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Thymol nanospheres as an effective anti-bacterial agent

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ABSTRACT

Among thymol, carvacrol, citronellal, eugenol and terpinen-4-ol, thymol showed the highest antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Thymol was then encapsulated into water dispersible submicron sized ethylcellulose/methylcellulose spheres, attaining the relatively high thymol loading level of 43.53% (weight of encapsulated thymol to weight of the thymol-loaded spheres). When tested against the same three bacterial strains, the encapsulated thymol gave comparable minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) values to the unencapsulated compound while mostly showing lower MIC and MBC values than the conventionally used preservative, methyl-*p*-hydroxybenzoate (methylparaben). The use of encapsulated thymol at 0.078, 0.156 and 0.625 mg ml^{-1} (0.52, 1.04 and 4.16 mmol^{-1}, respectively) in cosmetic lotion formulations provided total suppression of viable *E. coli*, *S. aureus* and *P. aeruginosa* growth (all initially seeded at 10^5 cfu ml^{-1}), respectively, over the three month test period, whereas unencapsulated thymol showed effective suppression for only 2–4 weeks. Effective bacterial suppression by encapsulated thymol was also observed when used in cream and aqueous gel cosmetic formulations.

1. Introduction

Plant essential oils are potentially useful sources of naturally derived antimicrobial compounds. Their antimicrobial activities against bacteria, viruses, fungi, parasites, and insects, have been reported (Bakkali et al., 2008). The use of essential oils to retard spoiling (Burt, 2004) and to impart unique aromatic characteristics to many commercial products has been reported (Castilho et al., 2012). Various anti-bacterial agents have been identified from natural essential oils (Prabuseenivasan et al., 2006; Sokovic and Griensven, 2006). Amongst the identified natural anti-microbial agents, thymol (2-isopropyl-5-methylphenol) has performed well in many reports, compared to other agents. The compound is the main constituent in essential oils from many herbs, such as Oregano, Thyme and winter savory (Sivropoulou et al., 1996; Piccaglia et al., 1993). Thymol is able to inhibit both Gram-positive and Gram-negative bacteria, including the potential pathogenic strains of *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Dorman and Deans, 2000). Thymol, and essential oils rich in thymol, have proven benefits in medical (Silva et al., 2011), food (Evans and Martin, 2000; Lambert et al., 2001; Sacchetti et al., 2005; Oussalah et al., 2006; Shapira and Mimran, 2007), agricultural (Lazar-Baker et al., 2010), veterinarian and pest control (Glenn et al., 2010) applications. In addition to thymol, other active components found in essential oils that have demonstrated antibacterial activity include its isomer carvacrol, as well as citronellal, eugenol, geranyl acetate and terpinen-4-ol (Dorman and Deans, 2000). However, direct comparison of antibacterial activity amongst these compounds is rarely reported.

The use of natural essential oils or compounds extracted from essential oils as the main anti-bacterial agents, however, still faces the problems of (i) the ease of degradation or chemical reactivity of many of these extracted compounds (Shoji and Nakashima, 2004), (ii) the limited water solubility of these materials (Shoji and Nakashima, 2004) and (iii) their short term availability for bioactivity due to their volatile character. Chemical modification of carvacrol (an isomer of thymol) into carvacrol disodium phosphate has been carried out to solve its low water solubility (Coimbra et al., 2011), whereas the derivatization of caffeic acid into caffeic acid phenethyl ester (a naturally occurring compound in bee propolis with strong antimicrobial and anticancer bioactivities) has been proposed to solve the instability of the compound (Coimbra et al., 2011).

