Antioxidant activity from limonene encapsulated by chitosan

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Abstract. Limonene has been reported to have the effect of inhibiting free radicals. The use of limonene is still limited because of its low solubility in water. Hence, limonene modification is needed. The solution offered to solve the problem is by encapsulating it within chitosan-NaTPP to protect from degradation and increase the solubility of chitosan in water, so that limonene coated in chitosan can increase the value of free radical inhibition activity. This study aims to obtain limonene isolation from orange peel, chitosan synthesis, chitosan modification, limonene-chitosan encapsulation, and followed by antioxidant test. The results show that limonene can be isolated from orange peel. Emulsion prepared by spontaneous emulsification method obtains limonene emulsion with particle size of 339.5 nm. Chitosan modification using NaTPP obtained micro particle size of 2249.7 nm. Encapsulation of limonene in chitosan particles carried out using freeze drying method obtain an encapsulation efficiency of 46%. Limonene-chitosan encapsulation has antioxidant activity with IC50 value of 116 ppm.

Keywords: limonene, nanoemulsion, encapsulation, chitosan

1. Introduction
Antioxidants can stop the formation of free radicals and the chain reactions that could result in cell damage or even death [1-3]. Yet, the process of oxidation plays an important role in the body’s defence against infection or in response to tissue damage such as cancer [4]. The free radical-scavenging activity of fruit[5], vegetable[6] and medicinal plant[7] extracts have been extensively studied. Essential oils rich of monoterpenes are recognized as food preservatives[8-10], and monoterpenic essential oils are natural antioxidants[11] that are active against certain cancers[12]. Indeed, a number of dietary monoterpenes have antitumor activity that can prevent the formation or progress of cancer and cause tumour regression.

Limonene is a kind of monocyclic monoterpen (136.24 kDa molecular weight) which found in the various food stuffs such as sour fruits, orange, carrot and coffee[13-15]. Limonene from essential oil of orange peel has percentage of more than 90%. Limonene has a well-established protective activity against many types of cancers[16].

In recent years, chitosan has received a lot of interest in the encapsulation of bioactive compounds owing to its status as generally recognition as safe (GRAS) due to its excellent biodegradability[17]. Chitosan has shown its capacity for the loading and delivering of sensitive bioactive compounds[18, 19], polyphenolic compounds[17, 20], and vitamins[21]. Chitosan can be made from shrimp shell. According to Hu et al. [20], one of the ingredients that is safe to use as coating is chitosan from the
result of extraction of waste skin from Crustacean animal groups. Chitosan has been widely used as a drug coating with the aim of optimizing the absorption of drugs in target cells. Chitosan also can be crosslinked with sodium tripolyphosphate (Na-TPP) to improve its solubility in water.

In this work, we reported the isolation of limonene and chitosan from waste materials, which are orange peel and shrimp shell, respectively. Moreover, we also examined the antioxidant activity of the limonene nanoemulsion encapsulated by modified chitosan.

2. Experimental Section

2.1. Material
Shrimp shells and orange peels were gained from Tembalang area. Distilled water, NaOH 4% (p.a merck), NaOH 40%, HCl (p.a merck), NaOCl, Na-TPP, glacial acetic acid 1% (p.a merck), methanol (p.a merck), DPPH, ascorbic acid (p.a merck), anhydrous natrium sulphate (p.a merck), and Tween 80.

2.2. Instrumentation
GC-MS (Shimadzu GC17A MSQP 5000), Spectrophotometer UV-Visible (PG Instrumental Limited Model T60U), Particle Size Analyser (PSA), Freeze dryer (LL1500), magnetic stirrer, rotary evaporator, vapour distillation set, and reflux set.

2.3. Limonene Isolation.
Orange peel needed for this experiment was about 10 kg in dry condition by aeration. For one distillation period, 1 kg of dry orange peel was distilled up to five hours. The distilled liquid was the isolated and anhydrous natrium sulfate (5 g for 12 mL distilled liquid) was added and then it was filtered. This isolated limonene was then characterized by GC-MS.

2.4. Chitosan Synthesis
2.4.1. Deprotonation. 25 g shrimp shell powder 60 mesh was added by 250 mL NaOH 4% (b/v). In this section, the solution was set up at 80°C for 1 hour. Furthermore, solution was filtered and washed using distilled water then dried at 60°C temperature.

