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Antibacterial Retention of Star Anise (*Illicium verum*) Essential Oil and Oleoresin in the Cellulose-PEG Composite

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Abstract

Star anise essential oil (EO) and oleoresin (OL) consists of various volatile compounds that can be easily damaged and affect their quality and function. The degradation is mainly caused by the deterioration and release of their active components. Encapsulating the extracts into a composite matrix can give slow release and minimize the contact between active compounds and surrounding. Cellulose-PEG composite has good compatibility and affinity, it can retain the extracts so does their biological activities. In this work, the essential oil and oleoresin were encapsulated into the cellulose-PEG composite with a ratio of 1:2 (µL EO and OL per mg of composite). The cellulose was obtained from extraction of star anise residue of EO and OL isolation. The extraction steps were pre-alkalization, alkalization, and three-stage bleaching. Antibacterial assay for the samples uses a combination of agar disc and well method, where the pure one uses the disc as absorbent and both the disc and composite are put in the well. The diameter of the inhibition zone against Staphylococcus aureus was observed once a week for eight week. The result shows that pure and composite-packed star anise essential oil and oleoresin samples have fluctuating antibacterial activity during the obeservation. Both packed EO and OL show a lower difference of the diameter of the inhibiton zone which indicated the composite can retain the antibacterial activity of the star anise essential oil and oleoresin.

Keywords: Star anise, antibacterial, essential oil, oleoresin, composite.

INTRODUCTION

Star anise (*Illicium verum*) is a herb mainly applied as medicine and spices, which mainly found in Asia. The traditional Chinese medication uses the herb as a cure for colds and fevers, reduces anxiety and insomnia, and act as a sedative. Biological activities possessed by this fruit are antibacterial, anticancer, anti-inflammatory, and antispasmodic. The active components are anethole, estragole, anisaldehyde, limonene, linalool, and alpha-pinene [1].

Essential oil is a star anise extract produced by steam distillation and hydrodistillation. It has light yellow colored and more intense aroma than the fruit. The antibacterial property of star anise oil works well on Staphylococcus aureus, Mycobacterium tuberculosis, Bacillus subtilis, and other bacteria [2].

Thick brown oily oleoresin is an output of star anise production by solvent extraction [3]. It contains essential oil, non-volatile compounds, fatty acids, pigments, and resins. The aroma is more balanced and pungent than oil, slightly solvent-odored at times, and the taste is as sweet as the oil and more complete [4]. Solvent-extracted oleoresin can act as a growth inhibitor in some bacteria, such as Staphylococcus aureus, Streptococcus pneumoniae, and Parathypoid bacillus [2].

The volatiles deteriorated by direct-indirect contact with air, light, temperature, oxygen availability, water content, compound structure, and chemical composition. The alteration of the product can defect the aroma, taste, and functions, even more, the user's well-being [6]. Turek et all (2013) investigated this phenomenon by model systems, gas chromatographic detection on changes of the substances, and varying the storage condition. Microencapsulation work by combining maltodextrin and soybean protein with star anise oleoresin and was exposed to high-temperature condition for 20-80 minutes. The results show that the encapsulated oil depletes the oleoresin amount and content [3].

Encapsulating both the essential oil and oleoresin into a material which is obtained from the residue of the extraction process is a beneficial yet sustaiable way to overcome the deteriorating of the essential oil and treating the biowaste from previous procedure. The waste is a cellulosic biomass that can be isolated to produce cellulose which may developed into

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composite material as a storage for the essential oil and oleoresin.

A composite is a combination of two materials or more which possess different chemical and physical properties than its ingredients. This material is robust, low-density, inexpensive, highly adhesive on its surface, and widely applicable [7]. The elements mainly consist of polymer matrix and synthetic filler (reinforced materials). There are two types of filler, organic and inorganic, this day organic ones, such as natural fiber, wood fiber, and starch, are preferred because of their affordable, low density, non-scraping, good thermal properties, and biodegradable [8].

Cellulose is an organic filler that can diversify the application of composite. Adding cellulose into the polymeric matrix improves permeability, mechanical properties, and thermal stability. It applies in various sectors without significant problems with environmental pollution. Cellulose extraction can use chemical methods. There are three main steps purification of the raw fiber material to remove non-cellulose, controlled chemical treatment to isolate nanocrystals, and use of chemical or ultrasound treatment to concentrate and treat CNF [9].