The chemical change in the structure of the compound, however, prevents the obtained derivatives from being classified as natural ingredients. This leaves encapsulation technology as a promising tool to make possible the effective use of these ingredients in an unmodified form. Reported encapsulation technologies for essential oils or active ingredients of essential oils include molecular inclusion complexation with host molecules, such as cyclodextrin

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(Del Toro-Sánchez et al., 2010; Marques, 2010; Ponce Cevallos et al., 2010), coacervation with various carbohydrates and proteins, such as starch (Glenn et al., 2010), gum arabic (Guarda et al., 2011) and corn zein (Xiao et al., 2011), coacervation with polymers, such as cellulose and polyvinyl pyrrolidone (Meunier et al., 2006), chitosan and angico gum (Paula et al., 2010), and encapsulation into liposomes (Liolios et al., 2009). Although an improvement in the water solubility and some controlled release properties were obtained from these reported technologies, a low loading level (less than 10% by weight) together with other drawbacks, such as the fragile nature with ease of breaking of the liposomes, the large size of 5–2000 μm of the loaded particles, and the uncontrollable equilibrium of the host–guest complexation, have contributed to the limited use of these materials as the main anti-microbial agents in various products. In fact, the use of these encapsulated essential oils in cosmetic formulations is scarcely demonstrated despite the consumers' demands for natural anti-microbial agents in these products.

In this report, the antibacterial activity of five commonly known active components from essential oils, carvacrol, citronellal, eugenol, terpinen-4-ol and thymol, against the Gram-positive bacteria, *S. aureus* and the Gram-negative bacteria, *E. coli* and *Pseudomonas aeruginosa*, were first compared. Then, the encapsulation of the most active of these five compounds into a blend of ethylcellulose/methylcellulose (EC/MC) sub-micron size spheres was performed, and the anti-bacterial activity of the obtained spheres was evaluated *in vitro* against the same three bacterial strains. Finally, the obtained nanospheres were tested as the main preservatives in three cosmetic formulations, water in oil emulsion, oil in water emulsion and aqueous polymeric gel.

2. Materials and methods

Methylcellulose (MC, Mn 40,000; D.S. (methoxy) 1.60–1.90) and ethylcellulose (EC, 48% ethoxyl content) were purchased from Aldrich (St. Louis, MO, USA). Citronellal, eugenol, terpinen-4-ol and thymol were purchased from Thai-China Flavors and Fragrances Industry (Nonthaburi, Thailand). Carvacrol was purchased from Aldrich (St. Louis, MO, USA). Tryptic soy broth (TSB), tryptic soy agar (TSA), Bacto™ peptone, tryptone-azolectin-Tween (TAT) broth and tryptone (pancreatic digest of casein) were purchased from Difco laboratories (Detroit, MI, USA).

2.1. Comparison of antibacterial activity

The three bacterial strains, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 9027, were purchased from the American Type Culture Collection (Manassas, VA, USA), and were maintained in TSA at 4 °C throughout the study and used as stock cultures.

To test for antibacterial activity, single bacterial colonies were selected from TSA plates and grown in TSB at 37 °C. The turbidity of the bacterial suspension was visually adjusted to that of a 0.5 McFarland standard using sterile 0.85% (w/v) sodium chloride (NSS), and then used to make a lawn on the surface of TSA plates by swabbing the complete surface area three times with a sterile cotton swab. Once set, an 8-mm diameter well was made in the center of each agar plate using a cork borer into which 15 μl of the test substance (carvacrol, citronellal, eugenol, terpinen-4-ol or thymol) was added. Plates were incubated at 4 °C for 4 h to allow diffusion of the test substance into the agar, and then at 37 °C for 18–24 h. The diameter of the zone of inhibition around each well was then measured. Each experiment was performed in triplicate.

