



## Formulation of peppermint oil nanoemulsion using conjugates of whey proteins with maltodextrin and its characterization

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Whey protein-maltodextrin conjugate is used as emulsifier and stabilizer to prepare peppermint (*Mentha piperita L.*) oil (PO) nanoemulsion. The mean particle size, zeta potential and poly dispersity index (PDI) of stable PO nanoemulsion (5% oil+8% conjugate+0.5% Tween 80) was 144.8±5.32 nm, -24.40±0.42 mV and 0.217±0.05 respectively and this formulation was not unstable to food processing conditions like pH 3.0 to pH 7.0, heat treatments and ionic strength 0.1 M to 1.0 M. The emulsion was stable at 25°C for 15 days and its particle size is 332.2±4.66 nm at 15<sup>th</sup> day of storage. Agar well diffusion method is used to assess the antimicrobial efficacy of PO (5%) dissolved in dimethyl sulphoxide (DMSO) and 5% PO nanoemulsion against microorganisms like *E. coli* ATCC 25922, *B. cereus* ATCC 14459, *Salmonella typhi* NCDC 6017 and *E. faecalis* NCDC 115. The formulation prepared in the present study will have the application in preservation of various foods against spoilage microorganisms.

**Keywords:** Antimicrobial efficacy, Essential oils, Milk protein-maltodextrin conjugate, Nanoemulsion, Peppermint oil

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Herbal products are used for self treatment from ancient times. Essential oils (Eos) are aromatic hydrophobic preparations of plants origin obtained by solvent extraction or expression or hydro-distillation or steam distillation<sup>1</sup>. US FDA (Food and Drug Administration) listed EOs and their extracts under 'Generally Recognized as Safe' (GRAS) status<sup>2</sup>. Traditionally, these oils are widely employed in cosmetics, perfumes and as traditional medicine. Peppermint is traditionally used in folk medicine all over the worlds both in western and eastern countries and also to enhance the shelf stability of foods because it inhibits microorganisms<sup>3</sup>. These oils are used to inhibit the growth of microorganisms responsible for food spoilage, dental caries and skin diseases<sup>4</sup>. Since ancient time, peppermint oil has been used for various purposes like to treat headache, common cold, neuralgia in addition to treat a variety of digestive complaints<sup>5</sup>. Nowadays, in the food systems preservatives from natural origin are preferred. Some of the EOs known for their applications as natural antimicrobial compounds in food as preservative<sup>6</sup>. The key issues to incorporate EOs directly in food products are their sensory properties (volatility and strong odour) and low solubility in food system<sup>7</sup>.

Nanoencapsulation of lipophilic compounds is an efficient and viable approach to overcome these limitations. Nanoemulsion based delivery system can be a best option to encapsulate and safeguard these hydrophobic molecules.

Nanoemulsions are water/oil or oil/water emulsions that are stabilized by surface active compounds<sup>10</sup>, with the average particle diameter from 100-500 nm<sup>8</sup>. The supremacy of nanoemulsions against conventional emulsions is bulk viscosity, optical transparency and physical stability that is attributed to their smaller particle size distribution<sup>9</sup>. Whey proteins, have potential to be used as emulsifiers in foods<sup>11</sup> due to their amphipathic nature<sup>12</sup>. However, whey proteins functional properties are questionable over certain processing treatments because of aggregation or precipitation at the pH near to their isoelectric point. Conjugation of proteins with polysaccharide results in enhancement of emulsifying properties of proteins particularly at the pH nearer to PI (isoelectric point) of the proteins. Protein-polysaccharide conjugates maintain solubility and molecular integrity of proteins<sup>13</sup>. Consequently, there is a wide opportunity for use of protein-polysaccharide conjugate as an emulsifier in comparison to protein alone.

