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Antimicrobial effectiveness of clove oil nanoemulsions against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in fresh beef samples

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Fresh meat is highly susceptible to food borne pathogens. Controlling these pathogenic microorganisms is important for maintaining food safety either by chemical or physical treatments. However, due to consumer preferences and health reasons there is demand for natural and better antimicrobial agents. There has been extensive work on natural alternative antimicrobial agents but the delivery of such agents to food systems is still a challenge. One alternative delivery system i.e., food grade nanoemulsions (NEs) as promising intervention. To explore this concept, various formulations of (NEs) with added clove oil concentrations ranging from 0.1-5% (w/w) were tested for controlling microbial load in fresh beef samples. Sterile raw meat samples were artificially inoculated with *Escherichia coli* O157:H7 and *Staphylococcus aureus* followed by spraying clove oil NEs. The samples were stored at room temperature and later enumerated for bacterial load after 60 min of treatment. After treatment there were significant reduction in bacterial loads present in fresh meat samples (10 ± 3 and 5 ± 2 CFU of *Escherichia coli* O157:H7 and *Staphylococcus aureus*) respectively when compared with control (TNTC). The data suggested that clove oil NEs may be an effective strategy to control microbes in fresh beef.
Keywords: Essential oils, food safety, antimicrobial agent, clove oil, nanoemulsions, beef.

INTRODUCTION

Approximately 600 million people suffer from the foodborne illnesses and 0.42 million deaths every year, due to consumption of contaminated foods (WHO, 2020). Fresh meat provides an ideal environment for microorganisms to grow, particularly *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes*, due to its high water activity and nutrient content (CDC, 2019). Complicated food supply chain, abattoir, butchery shops are considered to be the primary source of contamination (Jamali *et al.*, 2014; Mezal *et al.*, 2013). Several approaches have been used to control contaminations i.e., use of irradiation, carbon dioxide and application of culinary herbs. However, these methods have limitations in its effectiveness owing to higher cost and denaturation of proteins that lead to undesirable textural changes in final product (González-Alonso *et al.*, 2020; Kim *et al.*, 2020). There is a need to develop effective techniques to protect meat products from bacterial infections without affecting its physicochemical characteristics.

Plant essential oils (EO_S) have demonstrated a promising substitute in comparison to chemical preservation for controlling food borne bacteria. Clove (*Syzygium aromaticum*) belongs to the *Myrtaceae* family, traditionally used as flavoring agents and antimicrobial additives (Hyldgaard *et al.*, 2012; Manganyi *et al.,* 2015). Eugenol, the most essential ingredient in clove, has powerful biological effects like antioxidant, insecticidal, antimicrobial, anti-vomiting, antiseptic, antispasmodic, analgesic, anti-carminative and anti-phlogistic activities (d'Avila Farias *et al.*, 2014; Guan *et al.*, 2007; Pinto *et al.*, 2009). It also contains beta-caryophyllene, eugenyl acetate, alpha-humelene and many others bioactives (Majeed *et al.*, 2016). Clove oil has shown antibacterial efficacy in various studies against *Micrococcus luteus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Bacillus cereus* (Fu *et al.*, 2007; Hoque *et al.*, 2008). The antibacterial effects of EOs is due to the disruption of nucleic acid synthesis, energy metabolism, and cytoplasmic membrane disruption (Barbosa *et al.*, 2015). Many researchers have reported preservative

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ability of clove EOs to extend the shelf life of foods (Hosseini *et al.*, 2009; Ortiz *et al.*, 2018).

Antimicrobial efficiency of clove oil, cinnamon oil and garlic oil against *Listeria monocytogenes*, *Staphylococcus spp.* and *E. coli* was found ineffective by Babu *et al.* (2011) due to higher volatility and low water solubility of these EOs. There are multiple delivery systems like nanoparticles, microemulsions, nanoliposomes, dispersions are there to encapsulate different bioactive compounds, However, stability of these delivery systems is a major challenge. The focus of our study was to develop a food grade stable delivery system which can be applied easily on raw meat products, secondly, NEs have excellent encapsulation ability as compared to other delivery systems. Moreover, nanoemulsions (NEs) based delivery systems have been investigated and formulated to improve the stability and solubility of these EOs, like oregano oil nanoemulsions to combat foodborne pathogens (Bhargava *et al.*, 2015). In a related study, Xue *et al.* (2015) examined thyme oil nanoemulsions for antimicrobial activity against *Salmonella enteritidis* and found that they had a substantial reduction in bacterial growth compared to free thyme oil.

