which can cause food poisoning. This bacterium is commonly found in marine environments and can contaminate seafood, especially when consumed raw or undercooked. Symptoms of Vibriosis include diarrhea, abdominal cramps, nausea, vomiting, fever, and chills. In severe cases, it can lead to more serious health complications (Wang et al., 2022)

Both eucalyptus and neem contain compounds that have shown effectiveness against various pathogens. Eucalyptus oil has been noted for its ability to inhibit bacterial growth (Elangovan and Mudgil, 2023), while neem has been traditionally used in many cultures for its medicinal properties, including antibacterial effects (Jordi et al., 2024).

Eucalyptus oil, which is rich in the active compound 1,8-cineole, demonstrates potent antibacterial activity against a range of pathogens, including *V. parahaemolyticus*. The mode of action of eucalyptus oil involves disrupting bacterial cell membranes, leading to increased permeability and subsequent cell lysis. This disruption can occur through losing integrity of cell-membrane lipids and the inhibition of essential cellular functions, such as respiration and enzyme activity, ultimately resulting in bacterial death (Zhang et al., 2010). Additionally, eucalyptus oil has been shown to possess anti-inflammatory and antioxidant effects (Sebei et al., 2015).

Additionally, neem oil, derived from the seeds of the neem tree (*Azadirachta indica*), comprises a complex blend of more than 100 biologically active compounds, with azadirachtin being the most notable and extensively researched (Alzohairy, 2016). Azadirachtin exhibits significant antibacterial effects by interfering with bacterial cell division and inhibiting essential metabolic processes, thus disrupting the growth and reproduction of pathogens (Stan et al., 2021). Azadirachtin's mode of action include blocking the synthesis of chitin, a crucial component of bacterial cell walls, leading to cell lysis and death. Additionally, other compounds such as nimbin and nimbidin contribute to neem oil's antimicrobial properties by exhibiting antiviral and antifungal activities (Sarawaneeyaruk et al., 2015).

Utilizing these natural oils could provide a promising approach to enhance food safety in shrimp processing. By incorporating eucalyptus or neem extracts during the preparation stages, it may be possible to reduce the microbial

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load on shrimp and thereby lower the incidence of foodborne illnesses caused by *Vibrio* species (Wylie and Merrell, 2022).

Therefore, the present study aimed to assess the anti-Vibrio effects of eucalyptus and neem essential oils in shrimp samples that were experimentally inoculated and stored under refrigeration.

2. MATERIAL AND METHODS

The protocol of this study was approved by rhe scientific research committee, Faculty of Veterinary Medicine, Benha University, Cairo, Egypt, with an ethical approval number (BUFVTM 38-12-24).

2.1. Collection and preparation of shrimp

A total of 900 g of chilled, raw middle-size Peregrine Shrimp (*Metapenaeus stebbingi*) were obtained from El-Obour city central market, Qalyubia governorate. Samples were transferred in an ice box to the Animal Health Research Institute-Benha regional lab, where it was peeled, minced, and subjected to laboratory investigations.

1.1.2.

2.2. Collection of essential oils

Commercially prepared ready-to-use essential oils of eucalyptus (*Eucalyptus globulus*), and neem (*Azadirachta indica*) were collected from Nefertari Co. for oil production, Egypt. Hydro-distillation is used to extract the essential oils (EOs) from their sources with a purity of 99.9%. EOs were kept in dry-sealed dark glass vials at 4°C until use (Barros et al., 2022).

2.3. In vitro determination of the minimum inhibitory concentration (MIC) of the used essential oils

2.3.1. Preparation of *V. parahaemolyticus* strain

It was performed according to ISO 21872-1 (2017). Field strain of *Vibrio parahaemolyticus* stock culture was enriched in alkaline saline broth, followed by tenth-fold serial dilution and spreading onto Thiosulfate citrate bile and sucrose agar (TCBS) then incubated at 37°C for 24 hours for obtaining 10^3 CFU/ml working culture.