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In the present study, the antimicrobial nanoemulsions were formulated using peppermint (*M. piperita L.*) oil (PO). PO is insoluble in water and soluble in ethanol (70%). It is one of the most extensively used EOs in food, cosmetics and pharmaceuticals and exhibits antimicrobial, antifungal and antiviral activities. The antimicrobial properties of PO are mainly because of the cumulative effects of compounds like menthol, menthone, methyl acetate and limonene<sup>14</sup>. Whey protein (WP)-maltodextrin (MD) conjugates were prepared by dry heating of mixtures using maillard reaction conditions and used for PO emulsion preparation. The main objective of this study is to prepare stable peppermint o/w nanoemulsions using WP-MD conjugate as emulsifier and its characterization for particle size distribution and zeta potential by employing various food processing and storage conditions. Furthermore the antibacterial activities of encapsulated and unencapsulated PO were assessed against food spoilage organisms like *E. coli* ATCC 25922, *B. cereus* ATCC 14459, *S. typhi* NCDC 6017 and *E. faecalis* NCDC 115

## Materials and methods

### Materials

Whey protein concentrate (WPC-70) was purchased from Modern Dairy Pvt. Ltd. Karnal, Haryana. Peppermint oil was procured from Siva aromatics Pvt. Ltd., Delhi. Nutrient Agar, Brain Heart Infusion Broth, Maltodextrin (MD) and Agar were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai. Tween 80 (Polyoxyethylene sorbitan monooleate) was purchased from MERCK (Merck Specialities Private Limited, Mumbai). Microbial cultures were obtained from American Type Culture Collection (ATCC) and National Collection of Dairy Cultures (NCDC) of Dairy Microbiology, NDRI, Karnal.

### WP-MD conjugates preparation

The protein-maltodextrin conjugate was prepared using WPC (70) and MD in 1: 2 ratios<sup>15</sup> and used for preparation of peppermint oil nanoemulsion.

### Preparation of peppermint oil nanoemulsion

Emulsions were prepared using peppermint oil as the inner oil phase or core material at the concentration of 1.0 -10% and the WP: MD conjugate (1:2 w/w) at the concentration of 1.0 -10% was taken as coating material<sup>16</sup>. The oil and water phase were first mixed using magnetic stirrer under ambient temperature and further subjected to Ultra sonication

for 20 min at 5°C for conversion of mixture to nanoemulsions.

Stability of the prepared emulsion was assessed by slightly modified Dalev and Simeonova method<sup>17</sup>.

## Characterization of peppermint oil nanoemulsions

### Particle size distribution and zeta potential

Malvern nanoparticle analyzer (Temperature 25°C and humidity of 85% ) with disposable 4 side plain cuvettes was used to measure the poly dispersity Index (PDI), mean particle size (Z-average), and zeta potential of the nanoemulsions. The limits for the instrument is 0.3 nm to 8.0 µm. The freshly prepared nanoemulsion was diluted to 50 times for experiment.

### Stability of PO nanoemulsions under different processing parameters

The effect of different processing parameters like thermal treatments [pasteurization (63°C for 30 min), forewarming (80°C for 10 min), boiling (95°C for 10 min) and sterilization (121°C/15 min)], pH 3.0 to pH 7.0 and ionic strength 0.1M M to 1.0 M NaCl on prepared nanoemulsion were assessed that will be useful for their commercial applications. Fresh nanoemulsions were used for each treatment. Mean particle size and zeta potential of nanoemulsion were measured for every treatment.

### Antimicrobial assay

The selected microorganisms like *E. coli* ATCC 25922, *B. cereus* ATCC 14459, *S. typhi* NCDC 6017 and *E. faecalis* NCDC 115 were grown in broth like brain heart infusion (BHI) prior to evaluation of antibacterial activity.

### Antimicrobial activity of peppermint oil nanoemulsion

Agar well diffusion assay<sup>18</sup> method was used to screen the nanoemulsion at different concentrations (25 to 100 µL) for their antimicrobial activity against selected organisms with slight modifications. For negative and positive control, sterile saline and peppermint oil diluted in Dimethyl sulphoxide (DMSO) were used respectively. Antimicrobial activity of unencapsulated (oil in DMSO) oil and encapsulated (nanoemulsion) oil were assessed. Three mm or more diameter zones of Inhibition (excluding the well diameter) were considered.

### Statistical analysis

MS-EXCEL-2010 package was used as statistical tool for the analysis of the data. Results were interpreted as mean±standard error (SE) of three replicates.