Poly Ethylene Glycol (PEG) is a non-toxic and biocompatible polymer consists ethylene glycol units. The hydroxyl groups on cellulose give good compatibility and affinity to the PEG because of their adhesion between the fiber surface and polymeric matrix. Blending the extract into the composite system can improve the solubility and stability of the constituents, and so does its biological activities [10]. Mishra et al. [10] encapsulated lemongrass oil into a cellulose-PEG composite, thus a satisfactory result of retaining the aroma and flavor longer than the pure oil, yet possessing similar functional properties as the crude oil.

The storage method of essential oil and oleoresin affect the product efficacy because the retention of its aromatic compound is controlled and gives a longer shelf-life, encapsulation of the product into a polymer matrix can promote this idea [11]. In Mishra's research [10], citronella oil added to the cellulose-PEG-composite system can be well encapsulated, thus retaining its aroma longer and showing the same functional properties as its essential oil.

The antibacterial activity assay tested using combined difussion agar-well method. The agar diffusion method is done to observe the antimicrobial activity of plants extract by spreading an amount of bacterial inoculum on the agar surface [12]. An inhibition zone is a round-clear area around the point of the antibiotic substance where bacterial colonies do not grow on it. It determines the effectiveness of the antibiotics when the diameter of the samples is wider than the antibiotics in hindering the growth of bacteria [14].

Antibiotics or antimicrobial agents are classified based on the mechanism of their antimicrobial activity, such as agents that inhibit cell wall synthesis, cell membrane depolarization, protein synthesis inhibitors, nucleic acid synthesis inhibitors, and antimicrobial metabolic pathway inhibitors [15]. Essential oils are one the antimicrobial agents that can damage cell membranes, decrease the integrity of cell membranes, and cause leakage of intracellular macromolecules while inhibiting protein formation and biofilm synthesis [16].

The mechanism of this antimicrobial activity was observed by scanning electron microscopy (SEM) by tracking the changes in bacterial morphology. Li et al. (2019) noticed the changes in Gram-positive and Gram-negative bacteria's morphology on exposure to finger citron essential oil (FCEO). The bacteria changed and suffered severe damage with increasing the amount of added FCEO and the time of exposure to oil on bacteria. Bacteria also experience a decrease in growth rate, cell membrane lysis, leakage of intracellular substances, and cell death. Staphylococcus aureus is a Gram-positive bacterium that causes various diseases, such as skin and soft tissue infections, systemic infections, and other fatal diseases [17].

MATERIAL AND METHOD

General experimental procedures

Star anise's essential oil (EO) and oleoresin (OL) were obtained by hydrodistillation and solvent extraction. Cellulose was isolated from the residue of the oleoresin extraction procedure by chemical pretreatment and physical pulverization later. PEG 4000 was melted and mixed with the cellulose at a 1:1 ratio, then the essential oil and oleoresin were poured into two different parts of the cellulose-PEG mixture. The mixture was kept in a glass jar and tested for its antibacterial activity every week for 8 weeks. The antibacterial assay combines the agar disc and well method, where the agar well was used for composite-infused EO and OL, while the agar disc-well was applied for EO and OL. The EO and OL in encapsulated and non-encapsulated forms

are compared to its retention. The inhibition zone was measured as the result of the antibacterial activity of the samples by using an electronic digital caliper.

Plant materials

The dried flower of star anise (Illicium verum) was collected from a local farmer in Central Java, Indonesia in June 2021 and identified by the Laboratory of Taxonomy, Structure, and Development of Biology, Brawijaya University, Malang, Indonesia.

Essential oil extraction

A 40 grams of star anise was hydrodistilled for 8 hours, then separated the oil from the water-oil mixture using a separating funnel and dried with the sodium sulfate anhydrous. The oil was weighed, yielded 2.6617 grams (6.654%), and kept in the air-tight glass jar. The essential oil composition determined by GCMS Shimadzu QP-2010se in the Instrumentation Laboratory of Chemistry Department, Brawijaya University, Malang, Indonesia.

