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## Improving Oral Intake of Essential Oil Blends Using A Novel Formulation of Biodegradable Chitosan/Lecithin Nanoparticles

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### Abstract

A novel, safe and efficient method was developed to encapsulate a blend of essential oils (EOs) into biodegradable nanoparticles (NPs). The biodegradable and biocompatible nanoparticles were made from chitosan (CH) and lecithin (LE). The quality of the essential oils was verified using gas chromatography/mass spectrometry (GC/MS). The synthesis of nanoparticles included emulsification, followed by sonication, homogenization, and extrusion. Transmission electron microscopy (TEM) indicated that the nanoparticles were spherical in shape with sizes ranging from 25 to 70 nm, while dynamic light scattering (DLS) showed high negative zeta potentials. The stability of the final formula was evaluated in gastric and intestinal fluids. The chitosan/lecithin encapsulated EOs exhibited promising antimicrobial activity against the multi-drug resistant bacteria *Salmonella typhi*.

**Keywords:** biodegradable nanoparticles (NPs), chitosan/lecithin, antimicrobial activity, *Salmonella typhi*, Essential oils.

## تحسين الامتصاص الفموي لمزيج الزيوت الاساسية باستخدام توليفة جديدة لدقائق الكيتوسان الليسيئين النانوية القابلة للتحلل الحيوي

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### الخلاصة

تم تطوير طريقة جديدة وامنة واكثر فعالية لتغليف الزيوت الطيارة في دقائق نانوية من مواد قابلة للتحلل الحيوي . اذ تم تحضير الدقائق النانوية من الكيتوسان واللسيئين القابلة للتحلل الحيوي والمتوافقة حيويًا. وفيما يخص الزيوت الطيارة فقد تم التأكد من جودتها باستخدام جهاز كروماتوغرافي الغاز ومطياف الكتلة. تضمن تحضير مستحلب الدقائق النانوية استخدام جهاز الموجات فوق الصوتية وجهاز المجانس وجهاز القذف. اظهر الفحص بالمجهر الالكتروني امتلاك الدقائق شكل بيضوي وحدود حجم تراوحت ما بين 25 الى 70 نانومتر مع ثباتية عالية من خلال الفحص بجهاز الزيتا. وتم اختبار ثباتية التوليفة النهائية في محاليل معدية ومعوية. وقد اظهرت التوليفة فعالية ضد بكتيريا السالمونيلا ذات المقاومة المتعددة لمضادات الحياة

### Introduction

Nanoscience offers advances in many aspects related to drug delivery, disease diagnosis, and therapy [1]. Several studies have approved the protection and improvement of drug bioavailability and

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bioactivity of various bioactive components [2-4]. Nanoencapsulation of bioactive ingredients can improve their solubility and administration [5]. In this regard, various drug delivery systems, such as nanoliposomes, polymeric micelles, and nanoemulsions, have been invented. In most cases, this modifies their pharmacokinetics and protects them from enzymatic attack [6].

Great emphasis has been placed on colloids prepared from lipids and polysaccharides [7]. These materials are safe because they are biocompatible and biodegradable. Similarly, chitosan has been reported as a stabilizer for microemulsions when lecithin, a phospholipid, is used as an emulsifying agent. Chitosan is a polymer that can be obtained by alkaline deacetylation of chitin, and has a mucoadhesive property that can facilitate protein uptake by intestinal epithelial cells [8]. Coating lipid nanostructures with chitosan has been investigated to increase their stability and provide mucoadhesive properties to the coated nanoparticles (NPs). The negative charge of phospholipids and the positive charge of chitosan can interact with each other to produce a new delivery system to poorly soluble antitumor drugs [9, 10]. Impaired solubility of drug decreases its absorption in the gastrointestinal (GI) tract and its subtherapeutic concentrations in plasma. This limitation may have an impact on the development and applications of the pharmaceutical formulation of a poorly soluble drug. In soluble drugs, they can cause drug rash and anaphylactic shock, which are potential side effects of intravenous and intramuscular administration. Oral administration is the preferred and classic drug delivery method. Therefore, several oral nanoscale researches have been reported to enhance *in vivo* oral administration [11]. Alkaline deacetylation method has been used to obtain chitosan from chitin [12]. Emulsification and cross-linking, emulsification solvent diffusion, polyelectrolyte complex (PEC), reverse micellar, emulsion-droplet coalescence, spray-drying, emulsification and cross-linking, modified ionic gelation with radical polymerization, and desolvation methods have been used to prepare chitosan NPs. However, these methods have drawbacks, including the requirement of chemical agents for cross-linking, washing, and precipitation steps. Besides, the limitations of the stability of the use of organic solvents and the difficulties in scaling led to significant drawbacks in many cases of the nanocarrier applications [13].