## Results and discussion

### Process optimization of peppermint oil nanoemulsion and its characterization

Nanoemulsion with different ratios of Core (peppermint oil) and coating materials (WP-MD conjugates and Tween 80) were prepared and evaluated for their stability. Out of 5 different formulations (shown in the Table 1), only 2 formulations; peppermint oil nanoemulsion (1% PO+4% WP-MD conjugate+0.25% Tween 80) and peppermint oil nanoemulsion (5% PO+8% WP-MD conjugate+0.5% Tween 80) were stable. The visual observation of the nanoemulsion was shown in the Plate 1. Among the obtained stable nanoemulsion, PO nanoemulsion of 5% oil phase were selected for further characterization based on their antimicrobial properties because of high content of essential oil. The optimized formulation of peppermint oil nanoemulsion (5% PO+8% WP-MD conjugate+0.5% Tween 80) which is having pH of  $6.6 \pm 0.21$  had shown mean particle size of  $144.8 \pm 5.32$  nm, Zeta potential of  $-24.40 \pm 0.42$  mV and poly dispersity index of  $0.217 \pm 0.05$ . Higher storage stability may be due to the lower PDI value of nanoemulsion. The stability of prepared nanoemulsion may be ascribed to the better

surface properties of WP-MD conjugate as compared to whey protein alone. 1:2 (w/w) ratio of WP and MD was highly suitable for the formation of conjugates with better surface active properties.<sup>19</sup> In such system, proteins adsorbed at the oil-water interface and form a viscoelastic layer but polysaccharide stabilizes the emulsion by forming a thickened layer around the adsorbed protein layer<sup>20</sup>. The increased stability of the emulsions containing whey protein-maltodextrin conjugate as emulsifier in contrast to the emulsions containing only whey protein as emulsifier is mainly attributed to the fact that the conjugated protein may form a bulky polymeric layer at the interface and MD in the emulsion extruding towards the aqueous phase thus provides steric stability to emulsion and hence prevents droplet aggregation and coalescence. Usage of the WPI-MD conjugates in the preparation of thymol emulsion the conjugates formed more transparent, dispersible and heat stable emulsion in comparison to mixtures of WPI and MD<sup>12</sup>.

### Stability of selected nanoemulsions under different processing parameters

#### Effect of pH on the particle size distribution and zeta potential of stable 5% PO nanoemulsion stabilized by 8% WP-MD conjugate and 0.5% Tween 80

The particle size distribution of prepared nanoemulsions of different pH was determined and their results were shown in the Fig. 1. The control sample of nanoemulsion at pH 6.5 has shown the mean particle size of  $144.8 \pm 5.32$  nm. The mean particle size decreased significantly with increase in pH from  $221.9 \pm 7.45$  nm (pH 3.0) to  $146.3 \pm 3.86$  nm (pH 7.0) and at the pH 4.0, it is  $332.2 \pm 4.56$  nm. The different result in the particle size at pH 4.0 is attributed to the isoelectric point effect of WP-MD conjugate. It is observed that nanoemulsions were stable and no aggregation and coalescence was observed over the employed pH range (3.0-7.0) and the stability is due to the combined effect of Tween 80 and excellent surface active properties of WP-MD conjugates. The function of Tween-80 is to form a layer between oil and water interface which results in the reduction of interfacial tension and promote the formation of a small droplets with reduced PDI<sup>21</sup>. Stable thymol nanoemulsion was prepared using WPI-MD complex from pH 3.0 to 7.0<sup>12</sup>.

The zeta potential magnitude for PO nanoemulsions was found to decrease towards negative side as pH increased from 3.0 ( $+6.90 \pm 0.22$

Table 1 — Different concentration of core and coating material used for the fabrication of stable nanoemulsion

Core material (PO)	Coating material (WP-MD (1:2 w/w) conjugate)	Tween 80	Stability
1.0-10%	0.1-10%	0%	Unstable
1.0%	0.1-3.5%	0.1-0.25%	Unstable
1.0%	4.0%	0.25%	Stable
5.0%	0.1-7.5%	0.1-0.5%	Unstable
5.0%	8.0%	0.5%	Stable