Potential antimicrobial actions of nanoencapsulated EOs is due to the infusion of EO into phospholipid layer of bacterial cell membrane along with the release of EOs for an extended period of time from nanoemulsion matrix. Furthermore, Since the partitioning coefficient between the aqueous and lipid phases is higher, the antimicrobial effects are stronger (Chang *et al.*, 2013; Moghimi *et al.*, 2016). A terpenes NEs in fruit juices are found to inhibit spoilage microorganisms (Donsì *et al.*, 2011). A thymol emulsion exhibited pH- and food matrix-dependent reduction on *Escherichia coli* O157:H7 (Shah *et al.*, 2012). Despite these applications, there is dire need of work on the long-term stability and antibacterial effectiveness of EO nanoemulsions using fresh meat models. As a result, the purpose of this research was to formulate a food-grade clove oil NEs and test its efficacy against food-borne pathogens such as *Staphylococcus aureus* and *E. coli* O157:H7 in meat samples.

MATERIALS AND METHODS

***Chemicals*:** Clove oil was procured from Now Oils (GNC, USA). HI-CAP-100 modified food starch was generously donated by Ingredion Germany GmbH. Sigma-Aldrich provided the Sorbitol MacConkey and Mannitol Salt Agar.

***Preparation of clove oil NEs*:** Oil-in-water (O/W) NEs containing clove oil were formulated based on the method described by Iqbal *et al.* (2020) with small variations. Emulsions (O/W) were prepared by mixing aqueous phase containing HI-CAP (5% (w/w) as emulsifier. HI-CAP 100 is an OSA-modified straight-chain starch and is highly suitable for encapsulation applications. The dispersed phase contains varying concentration of clove oil (C_{OC}-1 (1%), C_{OC}-2 (2%), C_{OC}-3 (3%), C_{OC}-4 (4%), C_{OC}-5 (5%). At 25 °C, these phases were subjected to three passes of a 275 MPa high-pressure disruption device (Constant Systems Ltd, Northants, UK).

Measurement of droplet size: The Malvern Zetasizer Nano ZS90 was used to determine the particle size of O/W NEs using the light scattering process (Malvern Instruments Inc., Worcestershire, UK). The O/W NEs were diluted 50 times, and all measurements were taken in triplicate at 25°C with the refractive index set to 1.35.

Measurement of ζ- potential: To examine the ζ-potential of NEs, a ζ-potential analyzer (Zetasizer Nano ZS90, Malvern Instruments Ltd, Worcestershire, UK) was used. Prior analysis, nanoemulsions were diluted 50 times and all measurements were collected in triplicates at 25°C with refractive index fixed at 1.35.

Long term stability: The long-term stability of formulated NEs were investigated by determining mean droplet size of NEs. Nanoemulsions were stored at ambient temperature over the period of 5 months and droplet size was investigated with the interval of 30 days (Souilem *et al.*, 2017).

***Minimum Inhibitory Concentration (MIC) of clove oil NEs*:** Purified *S. aureus* and *E. coli* O157:H7 strains were obtained from laboratory collection center of School of Life Sciences, Forman Christian College University Lahore. In order to achieve an optical density of 1.0 at 600 nm, the inoculation each of a single colony of *S. aureus* and *E. coli* O157:H7 was performed on LB broth, followed by incubation at 37ºC for 17 and 12 hours, respectively. The spread plate method was used for inoculation of 100 μL of *S. aureus* and *E. coli* O157:H7. The MIC of clove oil NEs was calculated using the well diffusion process. The MIC was calculated by testing 14 separate NEs dilutions (0.1-5 percent). The pouring into wells of 90 μL (for each dilution) was performed, with subsequent incubation for 24 hours at 37ºC. An observation regarding the clearing zones was made, and diameters of inhibition zones were recorded. LB broth medium cultured under same conditions was used as blank (Franklyne *et al.*, 2019; de Meneses *et al.*, 2019; Natania and Setiawan, 2020).

***Antimicrobial analysis*:** Stock cultures of *E. coli* O157:H7 and *S. aureus* were grown in LB broth at 37 ºC for 17 h and 12 h respectively followed by washing with phosphate-buffered saline, before inoculation. A bacterial suspension of 100 μL was mixed with 9 mL PBS and applied to fresh meat for 20 minutes. NEs containing 0.9% (v/v) clove oil were added to one piece of the sample and held for 2 hours, while the other sample was used as a control (Bhargava *et al.*, 2015).