Therefore, a new self-assembling nanoparticle system incorporating lecithin and chitosan was invented by using the solvent injection method. The self-assembly method is based on the binding between negatively charged phospholipids and positively charged chitosan, resulting in the formation of nanostructures with good stability, proving their biodegradability and biocompatibility, very good mucosal adhesion, and insignificant cytotoxicity. This delivery system improved their access to drugs and extended their release times into the body [14, 15]. Several recent studies have investigated the efficiency of this novel nanocarrier in delivering different drugs *via* various routes, such as oral and transmucosal routes of administration [16-19]. But, it is assumed that hydrophobic molecules can only be loaded with the self-assembly technique with high encapsulation efficiency, while low drug loading was detected for hydrophilic drugs [20].

Essential oils are groups of low molecular weight compounds with variable concentrations that can be obtained from various plant extracts. Most of these compounds are present in low concentrations [21]. Recently, several studies have included the use of EOs in therapeutic, cosmetics, and nutritional products. These studies have focused on studying the therapeutic properties of EOs against a number of diseases, such as bacterial, fungal and viral infections, as well as cancer. However, EOs are unstable with respect to environmental factors, such as pH, O<sub>2</sub>, moisture, and light, which are associated with declined volatility of EOs, as well as poor water solubility [22], [23]. Therefore, most researchers have begun to encapsulate EOs to improve their stability and water solubility, keep them away from environmental interfaces, reduce their volatility, improve their bioactivity, and reduce their toxicity [24, 25]. Moreover, encapsulation can improve their modeling (releasing time in the body). Meanwhile, nanoencapsulation is an efficient method to modulate and increase the release of biologically active substances. Further, the nanoencapsulation technology improves the cellular uptake mechanism and improves the bio-effectiveness of EOs [26-28].

Morbidity and mortality among patients infected with multidrug resistant bacteria is one of the most important health issues [29]. The episodes of multidrug resistant *Salmonella typhi* that causes enteric fever has been increased worldwide, decreasing the susceptibility of bacteria to ciprofloxacin [30]. Therefore, the aim of this study is to develop a more effective antibacterial agent to resolve future incidents of multi-drug resistant *S. typhi* strains. Meanwhile, the bioavailability of an intramuscularly antibacterial agent is an important criterion to be considered during the development of a new

bioactive agent. To the best of our knowledge, this is the first time that a blend of EOs is encapsulated by chitosan lecithin NPs to increase its bioavailability as an oral delivery carrier, while achieving minimal degradation of the nanocarrier in the stomach and prolonged contact with bioactive ingredients in the intestine.

## Materials and methods

### Materials

Soy lecithin was purchased from Puritans Pride (Oakdale, NY). Trypsin-EDTA was purchased from US biological (USA). Food grade Chitosan 95% deacetylation was obtained from Paragon Specialty Products. Cola Fax and Cola DC were obtained from Colonial Chemical Inc. (South Pittsburgh, TN). The other materials were purchased from Sigma-Aldrich (USA).