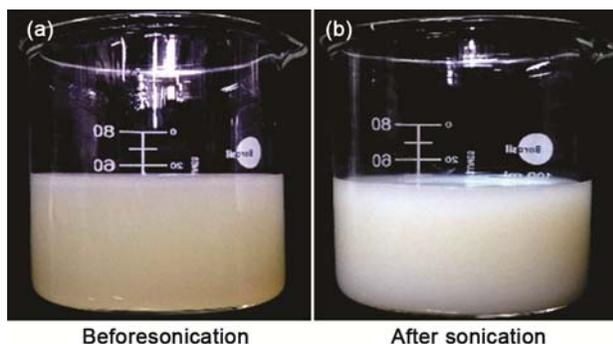


Plate 1 — Visual observation of PO emulsions before (a) and after (b) sonication

- (a) Before sonication  
(b) After sonication

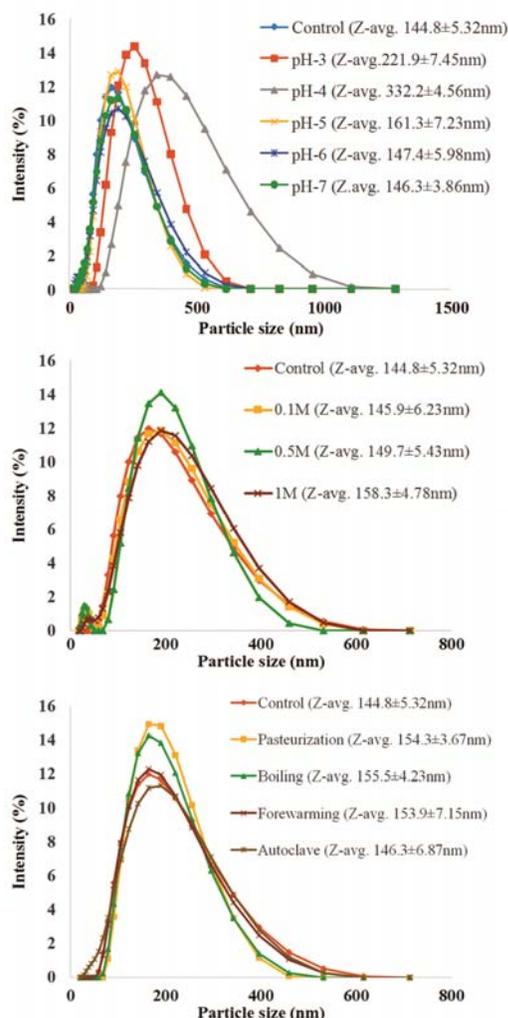


Fig. 1 — Effect of processing condition (pH, Ionic strength and Heat treatment) on particle size distribution of 5% peppermint oil nanoemulsion stabilized by whey protein-maltodextrin (1:2 w/w) conjugate and Tween 80.

mV) to 7.0 ( $-25.40 \pm 0.63$  mV) and values were tabulated in Table 2. The zeta potential at pH 4.0 ( $+1.10 \pm 0.52$  mV) shifted slightly to the positive side, whereas at pH 5.0 the zeta potential magnitude increased to the negative side ( $-15.80 \pm 0.61$  mV) and such variation is ascribed to shift in the isoelectric point of whey proteins to lower pH after it is conjugated with MD and thus stabilizes the emulsion droplets against aggregation at the isoelectric point of the proteins<sup>22</sup>.

#### Effect of ionic strength on the particle size distribution and zeta potential of stable 5% PO nanoemulsion stabilized by 8% WP-MD conjugate and 0.5% Tween 80

The particle size distribution of nanoemulsions at various ionic concentrations was shown in the Fig. 1. The nanoemulsions of different ionic

Table 2 — Effect of processing conditions (pH, ionic strength and heat treatment) on zeta potential and poly dispersity index (PDI) of 5% peppermint oil nanoemulsion stabilized by whey protein-maltodextrin (1:2 w/w) conjugate and Tween 80