Oleoresin extraction

A 10 grams of fresh star anise fruit were ground and extracted by a Soxhlet extractor. The process went on for 6 hours with 200 mL n-hexane as solvent. Later, the extract was evaporated in the rotary evaporator to remove the solvent then concentrated oleoresin was weighed and stored in the air-tight glass jar. The 3 grams of the oleoresin purified in the column chromatography consist of 4 layers of sand, aluminum oxide (8 cm), sand, and cotton placed consecutively from top to bottom. The eluent was a mixture of toluene and ethyl acetate with a ratio of 9:1. The pure oleoresin was determined as its constituent by GCMS Shimadzu QP-2010se in the Laboratory of Instrumentation, Department of Chemistry, Brawijaya University, Indonesia.

Cellulose extraction

A 10 grams of oleoresin residue was stirred in the ethanol 70%-water mixture with a ratio of 1:1 for 4 hours at room temperature. Then it was filtered off by filter paper, weighed, and yielded 37.13 grams. It has same color and shape as before the procedure. The pre-alkalized residue was mixed with 4% NaOH (w/v) solution and stirred for 4 hours at 80°C during this alkalization step. This step produced 33.17 grams of deep brown-black and smaller particles of residue. The delignification or bleaching procedure was done in 3 steps. In the first step, the residue was mixed for 4 hours at 70°C in a mixture of 50 mL of 5% H_2O_2 (v/v) solution and 50 mL of 3% (2,2,6,6-Tetraethylpiperidin-1-yl)oxyl or TEMPO (w/v) diluted into pure analyst ethanol solution. The first bleaching step produced brown clumped residue and the mass decreased to 19.1 grams. Then, the residue was mixed into the second solution consists of 50 mL of 10% H_2O_2 (v/v) and 50 mL of 5% NaOH. The second bleaching step was done for 4 hours at 70°C and produced 14.88 grams of light brown clumped residue. The third bleaching step of the residue was using a mixture of 50 mL of 10% H_2O_2 and 50 mL of 5% NaOH solution. The mixture was stirred with the residue at 70°C for 4 hours and obtained 15.19 grams of bright yellow clumped residue. The residue from the third bleaching step was dried in the oven at 50°C for 24 hours, ground, and kept in the air-tight glass jar.

Extract blending into the composite

To obtain a cellulose-PEG composite, 3 grams of PEG 4000 was melted, by heating the material at 60 °C, and mixed with 3 grams of cellulose (ratio 1:1 w/w). 0.75 mL EO was poured into the 1.5-gram slurry with ratio EO to cellulose-PEG composite 1:2 (v/w, µL EO per miligram composite). The mixture was stirred, then kept in the air-tight glass jar. The exact steps and material amount were repeated to produce OL-infused cellulose-PEG composite.

Antibacterial assay

Preparation of NA media was done by diluting a ratio of 0.1 g NA per 7 mL of distilled water. The sterilization of instruments and culture media did in an autoclave (121°C, 15 minutes). Laminar Air Flow (LAF) cabinets were disinfected with 70% alcohol and sterilized by UV light for 1 hour. The planting of S. aureus bacteria was started by compacting the NA medium in a test tube by tilting it at 30° and flowing air from the ventilation of the LAF cabinet. Furthermore, the used needle was sterilized using a Bunsen burner then a smear of bacteria was taken from the pure isolate and scratched on the surface of the solidified NA media. The media with S. aureus bacteria planted were incubated in an incubator for 24 hours at 37°C. After 24 hours of incubation, the bacteria can be used for antibacterial testing or stored in a refrigerator at a temperature below 15°C.

The NA media was prepared as an antibacterial test medium while all tools and media were sterilized using an autoclave. 2 mL of sterile 9% NaCl solution was used to prepare the rejuvenated bacterial suspension. 50 L of bacterial mother liquor was diluted into 5 mL of 0.9% NaCl solution homogenized in the same way as the previous method and obtained a bacteria suspension. 100 L of bacterial suspension solution mixed with ~20 mL of NA medium was poured into a petri dish while dragged to form a figure 8. The bacterial culture was then allowed to solidify.