2.3.2. Determination of in vitro antibacterial effect of the used oils

In vitro determination of the anti-vibrio effect of the used essential oils was performed by Agar well-diffusion technique according to CLSI (2012). The original oil was double-fold diluted by Tween-80 as an oil emulsifier to obtain 3.0% and 1.5% concentrations. Each of these concentrations was examined against the foodborne *V. parahaemolyticus* strain (10^3 CFU/ml) to investigate its antibacterial effect represented by a clear zone of inhibition before its application in shrimp meat samples.

2.4. Experimental design

2.4.1. Preparation of shrimp samples

Peeled shrimps were minced under hygienic conditions followed by arrangement in thin layers, and exposed to UV light to inhibit the most of commensal bacteria according to Soro *et al.* (2023), followed by inoculation of *V. parahaemolyticus* (about 10^3 CFU/g). After which, minced shrimp meat was grouped into six groups (150g/each group). It is worth noting that shrimp samples were examined bacteriologically and confirmed to be free from *V. parahaemolyticus* before the experimental inoculation and treatment. Groups were represented as follows:

Group 1: Untreated minced shrimp + *V. parahaemolyticus* (10^3 CFU/g) (Control positive)
Group 2: Treated minced shrimp with eucalyptus oil (1.5%) + *V. parahaemolyticus* (10^3 CFU/g).
Group 3: Treated minced shrimp with eucalyptus oil (3.0%) + *V. parahaemolyticus* (10^3 CFU/g).
Group 4: Treated minced shrimp with neem oil (1.5%) + *V. parahaemolyticus* (10^3 CFU/g).
Group 5: Treated minced shrimp with neem oil (3.0%) + *V. parahaemolyticus* (10^3 CFU/g).
Group 6: Untreated minced shrimp only (Control negative).

Treated samples were kept all refrigerated at 4 ± 1^{O} C until sensory and bacteriological examination at day zero (2hrs. post-treatment) was conducted, then periodically every 2 days up to the appearance of organoleptic spoilage during cold storage. Samples were subjected to the following examinations:

2.4.2. Sensory evaluation of examined shrimp samples

Shrimp mince samples were assessed for their sensory characteristics (color and texture) following Mörlein (2019) in scores (1 to 5), where 1- represented the worst while 5- represented the excellent mark.

2.4.3. Bacteriological examination

Samples were prepared according to ISO 6887-2 (2017), followed by counting of *V. parahaemolyticus* according to ISO 21872-1 (2017).

2.5. Statistical analysis

The statistical analysis was performed by using the Analysis of Variance (ANOVA) test on SPSS software v.20 according to Feldman et al. (2003). Reduction (%) = $\left(\frac{(R1-R2)}{R1}\right) x\ 100$, where R1 and R2 indicate the microbial count of control and treated samples, respectively.

3. RESULTS

As illustrated in Fig. 1, the eucalyptus oil exhibited broader zones of inhibition at concentrations of 1.5% and 3.0%, measuring 10 mm and 12 mm, respectively. In contrast, neem oil demonstrated smaller inhibition zones of 4 mm and 6 mm at the same concentrations. These findings suggest that eucalyptus oil possesses a greater potency against vibrio species compared to neem oil in the in vitro study.

Fig. (1). Inhibition zone of eucalyptus oil

The sensory scores of the treated shrimp mince showed a significant enhancement in the sensory quality that appeared as an elongation of the physical acceptability in relation to the control groups; which started to appear of spoilage characteristics after the 4th day of storage. Although all of the treated samples showed longer acceptability time in relation to the control groups, neem-treated groups (G4 and G5) had higher sensory scores than the eucalyptus-treated groups (G2 and G3) because of their neutral preferred odor intensity. It is worth noting that the enhancement effect was directly related to the concentration of the used oil (Fig. 2).

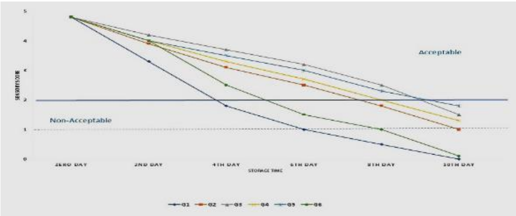

Fig. (2). Sensory profile of the examined fish groups in cold storage (4±1^{O}C). According to this figure, if the final quality score is 2, the sample's quality is marginally acceptable. If this score is less than 2, the sample is unacceptable. If this score is less than 1, the sample is apparently spoiled. G1: Control positive, G2: Eucalyptus 1.5%, G3: Eucalyptus 3.0%, G4: Neem 1.5%, G5: Neem 3.0%, G6: Control negative.