2.2. Preparation of thymol loaded particles

Thymol was encapsulated into a 1:1 (w/w) polymer-blend of MC and EC at a thymol to EC/MC polymer weight ratio of 1:1 by displacing the ethanol solvent with water, as reported (Sansukcharearnpon et al., 2010). To this end, the two polymers (125 mg EC and 125 mg MC) were dissolved in 25 ml of 75% (v/v) aqueous ethanol at 70 °C, allowed to cool to room temperature, and then the thymol (250 mg) was added and dispersed. After mixing, water was slowly dropped (0.75 ml/min) into the mixture to a final volume of 100 ml. To determine the encapsulation efficiency (EE) and loading capacity the suspension was filter centrifuged through a 100,000 Da MW cut off membrane (Amicon Ultra-15, Millipore, Billerica, MA, USA) at 9392 × *g* for 10 min, and the obtained clear supernatant was quantified for thymol content by UV absorption spectrophotometry, measuring the absorbance at a wavelength of 275 nm with reference to a freshly prepared calibration curve. The filtered spheres were also subjected to ethanol solubilization to extract the encapsulated thymol content, and the obtained solution was subjected to thymol quantification as above. The EE and thymol loading level were then determined as follows:

$$\%\,\mathrm{EE} = \frac{\text{weight of thymol in spheres}}{\text{weight of thymol used}} \times 100$$

$$\%\,\text{loading level} = \frac{\text{weight of thymol in spheres}}{\text{weight of thymol in spheres} + \text{weight of polymer}} \times 100$$

The aqueous suspension of thymol-loaded particles was subjected to analysis by dynamic light scattering (DLS) on a Zetasizer Nano S4700 (Malvern Instruments, UK), differential scanning calorimetry (DSC) using a METTLER DSC 822 (USA), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) using the JEOL JSM-6400 and JEM-2100 electron microscopes, respectively.

2.3. MIC and MBC determination

S. aureus, *E. coli* and *P. aeruginosa* were grown in TSB at 37 °C until a total bacterial count of 10^8 cfu ml^{-1} was reached. Then, the bacterial suspension was centrifuged at 8000 rpm (10,160 × *g*) for 10 min, washed with NSS and resuspended to 10^8 cfu ml^{-1} in modified peptone water. The obtained bacterial suspension was used for the evaluation of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each test compound.

The MIC of free thymol, the aqueous suspensions of thymol-loaded nanoparticles and methyl-*p*-hydroxybenzoate (methylparaben (pharmaceutical secondary standard) purchased from Sigma–Aldrich) were determined by the broth dilution method. To each test tube containing 3.0 ml of sterile (121° C for 15 min) modified peptone water was added the thymol, methylparaben or the aqueous suspension of thymol-loaded particles (concentration range of 10–2500 ppm), and the tubes were then inoculated with 200 μl of the above bacterial suspension, mixed and incubated at 37 °C for 0, 6, 12, 24 and 48 h before the turbidity of the broth was recorded at 600 nm. The broth was also subjected to total viable bacterial counts using the total plate count (TPC) method, spreading different dilutions of the broth onto TSA plates, in triplicate per dilution, incubating for 24 h at 37 °C, and then counting the total number of bacteria colonies. Each experiment was performed in triplicate.

2.4. Bacterial suppression in cosmetic formulations

S. aureus, *E. coli* and *P. aeruginosa* were grown in TSB at 37 °C until a total bacterial count of 10^8 cfu ml^{-1} was reached. Then, the

bacterial suspension was pelleted (centrifuged at 8000 rpm or 101,60 × *g* for 10 min), washed with tryptone sodium chloride (TSL), and then resuspended in TSL to 10^6 cfu ml^{-1}.

Three commercial cosmetic formulations, water in oil emulsion (cream), oil in water emulsion (lotion) and aqueous carbopol gel, were evaluated. The water in oil emulsion and oil in water emulsion were obtained from Garguar Lab (Patumtanee, Thailand). The propriety water in oil emulsion contains paraffinum liquidum, aqua, petrolatum, oleth-10, beeswax, sodium borate, sorbitansesquioleate, steareth-2, cetearyl alcohol, perfume, cetyl alcohol, propylene glycol, ceteareth-20 and alcohol, whilst the oil in water emulsion contains aqua, isopropyl palmitate, cetylalcohol, petrolatum, polysorbate 20, sorbitanstearate, propylene glycol, ethylhexylmethoxycinnamate, perfume, carbomer, disodium EDTA, glycerylstearate, lanolin and sodium hydroxide. The aqueous carbopol 940 P gel (pH 7) was obtained from Corel Pharma (Thailand).