Three wells or holes were made in each petri dish for samples, positive control, and negative control. A 0.003 grams of rifampicin solution was dissolved in 100 mL of sterile distilled water as a positive control. Pure distilled water was used as a negative control. The 50 uL of rifampicin solution (positive control) and sterile distilled water (negative control) were placed in the allocated wells. Composite samples were poured into wells. Oil and oleoresin samples used disc paper and placed into wells. Petri dishes were wrapped in plastic wrap and put in an incubator for 24 hours at 37°C. Bacterial cultures are measured for their zone of inhibition using a caliper. The diameter of the inhibition zone was measured with a digital caliper from the closest distance for bacteria to grow around the well.

RESULT AND DISCUSSION

From the hydro distillation, the obtained star anise oil has strong yet warm anise-scented, thin, and light-yellow colored. There are three components detected by GC-MS in the oil. They are 2-butoxyethanol, benzaldehyde, and trans-anethole. Their composition was 28.66%, 7.56%, and 63.78% consecutively. While the oleoresin has a pungent aroma with a slightly solvent smell, the color is dark green and thicker than the oil. It consists of 2-butoxyethanol 8.5%, benzaldehyde 5.15%, trans-anethole 75.02%, limonene 0.75%, zingiberene 0.92%, alpha-bergamotene 1.48%, trans-caryophyllene 0.89%, beta-bisabolene 1.02%, and benzopyrene 6.29%.

Cellulose extraction was done by treating the star anise residue from oleoresin extration procedure in multiple steps: pre-alkalization, alkalization, three-step delignification or bleaching, dan grinding. After the pre-alkalization process **(Figure 1a.)**, the residue retained its physical properties but significantly increased its weight to 37.13 grams which is three times than before.

Alkalization of the biomass produced black-powdery biomass **(Figure 1b.)** weight decreased to 9.21% because of the breaking of hydrogen bonds in non-cellulosic components, such as hemicellulose and lignin. Hydrogen bonds break in alkalization causing the roughness of the particle's surface, dark colored-product, and weight decline. Non-cellulosic components and impurities are easily diluted into the sodium hydroxide solution thus they turn darker because of the interaction between hemicellulose and lignin with sodium hydroxide.

The first bleaching stage **(Figure 1c.)** produces brown and agglomerated mass and a drastically decreasing weight of 43.34%. In stage two **(Figure 1d.)**, the cellulosic mass had lighter color (dark yellow), still clumped, and the weight was 22.09% lower than before. For the third and the last bleaching step **(Figure 1e.)**, the mass was way yellow light-colored yet still clotted and the weight increased to 2.08%.

Figure 1 shows the difference in biomass during cellulose extraction in each stage. Overall bleaching process gave weight reduction, with color changing from black to brown to dark yellow and finally light yellow. The mass reduction in the cellulose is caused by removing the remaining hemicellulose and lignin from the fiber. So, multistage bleaching can optimize hemicellulose and lignin removal so alkalization also takes place in this step can break the fiber bundle and gives a larger effective surface area.

(a) **(b)** **(c)** **(d)** **(e)**

Figure 1. Multi-stage cellulose extraction from Star Anise fruit residues: **(a)** pre-alkalization, **(b)** alkalization, **(c)** first bleaching, **(d)** second bleaching, and **(e)** third bleaching.

Dried cellulose is characterized by its morphology by Scanning Electron Microscope (SEM) in **Fig. 3**. The cellulose consists of flaky, layered, and irregular-sides particles. There are also fine granules and strands attached to the larger stubs. On the other part, there are layered stubs with a coarse surface. The size distribution shows that most of the cellulose freely ranged in 1.352 µm so it can be categorized as fine particles (1,000-2,500 nm). Fourrier-transform Infrared Spectroscopy (FTIR) Assay on the cellulose identified several groups which are the properties of the cellulose itself as in **Table 1** and **Figure 2**.

Table 1. FTIR Spectra of Cellulose from Star anise residue.