### Preparation of Chitosan-Lecithin Nanoparticles

Chitosan-lecithin encapsulated EO blends were prepared as follows. Solution A was prepared by dissolving 2.5 g of soy lecithin in 80 mL of 10 mM PBS buffer. It was then mixed with 20 mL (30% v/v) of the blend of EO and heated to 55°C. Solution B was prepared by dissolving 2.5 g of chitosan in 50 mL of 0.25M HCl with continuous stirring for 5 h. After that, the solution was filtered with 4 filter papers (3 pieces of polyethersulfone with filter code of PM30 and diameter of 76 mm and 1 piece of regenerated cellulose with filter code of YM10 and diameter of 76 mm) to remove undissolved chitosan. Thereafter, 50 mL of distilled water (DW) was added. This was subjected to constant stirring while heating to 55°C. When both solutions reached ~55 °C, they were combined by mixing 10 mL of solution A, after adding 0.05 g of Cola Fax and 0.25 ml of Cola DC, and 2 mL of solution B (50-55 °C) under vigorous stirring with heating at ~55 °C. After cooling the mixture, 0.06 g of each of Na<sub>2</sub>EDTA and potassium sorbate were added as preservatives. The resulting emulsion was the CH-LE encapsulated EOs, which was subjected to a combination of ultrasonication and homogenization (probe) to obtain an emulsion at nanosize. Ultrasonication was carried out at 20-kHz for 1.5 h at 37°C in vials containing 10-20 mL of the emulsion at 10 min pulses with 3 min breaks. Homogenization (SCILOGEX, D-160 Homogenizer) was run at 14x10<sup>3</sup>, 18 x10<sup>3</sup>, 26 x10<sup>3</sup>, and 30 x10<sup>3</sup> rpm for 3, 2, 1, and 1 min, respectively, with a pause in between.

### Transmission Electron Microscopy (TEM) Test

The morphology and size characterization of LE/CH NPs-Eos were tested by TEM (model CM120, Phillips Holland). A drop of sample was placed onto a carbon coated copper grid and stained with neutral phosphotungstic acid solution (1%) for 2 min. Excess stain was removed by wicking with filter paper before the analysis of the stained sample.

### Zeta potential and particle size measurement

The mean diameter size and zeta potential of the LE/CH NPs-Eos were tested with dynamic light scattering (DLS, Brookhaven instruments corporation, US). Besides, the surface charge and particle size distribution of NPs were determined by zeta potential analyzer (Brookhaven NanoBrook ZetaPlus, USA). Furthermore, DLS and zeta potential analyzer were used to study the effects of pH on the size and zeta potential of the prepared CH-LE/EO; they were studied after subjecting them to two values of pH (1.2 and 7.3) with time. Briefly, 100 µL of NP was dispersed in 1 mL DW, where all sample measurements were run in triplicate and the average values were reported.

### EO Blends

The EO blend containing peppermint, eucalyptus, tea tree, and clove oils was diluted with vegetable oil in a proportion of 3:7. The blends of EOs were characterized for their volatile components by headspace GC/MS analysis.

### Bacteria

Multi-drug resistant clinical isolates of *Salmonella typhi* were obtained from Al-kindy hospital in Baghdad and kept frozen at -20°C. For routine work, the bacteria were kept at 4°C and cultured by incubation at 37°C in Luria-Bertani broth overnight for 18 h [31].

### Determination of antibacterial activity of LE/CH –EOs NPs

The antibacterial activity of LE/CH NPs-EOs was tested by applying the colony counting method [32]. Bacteria were initially grown to the log phase and the turbidity of culture was adjusted to 5 × 10<sup>5</sup> cells/mL in Muller-Hinton (MH) broth. The bacteria were incubated for 18 h at 37 °C. The method first involved adding the bacterial strain to the media, in which the bacteria were grown at 37 °C for 18 h. One milliliter of growing bacteria was added to broth containing different concentrations of LE/CH –EOs NPs and incubated at an appropriate temperature. Then, 10 µL of bacterial suspension was

collected after 24 h and spread on a Plate Count Agar. The plate was incubated for 24 h and the total number of growing colonies was counted.