An aqueous suspension of the sample (encapsulated thymol, free thymol or methyl-*p*-hydroxybenzoate (positive control)) was added to the cosmetic formulation (cream or lotion or gel, 150 g) and mixed in a stomacher for 60 s. For the unencapsulated thymol, final concentrations of 0.078, 0.156 and 0.156 mg ml^{-1} (0.52, 1.04 and 1.04 mmol^{-1}, respectively) were used for formulations challenged with *E. coli*, *S. aureus* and *P. aeruginosa*, respectively, whilst for formulations with encapsulated thymol, the final thymol concentrations were 0.078, 0.156 and 0.625 mg ml^{-1} (0.52, 1.04 and 4.16 mmol^{-1}, respectively). The final concentration of methyl-*p*-hydroxybenzoate was 4.0 mg ml^{-1} (26.3 mmol^{-1}) in all cases. See Section 3.4 for the rational for the selection of these concentrations. Then 150 μl of the respective bacterial suspension (containing 10^6 cfu ml^{-1}) was thoroughly mixed into the cosmetic formulation in a stomacher to give a final bacterial concentration of 10^5 cfu ml^{-1}, and the mixture was incubated for 15–20 min. The mixed product was transferred to a sterile bag and kept at room temperature. Each mixed product was sampled at 0, 1, 2, 4, 8 and 12 weeks and the population of bacteria was counted. Ten grams of the sampling product were diluted with TAT buffer (90 ml) and mixed in a stomacher for 30 s. Then, 100 μl portions of various dilutions were spread on TSA plates and incubated at 37 °C for 24 h for TPC evaluation of the number of viable cells.

3. Results and discussion

3.1. Antibacterial activity of carvacrol, citronellal, eugenol, terpinen-4-ol and thymol

Firstly, the antibacterial activities of carvacrol, citronellal, eugenol, terpinen-4-ol and thymol against *E. coli*, *P. aeruginosa* and *S. aureus* were compared using the clear zone assay (Section 2.1). The results clearly indicated that thymol had the greatest antibacterial activity followed by that of its isomer carvacrol (Fig. 1). Eugenol and terpine-4-ol also showed antibacterial activity against the three tested bacterial strains but at a lower potency compared to thymol and carvacrol, whilst citronella could inhibit the growth of only *S. aureus* (Fig. 1). From these results, as the most potent antibacterial agent among the five tested compounds, thymol was selected for encapsulation.

3.2. Thymol encapsulation

Encapsulation of thymol using a 1:1 (w/w) ratio EC/MC as the polymeric shell material was performed by slowly increasing the water:ethanol ratio of the solvent medium and resulted in the formation of a milky white aqueous suspension. Analysis of the suspension by SEM (Fig. 2a) revealed the presence of almost

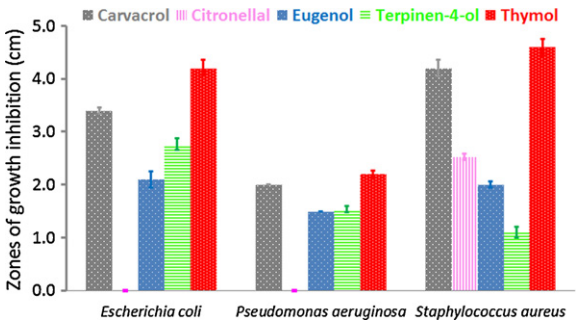

Fig. 1. Antibacterial activity of carvacrol, citronellal, eugenol, terpinen-4-ol and thymol, as assayed by clear zone method. Data are shown as the mean ± SD of three independent repeats.