Wavenumber (cm^{-1})	Identification
3335.91	Hydroxyl Group –OH
2918.03	Alkene –CH
2851.00	
1369.16	
1052.54	Primary alcohol –CO
1028.30	
895.66	Glycosidic bond

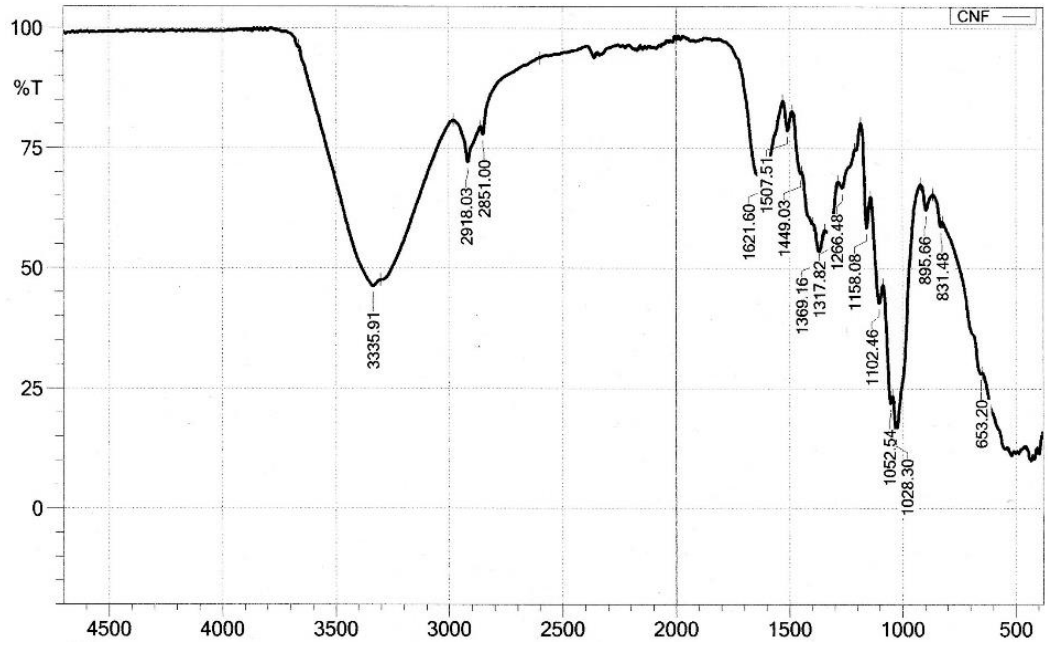
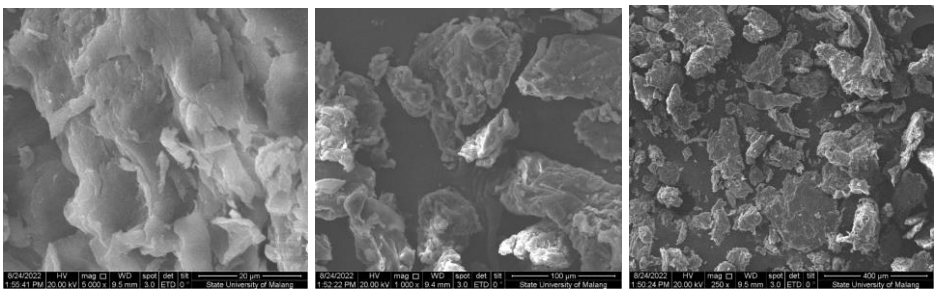


Figure 2. FTIR Spectra of Cellulose from Star anise residue.

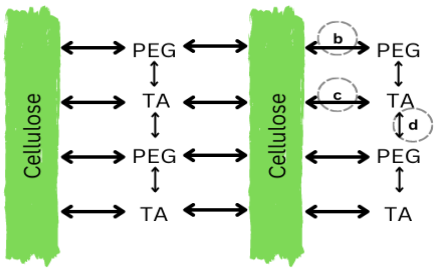


(a) **(b)** **(c)**

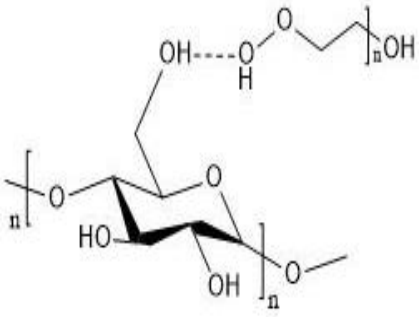
Figure 3. SEM imaging of the cellulose with magnification of: **(a)** 5.000x, **(b)** 1.000x, and (c) 250x