### ***In vitro* stability of chitosan-Lecithine EO nanoparticles**

The stability of LE/CH –EOs NPs was studied *in vitro* using simulated gastric and intestinal fluids (SGF and SIF, respectively) according to a previous method [33]. The SGF was prepared by mixing 2 g of NaCl, 7 mL of 36-38% HCl, and 3.2 g of pepsin, and the volume was made up to 1000 mL with DW. The pH was adjusted to 1.2 with 1M HCl. In the case of SIF preparation, 6.8 g of  $\text{KH}_2\text{PO}_4$ , 10 g of trypsin, and 5 g of bile salts were dissolved in 1000 mL DW, and the pH was adjusted to 7.4 with 1M NaOH. In order to determine the stability of LE/CH –EOs NPs, 3 mL of their solution was added to 3 mL of SGF or SIF and incubated overnight in a water bath at 37 °C. Particle size and zeta potential were measured at different time intervals and every simulation was replicated three times.

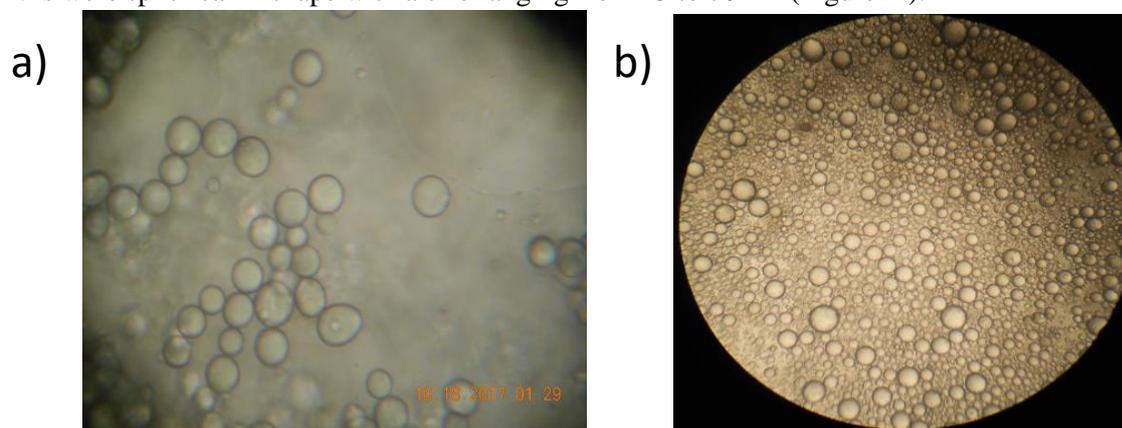
### **Results and discussion**

#### **Preparation of LE/CH NPs-EOs**

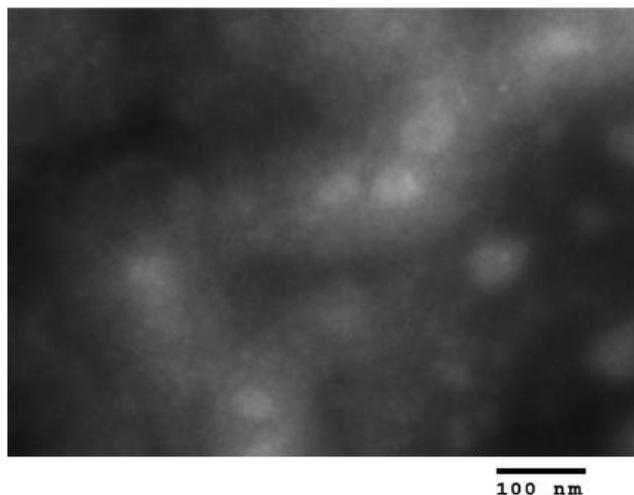
Blends of EOs were entrapped within LE/CH in a weight ratio of 5:1, using the principles of auto-assembly between LE and CH [34]. The LE/CH complex was successfully formed based on the electrostatic interactions between the negative charge of the phospholipid in LE and the positive charge of the amine group in CH. The method of preparation was simple and inexpensive, without the need for organic solvent and extreme conditions. Interestingly, at this setting, we replaced the extensive binding agent tripolyphosphate (TPP) with a natural ingredient, which is soy lecithin. Moreover, all ingredients used in this study were biocompatible and biodegradable. Generally, the construction that contained CH can provide mucoadhesion and enhance penetration due to its influence on the constricted connections between cells [35]. Therefore, LE/CH–EOs NPs are promising carriers for drug delivery *via* transmucosal routes, such as oral, pulmonary, or nasal.