spherical particles with a dry size average diameter of 420.0 ± 118.6 nm, whilst the TEM analysis (Fig. 2b) also revealed spherical particles. DLS analysis gave a just over two-fold larger average hydrodynamic size of the particles (865.9 ± 37.3 nm) with a fairly narrow size distribution range (PDI of 0.182), suggesting a fair degree of swelling of the MC/EC based particles in the aqueous environment. The process gave an EE of 77% with a relatively high thymol loading level of 43.53% (w/w). DSC analysis showed no melting peak of the thymol, typically seen at 52 °C, (data not shown), indicating the absence of any significant level of crystalline thymol in the particles. Thus, the thymol molecules in the spheres were in the solid solution state with the EC/MC polymer chains.

The water dispersible nanospheres of EC/MC loaded with thymol were formed during the slow change from an ethanol rich to a water rich medium, when the EC chains, which are soluble in ethanol but insoluble in water, arrange themselves such that the hydrophilic moieties (hydroxyl groups) are in maximum contact with the water while the hydrophobic moieties (ethoxyl groups) associate with themselves leading to their positioning inside of the forming particles with minimal water contact. The water insoluble thymol molecules automatically associate themselves with the hydrophobic interior of the particles. Some MC chains are likely to be entangled with the EC and be part of the spheres during the particle formation. Their presence usually makes the obtained spheres more easily and stably dispersible in water.

3.3. Antibacterial activity of the thymol-loaded particles

Here the agar dilution method was used to determine MIC and MBC values of all the tested materials (Kalemba and Kunicka, 2003). The MIC and MBC values of both free thymol and the EC/MC encapsulated thymol against *S. aureus* and *E. coli* were the same (Table 1), and thymol appeared able to kill the two bacterial strains very effectively even at these very low concentrations. Moreover, thymol was more effective than the popularly used naturally occurring preservative, methyl-*p*-hydroxybenzoate (methylparaben). However, for *P. aeruginosa* a higher thymol concentration was required to kill the bacteria than that required to inhibit their growth, and the MIC and MBC values against *P. aeruginosa* were greater for the encapsulated thymol compared to free thymol, meaning that the action of thymol toward this bacterial strain is somewhat retarded when the material is encapsulated. Since the EC/MC shell is not degradable under the experimental condition, it is speculated that diffusion of the hydrophobic thymol molecules from the spheres must be taking place prior to their action on the bacteria. Nevertheless, the encapsulated thymol was no worse than that for methylparaben against *P. aeruginosa* and was more effective at inhibiting the growth or killing *E. coli* and *S. aureus*. The low MIC and MBC values observed for the EC/MC encapsulated thymol encouraged further investigation for the long term use of encapsulated thymol as a

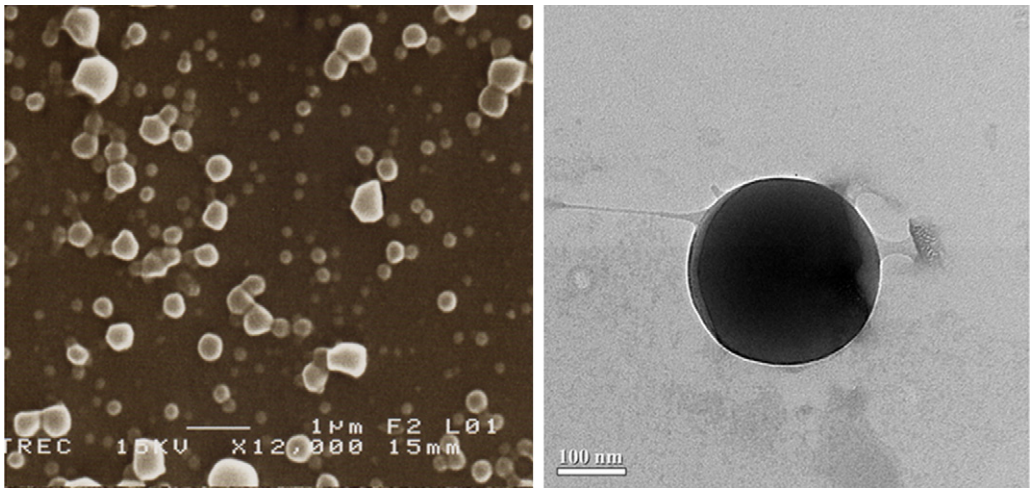

Fig. 2. Representative SEM (left) and TEM (right) images of thymol-encapsulated nanoparticles suspension.