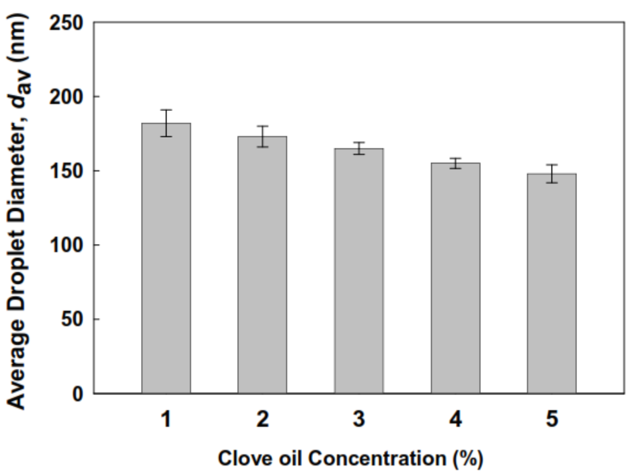
***Microbial assay*:** A sterile (Butterfield) phosphate buffer was used to prepare meat samples at 7 pH. A 225 mL of Butterfield phosphate buffer was used to dilute the 25 g meat samples (control and NEs treated meat) to achieve a dilution of $1{:}10^{-1}$. To screen *E. coli* O157:H7 in Sorbitol MacConkey Agar and *S. aureus* in Mannitol Salt Agar, 1 mL of suspension was inoculated by using pour plate method (Agarwal *et al.*, 2020; Shahbazi, 2019).

***Statistical analysis*:** Minitab 16 was used to measure mean values and standard deviations, and further analysis of means was completed by applying one-way analysis of variance. All of the values of experiments were taken in triplicates.

RESULTS

***Analysis of particle size and surface charge of O/W NEs*:** The clove oil NEs appeared translucent having mean particle size (d_{av}) of 148.2 ± 6 to 182 ± 9 nm with varying clove oil concentrations (Fig. 1A). There was significant difference in d_{av} of NEs containing lowest (1%) and highest (5%) oil concentrations in formulations. Negative charge was observed on the surface of the NEs and the average zeta potential was recorded between -20 to -32 mV (Fig. 1B).

(a)



(b)

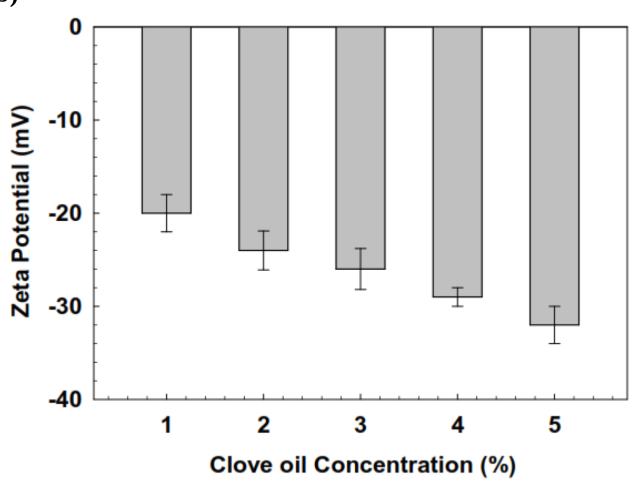


Figure 1. Physicochemical characterization of clove oil emulsions. (A) Droplet size distribution of O/W nanoemulsions, (B) ζ-potential of O/W clove oil NEs.

***Storage Stability*:** The storage stability of formulated emulsions with respect to clove oil content was also investigated over a period of 5 months. The Fig. 2 showed change in particles size over the period of time with varying concentrations of clove oil. However, except the Coc-1 and Coc-2, all formulated NEs showed stability for the course of five months without any significant (p > 0.05) coagulation (Fig. 2). After 30th and 60th day small increase in d_{av} was observed in all formulations due to Oswald ripening and coalescence. At day 90, the particle size of Coc-1 and Coc-2 increased significantly which might be due to the Ostwald ripening and coalescence, however the d_{av} of Coc-3, Coc-4 and Coc-5 showed non-significant increase in d_{av}. Finally, after 5 months, Coc-1 and Coc-2 showed phase separation, however Coc-5 with d_{av} 201 ± 11 remained stable without showing any significant phase separation.