#### **Morphological observation**

Before sonication, the prepared emulsion was observed under a light microscope. As shown in Figure-1, the particles were spherical with a diameter of about 30  $\mu\text{m}$ . Whereas after sonication, the morphological analysis of LE/CH–EOs NPs was performed by TEM. TEM imaging revealed that the NPs were spherical in shape with a size ranging from 25 to 70 nm (Figure- 2).



**Figure 1-** Light microscope images of LE/CH–EOs NPs (a: 40X, b: 10X) after ultrasonication at 20-kHz for 1.5 h at 37°C



**Figure 2-** TEM image of LE/CH–EOs NPs. were spherical in shape with a size ranging from 25 to 70 nm.

These results are comparable with previous results of CH-LE prepared by injecting LE in an alcoholic solution in water LE–CH–TPGS NPs as nanocarriers of epicatechin to enhance its anticancer activity in breast cancer cells [36]. Also, Figure-2 shows that the produced NPs are in a good assembly state due to the strong adsorption between CH and phospholipid of LE [34].

#### ***In vitro* behavior of LE/CH–EOs NP formula in SGF and SIF buffers**

The effects of both gastric and intestinal conditions on the stability of LE/CH–EOs NPs was evaluated using SGF and SIF buffers by determining particle size and zeta potential. It was found that the size of LE/CH–EOs NPs gradually decreased with time in SGF. This can be explained by the presence of CH in the nanostructure. In SGF (low pH) buffer, a large number of  $\text{NH}_2$  groups in CH were protonated and this subsequently increased the reaction rate with phospholipid in LE and made LE/CH–EOs NPs more stable in gastric enzymes. At a pH value of 1.2 (SGF buffer), the protonation of  $\text{NH}_2$  was increased and this led to an increased interaction between phospholipid and CH. Moreover, the structure of CH became an expanded form that aids in the strong affinity of phospholipid, which leads to the formation of more and more nanostructures while decreasing the degree of release of bioactive substances [37].

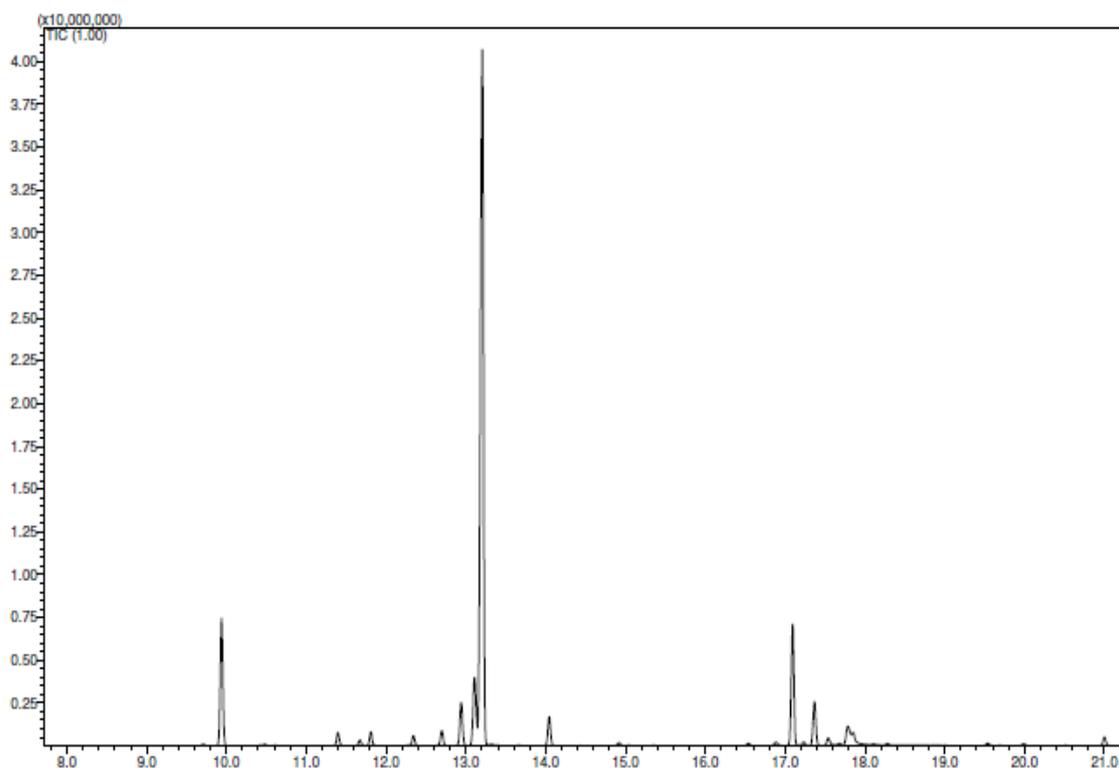
In the case of SIF (pH 7.3), the size of nanostructure was observably increased and subsequently decreased. This can be explained by decreasing the connection between phospholipid and CH. Media tend to enter into the particles and lead to increase their diameter due to the weak electrostatic force between CH and phospholipid, and this may reduce the cationic group in CH [34]. Further, the pancreatic enzyme can hydrolyze phospholipid whereas bile salt can stimulate lipid solubilization [38]. Therefore, it is possible to assume that both effects are associated with a reduction in the construction of nanostructures. In line with these findings, a previous study demonstrated that the presence of CH as coating agent for nanoliposomes could improve their stability in SGF compared to SIF [33].