The mixed essential oil in the composite had different colors and textures. It was yellowish and lumpy but the oleoresin mixed one was green and had a smooth texture. The interaction between cellulose, PEG, and trans-anethole showed in **Figure 4**. In the cellulose-PEG composite, the hydroxyl groups of both materials had interaction as in **Figure 4a**. that gave adhesion on their surfaces. Trans-anethole in the essential oil and oleoresin can be absorbed on the cellulose's surface and retained on the cellulose and PEG parts that have a hydroxyl

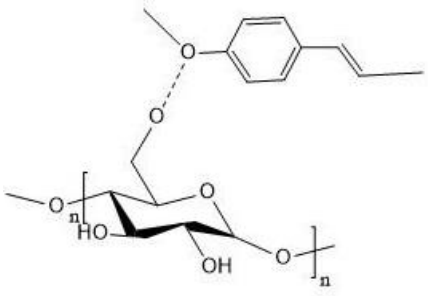
group. In **Figure 4b**., the interaction between cellulose and trans-anethole may occured between the hydroxyl group of cellulose and the methoxy group of trans-anethole. PEG and trans-anethole interaction described in **Figure 4c**., took place in the hydroxyl group of PEG and the methoxy group of trans-anethole. Trans-anethole that is absorbed in the hydroxyl group is harder to be released to the environment, so the content of the active ingredient in the oil and oleoresin can be stably decreased. This slow-released phenomenon can be assumed that packed essential oil and oleoresin of star anise can give longer retention of its biological activity. Retention or captivation of active ingredients can prolong the shelf-life of the extracts.



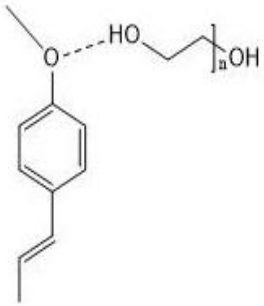
(a)



(b)



(c)



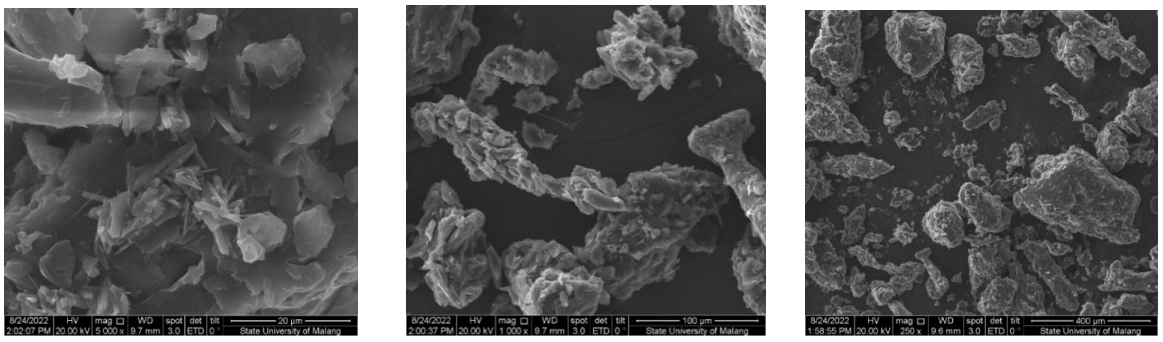
(d)

Figure 5. Interaction between cellulose, PEG, and trans-anethole.

The morphology of the composite was observed by SEM in the **Figure 5**. The particles are slab, rod, and flakes-shaped smooth on the surface, and sharp-edged. The flakes are mainly attached to the larger slabs and rods. In a bigger magnifying, the slabs and rods have a rough and scaly surface which indicates the PEG is attached to the cellulose's surface. Most of the composite consists of an unevenly distributed particle with frequently ranged in 1.376 µm. FTIR analysis was done to characterize the difference between cellulose and composite. Most of the peaks has shifted to a higher number. It indicates the change in the property of cellulose caused by PEG. The shifting of OH and CH bonds signifies the interaction between cellulose and PEG and the bending of their hydroxyl groups denoted at 1624.27 cm^{-1}.

Table 2. FTIR Spectra of Cellulose-PEG Composite.