2.4.2. Demineralisation. Demineralisation was done by dissolving 5 g of the dried deprotonation powder into 75 mL HCl 1 M (v/v) and was then stirred for 1 hour at room temperature and filtered. The residue was washed by distilled water and dried at 60°C temperature.

2.4.3. Depigmentation. The demineralisation result was dissolved in 30 mL NaOCl 4% and stirred for 1 hour at room temperature then filtered. The residue was then washed by distilled water and dried at 60°C temperature. This result called chitin was characterized by FTIR spectrophotometer.

2.4.4. Deacetylation. 1.5 g chitin was dissolved in 22.5 mL NaOH 40% while refluxed at 80°C for 1 hour, filtered and washed by distilled water then dried at 60°C. This result called chitosan was characterized by FT-IR Spectrophotometer.

2.5. Chitosan modified preparation
Chitosan (0.1 g) was dissolved in 100 mL glacial acetic acid 1%. At the same time, 0.25 g Na-TPP was dissolved into 100 mL distilled water. The Na-TPP solution was added into chitosan solution dropwise while stirred for 1 hour. Afterward, the suspension was centrifuged at 600 rpm for 1 hour. The result was freeze dried and characterized by PSA and SEM.

2.6. Limonene emulsion
Emulsification process was carried out using spontaneous emulsification technique [22]. Emulsification system consist of organic phase (limonene and ethanol 70%) and water phase (water and non-ionic surfactant). Organic phase was made by dissolving limonene into ethanol 70% so it reaches 20° brix
total solid dissolve. Spontaneous emulsification technique was done by dropped organic phase into water phase while stirred it. The surfactant used was tween 80. Thin nanoemulsion was then characterized by PSA.

2.7. Limonene emulsion encapsulated by chitosan
Modified chitosan as coating agent was added into limonene nanoemulsion with a ratio of 1:1 (v/v). The mixture was then homogenized for 5 minutes, hydrated at 4°C for 18 hours and rehomogenized for 30 seconds. After that, the result was freeze dried. The encapsulation efficiency (%EE) was calculated using UV-Vis Spectrophotometer.

2.8. Antioxidant Activity by DPPH Scavenging Activity
Concentration variation of Limonene encapsulated by chitosan was made by dissolving it into methanol solution. The concentration was made into 100, 200, 300, 400, 500 ppm. Then, 3 mL of DPPH solution (made by 2 mg DPPH in 50 mL methanol) was added for every concentration of 1.5 mL sample and incubated for 30 minutes in dark room. Ascorbic acid was used for positive control with various concentration of 10, 20, 30, 40 and 50 ppm. Antioxidant activity was measured using UV-Vis Spectrophotometer at 517 nm wavelength.

3. Result and Discussion

3.1. Limonene isolation
Limonene was separated from orange peel using steam distillation. The elucidation of the limonene compound was performed using GC-MS to show the limonene compound with molecular weight of 136. Fig.1 shows the mass spectra of limonene while Fig 2 explain its fragmentation.
3.2. Emulsion Limonene

Emulsion was prepared by a spontaneous emulsification nano method that occurs when the organic phase and the water phase are mixed. The organic phase is a uniform solution of organic solvents (ethanol 70%) and limonene. Bouchemal et al. [22] stated that the composition of the emulsion at optimum condition has a total value of 31brix solids. Brix shows the total value of solids of solutes and solvents. To reach a total solid of 31 brix, it takes 20 grams of limonene in 80 grams of ethanol. The aqueous phase was prepared by water with a non-ionic surfactant. Non-ionic surfactants are most commonly used to stabilize emulsions[23], tween 80 as non-ionic surfactants have high hydrophilic/lipophilic balance values (HLB) that can be stable in oil and water emulsion systems[24].

![Figure 3. emulsion limonene spectra](image)

Fig. 3 shows that there is one peak with an average particle size of 339.5 nm with a percentage of particles formed as much as 75% and a polydispersity index of 0.434. The polydispersity index indicates the homogeneity quality of a dispersion. The small polydispersity index value shows a narrow particle size distribution (PI <0.5) to indicate that the particle size is increasingly homogeneous. Based on the polydispersity index value, the limonene emulsion formed has a narrow particle size distribution to shows that the emulsion of limonene formed has been homogeneous.