preservative in cosmetic formulations at these relatively low concentrations.

3.4. Encapsulated thymol as preservative in cosmetic formulations

The upper concentration limit of the popular preservative methylparaben that is routinely used is 4.0 mg ml^{-1} (~26.3 mol^{-1}), and so this concentration was used as a positive control in the three different cosmetic formulations tested here. Since the objective of using a preservative in cosmetic formulations is to inhibit the growth of microorganisms, we decided to test both the free and the MC/EC encapsulated thymol in the various cosmetic formulations at a final concentration in between the MIC and MBC values. Therefore, the free thymol was tested at the concentrations of 0.078, 0.156 and 0.156 mg ml^{-1} (0.52, 1.04 and 1.04 mmol^{-1}), and the encapsulated thymol was tested at the concentrations of 0.078, 0.156 and 0.625 mg ml^{-1} (0.52, 1.04 and 4.16 mmol^{-1}) for formulations challenged with *E. coli*, *S. aureus* and *P. aeruginosa*, respectively. Note that these concentrations are some 52.6-, 25.6- and 12.8-fold lower than that of the methylparaben by weight, or 52.0-, 25.2- and 12.6-fold lower by molarity. Cosmetic formulations (lotion, cream and gel) with the added with the tested preservative (free thymol or encapsulated thymol or methylparaben), were mixed with the respective bacterial suspension at an initial bacterial level of 10^5 cfu ml^{-1}, and kept for a period of 3 months while performing bacterial counts periodically.

In the cosmetic lotion, it was obvious that the encapsulated thymol at a concentration of only 0.078 and 0.625 mg ml^{-1} could effectively inhibit the growth and multiplication of *E. coli* and *P. aeruginosa*, respectively, whereas free thymol, at the same concentration, could not (Fig. 3a and b). Rather, the number of bacteria in the lotion containing free thymol was reduced for only the first four or two weeks (for *E. coli* and *P. aeruginosa*, respectively), compared to that with the encapsulated thymol that showed a 100% reduction of the *E. coli* and *P. aeruginosa* populations (or at least to below the limit of detection (LOD) of the assay) during the whole 3 month period. It was speculated that without any polymeric shell protection, the free thymol molecules could either be degraded or volatilized from the lotion, and so the free thymol showed effective *E. coli* control for only a short period. In contrast, with the EC/MC polymeric shell around the thymol molecules, degradation and volatilization are minimized and, therefore, a prolonged antibacterial activity was observed. Interestingly, both the free thymol and encapsulated thymol at a concentration of 0.156 mg ml^{-1} in the cosmetic lotion could suppress the growth of *S. aureus* for the whole 3 month period (Fig. 3c). This bacterial strain might already be stressed or, for whatever reason, be more susceptible to thymol and so only a small amount (low concentration) of thymol was enough to inhibit its growth. Therefore, although some degradation and volatilization of free thymol had occurred, the thymol left in the lotion was enough to suppress the growth of *S. aureus*. Alternatively, all of the *S. aureus* cells were killed (rather than reduced to below the LOD) in the initial period before the thymol concentrations were reduced over time.