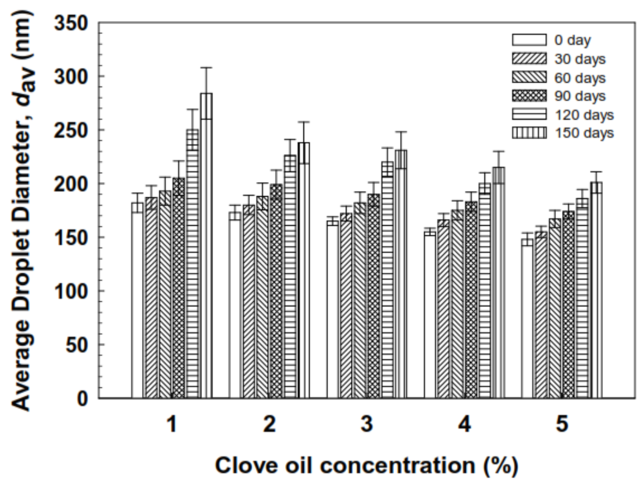


Figure 2. Effect of storage temperature on physical stability of clove oil NEs.

***Minimum Inhibitory Concentration*:** Clove oil NEs had a minimum inhibitory concentration (MIC) of 0.9%. An increase in the concentration of clove oil NEs resulted in an increase in well diameter, as shown in Fig. 3, suggesting a dose dependent effect. HI-CAP 100, on the other hand, has no impact on bacterial development. Clove oil NEs had constant antibacterial activity against *E. coli* O157:H7 at 4% and 5%, respectively, with a 3.0 mm inhibition region. In contrast, *S. aureus* displayed similar results in MIC (Fig. 3). Results were consistent with the findings of Bhargava *et al.* (2015) who used oregano oil to monitor *Salmonella typhimurium*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on fresh lettuce sample.

***Antimicrobial effect of clove oil loaded NEs on meat*:** Microbial count difference of control and treated group exhibited a considerable reduction in bacterial count of *E. coli* O157:H7 and *S. aureus* in NEs loaded meat. Number of colonies of *E. coli* O157:H7 and *S. aureus* after application of nanoemulsions were 10 ± 3 and 5±2 colonies respectively while in control too numerous to count (TNTC) was observed after 24 h (Fig. 4).

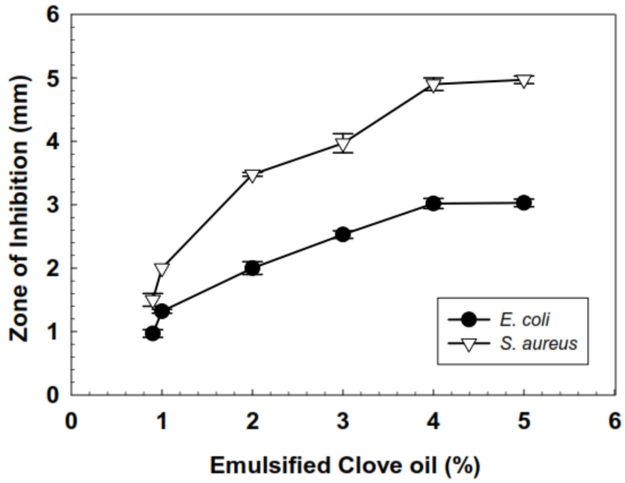


Figure 3. Antibacterial activity of clove oil NEs against *E. coli* and *S. aureus.*

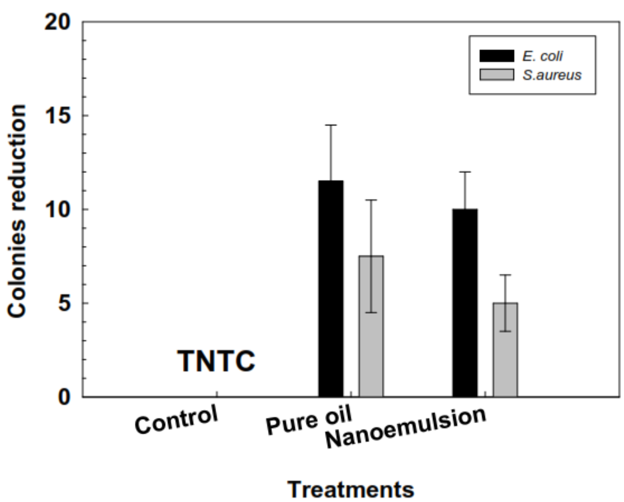


Figure 4. Antibacterial effect of various treatments against *E. coli* and *S. aureus* on fresh beef samples. TNTC (too numerous to count), the reduction was in CFU/mL.