Wavenumber (cm^{-1})	Identification
3431.47	Hydroxyl Group –OH
2883.80	Alkene –CH
2739.75	
1279.31	
1052.54	Primary alcohol –co
1028.30	
1456.57	
1359.18-1340.64	PEG
1279.31-1239.38	
841.46	
956.99	Glycosidic bond
1101.4	Secondary alcohol
1059.88	Ether



(a) 5000x **(b) 1000x** **(c) 250x**

Figure 4. SEM imaging of the cellulose-PEG composite with magnification of: **(a)** 5.000x, **(b)** 1.000x, and **(c)** 250x**.**

The inhibition zone (**Table 2.**) from the antibacterial assay of the samples against *Staphylococcus aureus* is shown in Table C. Both pure and packed essential oil and oleoresin have the lowest inhibition zone diameter at the last week of trial, yet the positive control or rifampicin always being larger than the other samples for all weeks. Thus, the antibacterial activity of the essential oil and oleoresin is weaker than rifampicin as an antibacterial agent. The diameter of pure and packed essential oil is up and down from the first to the last week. In the first week, the pure sample has a higher diameter than the packed one, then decreasing continuously at weeks 2, 3, 4, 6, and 8 when packed sample show declining at weeks 2, 4, 5, and 7. The diameter of the inhibition zone of the pure essential oil is decreased more than that of the packed. The diameter of the pure sample in the last week is 26.78%, which is lower than the first week, while the packed sample is 11.61%. Generally, the packed oil in the composite retains the antibacterial activity better than the pure oil itself.

The inhibition diameter of the pure oleoresin declined at 3, 5, 6, 7, and 8 weeks, while the inhibition diameter of the packed-oleo declined at 2, 3, 5, 6, and 8 weeks. The packed oleo has a significant decrease than the pure sample, but the deviation of the inhibition zone is decreasing for every week. Compared between the first to the last week, packed oleo has a lower percentage with a diameter of 5.21% smaller than pure oleoresin (28.80%). Generally, pure oleoresin has a drastically lower inhibition zone than the packed sample.

The declining of the inhibition zone diameter of the pure and packed star anise and oleoresin was caused by the released active components to the environment due to air contact between them. Essential oil components have similiar structure and can be easily converted into each other by oxidation, isomerization, cyclization, and dehydrogenation reaction. So, the stability of the extracts must be kept in mind because the alteration of the active componen can effect the quality of the product [6].

As the active component declining, the inhibition zone generated by the oil and oleoresin was declining as well as their antibacterial activity. Active components in the essential oil and oleoresin are the key in microorganism destruction by damaging cell membrane while decreasing its integrity, causing leakage of intracellular macromolecules, and inhibiting protein formation and biofilm synthesis [16].

The inhibition diameter of the composite-packed essential oil and oleoresin has more stable drop than the pure one. It certainly that cellulose-PEG composite give significant effect on the antibacterial activity of the extracts. According to Mishra (2018), packed essential oil in the the cellulose-PEG composite can retain the active component of lemongrass oil for 120 days by maintaining the stability of the active component release.

Table 2. The inhibition zone from the antibacterial assay of the samples against Staphylococcus aureus

Week	Inhibition Zone Diameter, mm				
	Essential Oil (EO)	EO in composite	Oleoresin	Oleoresin in composite	Positive Control (Rifampicin)
1	15.57	13.62	12.71	13.06	17.89
2	13.74	12.38	12.75	12.17	17.74
3	12.51	12.50	11.53	11.82	14.78
4	11.74	11.71	11.60	12.43	13.85
5	11.75	10.82	10.95	11.87	13.55
6	10.44	12.29	9.69	11.82	13.47
7	11.77	11.65	9.48	11.89	14.59
8	11.40	11.72	9.05	12.38	15.32
%Deviation of week 1 and 8	26.78%	11.61%	28.80%	5.21%	-

CONCLUSION

Antibactrial activity of pure and packed star anise essential oil and oleoresin are dropped over the time. This is indicated by the declining of the inhibition diameter in the antibacterial assay which had done for 8 consecutive weeks. Both packed essential oil and oleoresin has smaller %deviation of the inhibition diamater declining (from first to eighth week) than the pure extracts. Thus, the cellulose-PEG composite can retain the antibacterial activity and posses the ability as a storage for the extracts.

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