#### **GC/MS analysis**

Headspace GC/MS was used to check the chemical composition of EO blend. The major compounds in the blend, their retention times, and comparative percentage of the total peak areas are presented in Table- 1 and Figure- 3. While these only represent a fraction of the headspace EOs, the 'footprint' of volatiles can be compared to the GC/MS headspace for reference oils and blends. The EO blend contained 27 different compounds, six of which were the principal compounds that showed a concentration greater than 5%. They were eucalyptol,  $\alpha$ -pinene, I-menthone, cymene, limonene and menthone (40.513, 17.264, 8.575, 8.2471, and 6.0032%, respectively). Eucalyptol and  $\alpha$ -pinene were the two main compounds found in eucalyptus and peppermint oils, respectively, and are the most abundant oils used in making the blend results consistent with those reported by Althea [39].

**Table 1-**Headspace GC/MS of major chemical compounds in the blend of EOs

Retention Time	Compounds	Concentration; %
9.94	$\alpha$ -pinene	17.264
12.942	cymene	8.2471
13.11	limonene	6.0032
13.205	eucalyptol	40.513
14.043	$\gamma$ -terpinene	3.221
17.095	I-menthone	8.575

**Figure 3-** Headspace GC/MS of major chemical compounds in blend of EOs.

### Colony-counting method

The colony-counting method is a simple and very efficient approach in determining the effects of any agent used for antibacterial activity [32]. The results showed a significant decrease in the growth of *S. typhi* after treatment with the nanoparticles under test; approximately a 90% decrease in bacterial count of colonies was observed after 24 h exposure to LE/CH–EOs NPs, while there was no effect (<10%) on bacterial count of colonies after exposure to the antibiotic ciprofloxacin. A possible explanation for this antibacterial activity is the interaction of polycationic CH with negatively charged cell membrane of bacteria, which can lead to intracellular leakage and death of bacteria due to changes in permeability and structure of cell membrane [40]. These results are somewhat consistent with those reported by Kazem and Zidan [41] who found a promising inhibition capacity of a biodegradable and biocompatible nano-formula as an antimicrobial agent.

### Conclusions

In this study, an original, non-toxic, and effective method was established to encapsulate a blend of antimicrobial EOs in biodegradable and biocompatible LE/CH–EOs NPs. These NPs were prepared in the form of an emulsion, which showed a marked antibacterial activity. They were obtained by self-assembly as a result of the electrostatic interaction between the positive charge of CH and the negative charge of lipid in LE. Further, this formula of LE/CH–EOs NPs was stable in the simulated GI environment, making it suitable for oral administration.

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### ETHICAL CLEARANCE

This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific Research, Iraq.

### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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