Treatments	Zeta potential (mV)	PDI
Control	$-24.40 \pm 0.42$	$0.217 \pm 0.05$
Pasteurization (63°C/30 min)	$-20.70 \pm 0.61$	$0.226 \pm 0.03$
Forewarming (80°C/10 min)	$-20.50 \pm 0.35$	$0.234 \pm 0.02$
Boiling (95°C/10 min)	$-20.20 \pm 0.44$	$0.235 \pm 0.04$
Autoclave (121°C/15 min)	$-21.50 \pm 0.23$	$0.265 \pm 0.06$
0.1 M NaCl	$-19.6 \pm 0.15$	$0.247 \pm 0.03$
0.5 M NaCl	$-17.90 \pm 0.33$	$0.253 \pm 0.03$
1.0 M NaCl	$-16.10 \pm 0.74$	$0.265 \pm 0.04$
pH-3.0	$+6.90 \pm 0.22$	$0.269 \pm 0.02$
pH-4.0	$+1.10 \pm 0.52$	$0.305 \pm 0.04$
pH-5.0	$-15.80 \pm 0.61$	$0.245 \pm 0.05$
pH-6.0	$-20.1 \pm 0.11$	$0.225 \pm 0.01$
pH-7.0	$-25.40 \pm 0.63$	$0.231 \pm 0.03$

Values are Mean  $\pm$  Standard Error (n=3)

concentrations (NaCl: 0.1-1.0 M) were found to be stable against aggregation or coalescence or phase separation. As ionic concentration increases, particle size did not showed significant increase, i.e., from  $144.8 \pm 5.32$  nm (0.0 M NaCl) to  $158.3 \pm 4.78$  nm (1.0 M NaCl). In a study, protein stabilized corn o/w nanoemulsions were reported to be stable over NaCl concentration upto 1000 mM against droplet aggregation and creaming and that stability is due to dominance of repulsive forces over attractive forces among the droplets<sup>20</sup>. Similarly, insignificant difference was found between nanoemulsions of NaCl concentration ranging from 0.0mM to 50.0mM. Stability of emulsions at higher NaCl concentration is because of the steric repulsion dominance over electrostatic repulsion, in presence of maltodextrin<sup>12</sup>.

The zeta potential magnitude of PO nanoemulsions of different ionic concentration was shown in Table 2. The decrease in zeta potential magnitude was observed with increase in ionic concentration. The reduced electrostatic repulsive forces among the particles might be the possible reason. The zeta potential magnitude decreased from  $-24.40 \pm 0.42$  mV (0.0 M NaCl) to  $-16.10 \pm 0.74$  mV (1.0 M NaCl). Normally, addition of salt results changes in the surface properties because of the reduction in the electrostatic interactions among polysaccharide-polysaccharide and protein-polysaccharide present at the surface and in the bulk<sup>24</sup>. The obtained results commemorate the findings of other authors. At all ionic concentrations, the emulsion prepared using

maltodextrin and carrageenan along with WPC exhibited zeta potential towards negative side and further it decreased on increasing the ionic strength up to 500 mM<sup>25</sup>. This effect has been correlated with electrostatic attraction between the droplets along with ion-binding effect. At lower salt concentrations, the repulsive forces between the droplets were relatively stronger that helps to prevent the droplets to come close with each other and thus prevent aggregation, but beyond a critical salt concentration, the repulsive forces is too weak to overcome the attractive forces acting between the droplets that results in aggregation of the droplets<sup>26</sup>.

**Effect of thermal treatments on the particle size distribution and zeta potential of stable 5% PO nanoemulsion stabilized by 8% WP-MD conjugate and 0.5% Tween 80**

The effect of thermal treatments on the distribution of particle size of the PO nanoemulsions was shown in the Fig. 1. It was observed that formulated PO emulsion was stable to different thermal conditions like pasteurization, forewarming, boiling and sterilization. No significant change in the distribution of particle size of PO nanoemulsion over employed thermal treatments was observed and the stability of nanoemulsion is attributed to the emulsifying properties of conjugates. Enhanced surface properties of WP-MD conjugate were also observed during the preparation of other oils emulsions<sup>27</sup>. This may be due to the higher concentration of protein constituent at interface and the hydrophilized polysaccharide projected in aqueous phase that gives steric stabilization. Also, these conjugates effectively prevent the whey proteins aggregation during thermal treatment at different pH and ionic concentrations and prevent denaturation of whey protein<sup>28</sup>.