In the aqueous gel formulation (carbopol gel) the MC/EC encapsulated thymol (0.078 mg ml^{-1}) suppressed the growth of *E. coli* for the whole 3 months, while free thymol was effective for only 8 weeks (Fig. 3d). However, the initial reduction in the viable *E. coli* numbers in the carbopol gel with the encapsulated thymol was slower than that with free thymol, in that it required 2 weeks to suppress the bacteria to below the LOD with the encapsulated thymol compared to only 1 week with the free thymol. One plausible explanation for this is that the stiffness of the polymeric gel network could retard the rate at which the thymol-loaded particles contacted with the bacterial cells and/or the release of thymol from the spheres and their diffusion to the bacterial cells was slowed down due to the low solubility of thymol in the aqueous gel environment. In other words, thymol molecules would stay in the hydrophobic core of the spheres and only limited numbers of them would be

Table 1
MIC and MBC values of free thymol, encapsulated thymol and methyl-*p*-hydroxybenzoate (methylparaben).

Bacterial strain	MIC (mg ml^{-1})				MBC (mg ml^{-1})		
	Free thymol	Encapsulated thymol	Unloaded spheres	Methylparaben	Free thymol	Encapsulated thymol	Methylparaben
E. coli	0.010	0.010	n.a.	0.625	0.156	0.156	>1.250
S. aureus	0.156	0.156	n.a.	0.020	0.313	0.313	1.250
P. aeruginosa	0.039	0.313	n.a.	0.625	0.313	1.250	1.250

Data are shown as the mean values derived from three independent repeats.
n.a. = no detected activity at up to 10.0 mg ml^{-1}.