The anti-vibrio effect of the used oils (Table, 1), eucalyptus-treated groups showed a higher antibacterial effect than neem-treated ones. Anti-vibrio effect of the used oils appeared to be dose-dependent where higher concentrations (3.0%) made higher reduction effects. The initial count of *V. parahaemolyticus* at the zero-day was 3.2 $\log_{10}$ CFU/g that reached 2.0, 1.5, 2.5, and 1.8 $\log_{10}$ CFU/g for the eucalyptus (1.5% and 3.0%) and neem (1.5% and 3.0%), with maximum reduction % of 37.5, 53.1, 21.9 and 43.8, respectively.

Table 1 Mean values of *V. parahaemolyticus* count ($\log_{10}$ CFU/g ± SE) of the examined groups during storage at 4±1^{o}C

Day	G1	G2	R%	G3	R%	G4	R%	G5	R%	G6
Zero day	$3.2^{a} \pm 0.1$	$3.2^{a} \pm 0.1$	--	$3.2^{a} \pm 0.1$	--	$3.2^{a} \pm 0.1$	--	$3.2^{a} \pm 0.1$	--	ND
2nd day	$3.6^{a} \pm 0.1$	$2.8^{d} \pm 0.1$	12.5	$2.5^{d} \pm 0.1$	21.9	$3.0^{c} \pm 0.1$	6.3	$2.6^{b} \pm 0.2$	18.8	ND
4th day	$4.5^{a} \pm 0.1$	$2.4^{d} \pm 0.1$	25.0	$2.1^{d} \pm 0.1$	34.4	$2.8^{c} \pm 0.2$	12.5	$2.3^{b} \pm 0.2$	28.1	ND
6th day	xx	$2.2^{c} \pm 0.2$	31.3	$1.7^{c} \pm 0.2$	46.9	$2.6^{a} \pm 0.3$	18.8	$2.0^{b} \pm 0.1$	37.5	ND
8th day	xx	$2.0^{c} \pm 0.1$	37.5	$1.5^{c} \pm 0.1$	53.1	$2.5^{a} \pm 0.3$	21.9	$1.8^{b} \pm 0.1$	43.8	ND
10th day	xx	2.3 ± 0.2	28.1	1.8 ± 0.2	43.8	Xx	Xx	xx	xx	ND

Results were presented as mean±SE of triple trials. ^{abcd} different superscript letters within the same row mean statistically significant difference when ($P \leq 0.05$). R%: Reduction % xx. means that the samples showed signs of spoilage

4. DISCUSSION

Shrimp is a highly valued seafood that plays a significant role in global diets due to its rich nutritional profile and numerous health benefits. As one of the most commonly consumed shellfish, shrimp is not only delicious but also offers a low-calorie, high-protein option that is ideal for consumer's health. Additionally, shrimp is packed with vital vitamins and minerals, including iodine, selenium, and omega-3 fatty acids, which support thyroid function, boost immune health, and promote cardiovascular wellness (Liu et al., 2021).

Vibrio parahaemolyticus is a significant seafood-associated pathogen that poses a serious health hazard due to its prevalence in marine environments and its association with foodborne illnesses (Broberg et al., 2011). This halophilic bacterium is commonly found in raw or undercooked seafood, particularly shellfish, and is known to cause acute gastroenteritis in humans. Symptoms of infection include diarrhea, nausea, vomiting, and abdominal cramps, which can lead to severe dehydration, especially in vulnerable populations such as the elderly or those with compromised immune systems (Ndraha et al., 2022). The rise in seafood consumption, particularly raw dishes, has contributed to an increase in reported cases of *V. parahaemolyticus* infections globally. This pathogen's ability to thrive in warm coastal waters further exacerbates the risk of contamination, making it a critical concern for public health authorities (EFSA, 2024).