The influence of thermal treatments on the zeta potential magnitude of PO nanoemulsion was shown in Table 2 and it was observed that there is no significant change in the zeta potential magnitude of the nanoemulsion with applied thermal treatment. But it was found that there is a slight decrease in the zeta potential magnitude after heat treatment. Zeta potential magnitude of curcumin nanoemulsion decreased after pasteurization and boiling<sup>29</sup>.

**Effect of storage time on the particle size distribution and zeta potential of stable 5% PO nanoemulsion stabilized by 8% WP-MD conjugate and 0.5% Tween 80**

The influence of storage on the particle size of nanoemulsion at 25°C was displayed in the Fig. 2. The

PO nanoemulsion was stable during storage and the particle size increased from 144.8±5.32 nm (0<sup>th</sup> day) to 332.2±4.66 nm (15<sup>th</sup> day). There was no clear phase separation or creaming was found during the storage period. In a similar study, it was reported that peppermint oil (with medium chain triacyl glycerol) nanoemulsion stabilized by modified starch was stable to 30 days storage period<sup>30</sup>. In the literature, it was reported that o/w emulsions stabilized by NaCN–MD conjugate showed increased stability compared to emulsions stabilized by NaCN alone over 20 days storage under accelerated shelf life testing conditions<sup>31</sup>.

**The antimicrobial activity of PO nanoemulsion stabilized by WP-MD conjugate and Tween 80**

Agar well diffusion method was used to assess the antimicrobial activity of bulk PO (5%) diluted with dimethyl sulphoxide (DMSO) and 5% PO nanoemulsion against different microorganisms like *E. coli* ATCC 25922, *B. cereus* ATCC 14459, *S. typhi* NCDC 6017 and *E. faecalis* NCDC 115.

Inhibition zone of different concentration of bulk PO (5%) dissolved in dimethyl sulphoxide (DMSO) and 5% PO nanoemulsion against different microorganisms were shown in the Table 3 & Fig. 3. As it is evident from the zone of inhibitions, there is no significant difference in the antibacterial activity of bulk PO and PO nanoemulsion against the experimental organisms. It is also observed that growth of *S. typhi* NCDC 6017 was not inhibited at 25 and 50 µL of bulk PO (5%) dissolved DMSO and 5% PO nanoemulsion, whereas other test organisms like *E. coli* ATCC 25922, *B. cereus* ATCC 14459 and *E. faecalis* NCDC 115, showed zone of inhibition at these lower

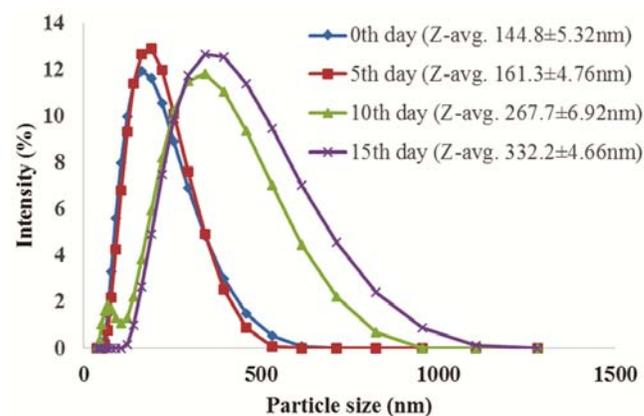


Fig. 2 — Effect of storage time on particle size distribution of 5% peppermint oil nanoemulsion stabilized by whey protein-maltodextrin (1:2 w/w) conjugate and Tween 80.

Table 3 — Zone of inhibition of 5% peppermint oil (PO) nanoemulsion and 5% bulk peppermint oil dissolved in dimethyl sulphoxide (DMSO)

Microorganism	Diameter of zone of inhibition(mm)		
	Concentration of sample (μL)	DMSO+P O PO nanoemulsion	
<i>B. cereus</i> ATCC 14459	25	3.0±0.24	3.5±0.19
	50	6.2±0.18	6.6±0.21
	75	11.0±0.22	11.5±0.13
	100	15.3±0.30	15.3±0.3
<i>E. faecalis</i> NCDC 115.	25	3.7±0.19	4.0±0.43
	50	7.0±0.26	7.4±0.32
	75	11.2±0.21	11.8±0.35
<i>Salmonella typhi</i> NCDC 6017	100	17.6±0.33	18.0±0.24
	25	-	-
	50	-	-
	75	2.7±0.49	3.0±0.52
<i>E. coli</i> ATCC 25922	100	5.0±0.55	5.5±0.51
	25	4.7±0.31	5.0±0.13
	50	7.9±0.17	8.3±0.11
	75	12.1±0.19	12.8±0.32
	100	17.1±0.22	17.6±0.21