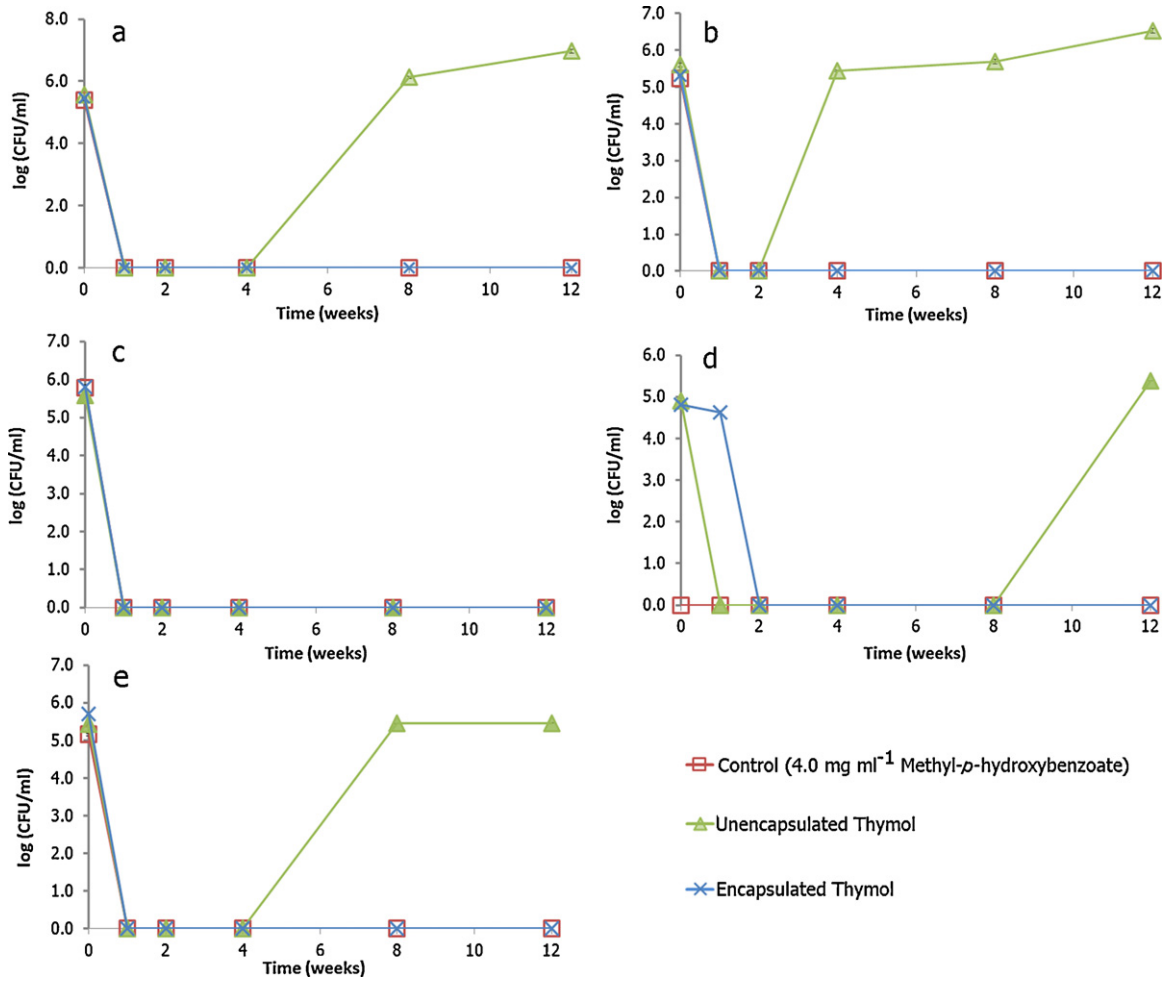

Fig. 3. Number of viable (a and d) *E. coli*, (b) *P. aeruginosa* and (c and e) *S. aureus* cells after up to 3 months in cosmetic applications based upon a (a, b and c) oil in water lotion, (d) aqueous carbopol gel and (e) water in oil cream. The concentration of methyl-*p*-hydroxybenzoate (control) was 4.0 mg ml^{-1}, and thymol was at 0.078, 0.156 and 0.156 mg ml^{-1} for formulations challenged with *E. coli*, *S. aureus* and *P. aeruginosa* (all at 10^5 cfu ml^{-1}), respectively.

released (probably *via* diffusion mechanism) into the gel and be vaporized. For free thymol, the longer observed duration of the suppression of bacterial numbers below the LOD in the carbopol gel formulation (8 weeks) compared to that observed in the lotion formulation (4 weeks) (Fig. 3a and d) was likely to be the result of the retardation of thymol diffusion and volatilization by the polymeric gel network in the gel formulation.

In the cream formulation, only the encapsulated thymol (0.078 mg ml^{-1}) could suppress the growth of *S. aureus* for the whole 3 months, whereas the free thymol was effective for only 4 weeks (Fig. 3e). That the free thymol could totally suppress the number of *S. aureus* below the LOD in the lotion for all 3 months (Fig. 3c), yet could suppress the same bacteria in the cream formulation for only 4 weeks, clearly indicates that the cosmetic formulation directly affects the thymol sensitivity of the bacteria.

4. Conclusions

When tested by the clear zone agar dilution method against *E. coli*, *P. aeruginosa* and *S. aureus*, thymol showed the greatest antibacterial activity against all three bacterial strains tested, followed by its isomer carvacrol, and then citronellal, eugenol and terpinen-4-ol. Encapsulation of thymol into the nanospheres fabricated from a 1:1 (w/w) blend of EC and MC gave thymol-loaded spherical nanoparticles with an average dry diameter of 420 ± 118.6 nm at a loading capacity of 43.53% (100 × weight of encapsulated thymol/total weight of the thymol loaded spheres). The MIC and MBC values of these thymol loaded nanospheres against *E. coli*, *P. aeruginosa* and *S. aureus* were in the same range as that of the unencapsulated thymol, thus indicated that encapsulation did not deactivate the antibacterial activity of the material. The encapsulated thymol particles dispersed excellently in water. Tests in lotion, gel and cream formulations challenged with the three bacteria, indicated that the encapsulated thymol was an effective preservative, as good as the traditional methylparaben, even when used at 12–52-fold lower concentrations (by mass or molarity).

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