The natural preservation of seafood has gained significant attention as a means to enhance shelf life while maintaining quality and safety, particularly through the use of essential oils (Baptista et al., 2020). These oils, derived from various plants, possess antimicrobial properties that inhibit the growth of spoilage microorganisms and pathogens in seafood (Pierozan et al., 2024). For instance, essential oils such as those from eucalyptus and neem have been shown to effectively reduce bacterial counts and extend the freshness of fish and shellfish (Jara-Medina et al., 2024).

Regarding the Egyptian national standard of chilled shrimp (EOS No. 5021, 2020), shrimp must be free from *V. parahaemolyticus*, with a maximum of seven days' shelf-life. The present results revealed enhanced sensory quality with an extended shelf-life of up to ten days of storage. In addition, the significant antibacterial effect of the used oils appeared as obvious reductions in the *V. parahaemolyticus* counts in the treated groups; The current improved sensory quality and shelf life of the treated groups either by eucalyptus or neem oils may be attributed to their notable antioxidant effect, as well as their antibacterial potential that help in keeping the favorable sensory quality of seafood (Sebei et al., 2015). Our results came in line with the recorded results by Jara-Medina et al. (2024) who found that eucalyptus extract exhibited a significant inhibition of *Vibrio* strains, achieving up to 75% growth inhibition at a concentration of 200 µg/mL. In contrast, while neem oil also possesses antibacterial properties, its effectiveness against *V. parahaemolyticus* has been reported to be less pronounced than that of eucalyptus oil, particularly in terms of biofilm inhibition (Chen et al., 2024). Besides that, one study demonstrated that neem leaf extract significantly inhibited the growth of *V. parahaemolyticus*, with a minimum inhibitory concentration (MIC) of 62.5 mg/mL, indicating its effectiveness in controlling this pathogen in food products (Mahmoud et al., 2024). Additionally, another investigation reported that neem nano-emulsion exhibited promising antibacterial activity against various *Vibrio* species, including *V. parahaemolyticus*, by disrupting bacterial cell membranes and leading to cell lysis (Ravi et al., 2019). Furthermore, a review emphasized the broad-spectrum antimicrobial properties of neem extracts, noting their effectiveness against antibiotic-resistant strains of *V. parahaemolyticus* (Wylie and Merrell, 2022). So, the current study findings suggest that neem oil and its derivatives can be effectively utilized to enhance food safety by targeting harmful bacteria like *Vibrio parahaemolyticus* in seafood products.

Records consistently demonstrated that eucalyptus oil exhibits superior antimicrobial activity compared to neem oil across various applications. For instance, Massey and Mehtab (2023) indicated that eucalyptus oil demonstrated a maximum zone of inhibition of 10 mm against *S. aureus*, while neem oil only achieved a zone of 2 mm under similar conditions, highlighting the greater efficacy of eucalyptus oil. Additionally, another comparative evaluation found that eucalyptus oil had significantly better antibacterial activity against multidrug-resistant pathogens compared to neem oil, which showed only moderate activity (Shenoy et al., 2014). The active component 1,8-cineole, which makes up the majority of eucalyptus oil, has strong antibacterial qualities against a variety of foodborne bacteria, including *V. parahaemolyticus*. The way eucalyptus oil works is by breaking down the membranes of bacteria, which increases permeability and causes cell lysis. Bacterial death may follow from this disruption, which can be caused by the loss of cell-membrane lipid integrity and the inhibition of vital cellular processes including respiration and enzyme activity (Zhang et al., 2010). Additionally, a complex mixture of more than 100 biologically active substances may be found in neem oil. Of these, azadirachtin is the most well-known to block vital metabolic functions and interfere with bacterial cell division. It works by preventing the production of chitin, which is essential for bacterial cell walls and causes cell lysis and death (Sarawaneeyaruk et al., 2015).

5. CONCLUSIONS

The application of eucalyptus and neem essential oils represent a promising frontier in seafood preservation. Both oils offer significant advantages in enhancing food safety without compromising the sensory qualities of shrimp products; where eucalyptus oil showed more potent anti-vibrio effect than neem oil with higher reduction effects; therefore, it is recommended to use eucalyptus and neem oils (1.5% and 3.0%) as safe food additive to the seafood for safer and higher quality seafood.

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