DISCUSSION

In this research the increase in d_{av} was attributed to the use of natural starch emulsifier that is aligned with findings of previous research (Majeed et al., 2016). In comparison decreased in d_{av} was reported in several formulations where nonionic surfactants (e.g., Tween 80, Span 80) were used (Anwer *et al.,* 2014; Shahavi *et al.,* 2016, 2019). The adsorption of this negative charge onto the emulsified surface may be induced by either free fatty acids or other polar constituents that are present in the oil phase (Bhargava *et al.,* 2015). The negative charge may also be contributed by carboxylic group present in emulsifiers (Iqbal *et al.,* 2020). For commercial use of antimicrobial agents, long term stability is a major requirement. However, phase separation, flocculation, Ostwald ripening, creaming, sedimentation, and coalescence are the main mechanisms of NEs instability (Karthik *et al.* 2015).

Clove oil has been proven effective against many pathogens (Condò *et al.*, 2020), However, due to its low water solubility, its use in the food processing industry is limited. Fabrication of stable oil in water NEs with food grade natural emulsifiers is a key to increase the solubility and surface area of oil. HI-CAP 100, a modified starch effectively stabilized NEs with long term stability (Khan *et al.,* 2020). The high stability with small d_{av} (148.2 ± 6 nm) can be explained by strong electrostatic repulsions due to high value of ζ-potential (-30 mV) of NEs. The long-term stability is way forward for commercial use of clove oil nanoemulsions as antimicrobial agents in food industry. Mostly Tween 80, a synthetic food grade emulsifier was used to formulate essential oil nanoemulsions, which has showed only one-month stability (Bhargava *et al.,* 2015; Condò *et al.,* 2020) which hinders its use for commercial purposes.

It was observed that, due to lipopolysaccharide shield in gram negative bacteria, they are more resistant to EOs than gram positive bacteria against antibacterial hydrophobic compounds. (Hyldgaard *et al.,* 2012). Hence, small particle size with reduced hydrophobic property can overcome the lipopolysaccharide barrier easily resulting increased antimicrobial activity of EOs. However, results showed that despite of same MIC the inhibition zone of *E. coli* O157:H7 was smaller than the *S. aureus*, this can be explained by lipopolysaccharide protection of Gram-negative bacteria because the eugenol, major constituent in clove essential oil, permeabilize the cell membrane (Hyldgaard *et al.,* 2012). Furthermore, NEs hydrophilic surfaces, combined with their small particle size, enable them to penetrate cell membranes, allowing EO from NEs to come into contact with the action site (Nazzaro *et al.,* 2013; Sharif *et al.,* 2017). In addition, unlike EOs solubility in water, NEs cause EOs to diffuse in the aqueous region at higher concentrations (Donsì *et al*., 2011).

The EOs have potentials to reduce the bacterial account and many research findings agrees with our results (Murbach *et al.,* 2014; Salvia *et al.,* 2015). One of the main reasons for enhanced effectiveness has been linked to the enhanced activation kinetics of these NEs compared to their traditional coarse emulsions. Also, the essential oil from the NEs gets infused on the surface, potentially altering the phospholipid layer of the microbial cell membrane. Moreover, the prolonged release of EOs from NEs brings about the partitioning of aqueous and the lipid phases, which in return imparts enhanced levels of antimicrobial activity.

Conclusion**:** Current findings show antimicrobial efficiency of clove oil when used in emulsified form. Clove oil was nonencapsulated with food grade emulsifier HI-Cap 100 by using high speed homogenizer. Varying concentrations of clove oil were used to optimize the stable formulation. Promising stability of more than 5 months were observed at 5% clove oil. In addition, clove oil NEs have successfully reduced the bacterial colonies of *E. coli* O157:H7 and *S. aureus* on raw beef samples. However, further research investigation would be useful to assess the impact on meat cooking properties that may altered due to the application of emulsified clove oil.

Conflict of Interest**:** The authors declare no conflict of interest for current research article.

Author's Contribution Statement**:** RI and ZM conducted experiments, ZM and NK evaluated the research results. RI, ZM and NK writes the initial results, whereas ZM and NK conceived the idea and finalized the manuscript.

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