3.3. Synthesis of chitosan from chitin

FTIR spectra of chitin and chitosan are shown in Fig 4. And Fig 5. The peak loss at 1265.30-1319.31 cm$^{-1}$ on FTIR of chitosan shows the loss of C-N amide functional group. This indicates the success of the deacetylation reaction in chitin to produce chitosan. Chitosan-forming reaction from chitin deacetylation is shown in Fig. 6. The deacetylation process in the strong base causes the loss of the acetyl group in chitin by breaking the bonds between the carbon in the acetyl group and nitrogen in the amine group. High concentration of NaOH can produce chitosan with high deacetylation levels.
Figure 4. FTIR spectra of chitin.

Figure 5. Spectra FTIR chitosan.

Figure 6. The deacetylation mechanism.
3.4. Modified Chitosan

Chitosan has the desired biocompatibility characteristics and its ability to increase membrane permeability so that chitosan is most promising because it has the ability to form membranes [25]. Physical modification of chitosan includes changes in particle size or chitosan grains to be smaller for wider use. Utilization of physical modification results in the size of nanoparticles[26].

Nanoparticles have very specific properties, with multiple surface area can increase the occurrence of more chemical reactions. A substance can be absorbed directly into the bloodstream where it is needed, this process is more effective than broken down during the digestive system. Nanoparticles are bioavailability i.e. very small size more freely enter the parts of the body so that nanoparticles will be more easily absorbed by the cells individually[27]. The most common method in making nanoparticles is by ionic gelation method using a magnetic stirrer. The ionic gelation method can be carried out by mixing chitosan polymer with polyanion sodium tripolyphosphate which produces the interaction between positive charge on chitosan amino group with payload of tripolyphosphate. Tripolyphosphate is used since it is considered as the best crosslinking agent[28]. Chitosan modification was carried out using Na-TPP. The goal at this stage is to increase the solubility of chitosan in water. Na-TPP when dissolved in water will give hydroxyl ions as electron donor groups [29]. Interactions that arise between chitosan and NaTPP cause the distance between chitosan chains to be tenuous so that chitosan polycation allows interacting with limonen. Modern particle calculations generally use image analysis or some kind of particle counting such as Particle Size Analyser (PSA).

![Spectra of particle size analysis chitosan](image)

Figure 7. Spectra of particle size analysis chitosan.

PSA analysis indicates a peak to show an average particle size of 2249.7 nm with a percentage volume of 80% and the polydispersity index of 0.772. Based on this data, resulted chitosan is categorized as micro-sized. Chitosan microparticles are not yet complete because the polydispersity index (PI <0.5) is still large so that the distribution of microparticles is not homogeneous.

3.5. Limonene encapsulation

Encapsulation of limonene in chitosan particles was carried out using freeze drying method [30]. Encapsulation resulted in the form of suspension and after centrifugation will produce yellowish white deposits. The success of encapsulation can be calculated based on the % encapsulation efficiency (%EE) calculation. Encapsulation efficiency is one of the parameters that can be used to find out how many substances are coated in the matrix in the encapsulation process. The absorbance results of limonene encapsulation are shown in Fig. 8 to obtain an equation as: \( y = 0.010x - 0.168 \). At a concentration of 100 ppm, limonene encapsulation chitosan sample shows an absorption of 0.372 to obtain %EE of 46%. This means that 46% of the limonene is able to be encapsulated in chitosan. This data indicates that limonene encapsulated in chitosan is categorized as low encapsulation (less then 50% of the amount encapsulated).
3.6. Antioxidant Activity Test

Antioxidant activity of limonene encapsulation was examined using DPPH scavenging activity method. By adding DPPH free radical, we know how potential does limonene encapsulation as antioxidant compound. Encapsulation limonene has the effect of antioxidant activity with an IC$_{50}$ value of 116 ppm. This value means that it requires an encapsulation limonene chitosan concentration of 116 ppm to be able to reduce 50% of DPPH radicals.

4. Conclusion

Limonene can be isolated from orange peel. Emulsion prepared by spontaneous emulsification method obtains limonene emulsion with particle size of 339.5 nm. Chitosan modification using NaTPP obtained micro particle size of 2249.7 nm. Encapsulation of limonene in chitosan particles carried out using freeze drying method obtain an encapsulation efficiency of 46%. Limonene-chitosan encapsulation has antioxidant activity with IC$_{50}$ value of 116 ppm.

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