Values are Mean±Standard Error (n=3)

concentrations. Peppermint oil nanoemulsion and bulk peppermint oil exhibit same MIC against *L. monocytogenes* and *S. aureus*<sup>30</sup>. Peppermint oil exhibit MIC of 0.625 mg/mL against *S. aureus*<sup>14</sup>. EO of peppermint exhibited highest antibacterial activity with 12.00 mm mean zone of inhibition among leaf water extracts and EO of *Mentha piperita* L. against bacteria like *B. subtilis*, *P. aureus*, *E. coli*, *S. typhi* and *S. aureus*<sup>32</sup>. However, EOs antimicrobial mechanism is not fully understood. In the literature, it is proposed that antimicrobial mechanism was due to lipophilic compounds which disrupt the cell membrane<sup>33</sup>. The toxicity of the Eos is affected by their degree of Hydrophobicity<sup>34</sup>. Nevertheless, after being incorporated into oil-in-water emulsions, the compositions of the whole system influence the antimicrobial activity of EOs. Contradictory results have been presented in the literature. Nanoparticles of eugenol and trans-cinnamaldehyde using poly (vinyl alcohol) as the stabilizing agent, have enhanced inhibiting effect against *Salmonella* species. and *Listeria* species.<sup>35,9</sup>. In contrast, No change in the antimicrobial activity was observed for different droplet size lemon myrtle oil (LMO) emulsions, proposing that the active ingredients in the emulsions influence the antimicrobial property of nanoemulsions instead of the nano sized droplets<sup>36</sup>.

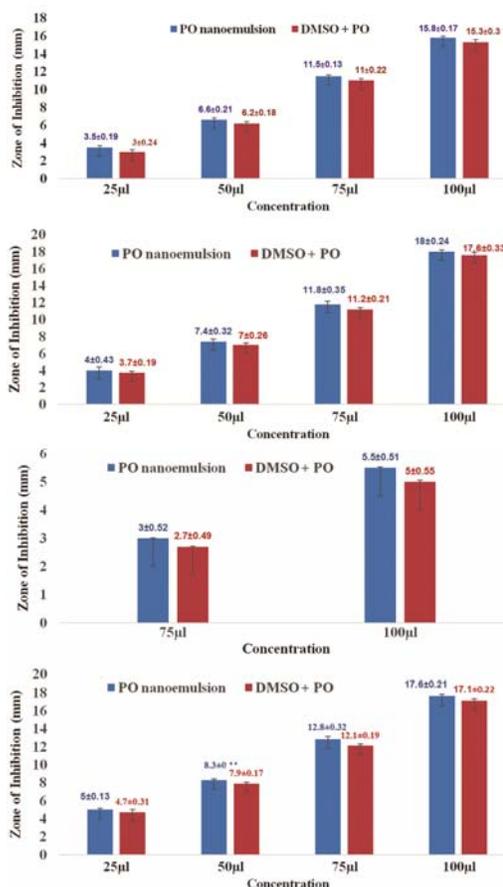


Fig. 3 — Zone of inhibition of 5% peppermint oil (PO) nanoemulsion and 5% bulk peppermint oil dissolved in dimethyl sulphoxide (DMSO) against *B. cereus* (ATCC 14459), *E. faecalis* (NCDC 115), *Salmonella typhi* (NCDC 6017) and *E. coli* (ATCC 25922) respectively.

## Conclusion

In the present study the process for the preparation of peppermint oil nanoemulsion using WP-MD conjugate was optimized. The prepared nanoemulsion is very stable and can withstand even harsh processing conditions. Further, this PO formulation inhibits both spoilage and pathogenic microorganisms. The present investigation may aid in the development of a more successful antibacterial system that can be used for the preservation of foods and thus may address the food quality and safety challenges.

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## Conflict of interest

None

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