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Preparation and Characterization of Sodium Alginate-Based Edible Film with Antibacterial Additive using Lemongrass Oil
(Penyediaan dan Pencirian Filem Boleh Dimakan Berasaskan Natrium Alginat dengan Bahan Tambah Antibakteria menggunakan Minyak Serai)

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ABSTRACT

Sodium alginate films at various concentrations of glycerol (0.4, 0.6, and 0.8% v/v) were prepared and characterized. The thickness, water solubility (WS), water vapor transmission rate (WVTR), tensile strength (TS), elongation at break (EB) and Young's Modulus (YM) of the films were affected by the amount of glycerol added. Among these films, the film containing lower glycerol (0.4 % v/v) presents better WS, WVTR, and the highest TS compared to other concentrations. Sodium alginate films containing lemongrass essential oil (LEO) were prepared to examine its antibacterial properties on four common foodborne pathogens: B. subtilis*,* S. aureus, E. coli*, and* P. aeruginosa *using Disc-diffusion assay. The highest inhibition was shown by* E. coli *(21 mm/susceptible),* B. subtilis *(18 mm/intermediate),* S. aureus *(16 mm/intermediate), and* P. aeruginosa *(13.5 mm/resistant). The incorporation of lemongrass essential oil as a natural antibacterial agent in the film formulation has developed its potential to be used as an active packaging with improved physical properties, especially water barrier properties.*

Keywords: Antibacterial properties; edible film; lemongrass essential oil; mechanical properties; sodium alginate

ABSTRAK

Filem natrium alginat pada pelbagai kepekatan gliserol (0.4, 0.6 dan 0.8% v/v) disediakan dan dicirikan. Ketebalan, keterlarutan air (WS), kadar penghantaran wap air (WVTR), kekuatan tegangan (TS), pemanjangan pada waktu rehat (EB) dan Young's Modulus (YM) filem adalah dipengaruhi oleh jumlah gliserol yang ditambah. Antara filem ini, filem yang mengandungi gliserol rendah (0.4% v/v) menunjukkan WS, WVTR yang lebih baik dan TS tertinggi berbanding dengan kepekatan yang lain. Filem natrium alginat yang mengandungi minyak pati serai (LEO) disediakan untuk mengkaji ciri anti-bakteria pada empat patogen bawaan makanan biasa: B. subtilis*,* S. aureus, E. coli *dan* P. aeruginosa *dengan menggunakan ujian disk-difusi. Perencatan tertinggi ditunjukkan oleh* E. coli *(21 mm/rentan),* B. subtilis *(18 mm/perantaraan),* S. aureus *(16 mm/perantaraan) dan* P. aeruginosa *(13.5 mm/tahan). Penggabungan LEO sebagai agen anti-bakteria semula jadi dalam pembuatan filem telah mengembangkan potensi sebagai pembungkusan aktif, tetapi cirinya dapat diubah, terutama sifat penghalang air.*

Kata kunci: Ciri antibakteria; ciri mekanikal; filem boleh makan; minyak pati serai; natrium alginat

Introduction

Generally, food packaging serves for the preservation of various categories of foods and raw materials from oxidative and microbial action and increases the product shelf-life (Tharanathan 2003). Edible films have received global attention since it is biodegradable for wrapping or coating food materials and can be consumed together with food in contact or removed before consumption (Erkman & Barazi 2018). Indeed, it offers a good moisture and oxygen barrier, inhibits moisture exchange between food products and the atmosphere, and prevents the occurrence of unwanted chemical and enzymatic reactions and microbial growth (Janjarasskul & Krochta 2010). A typical constituent of edible film is film forming material, plasticizer, and additives, including proteins, polysaccharides, lipids, and their combination (Han 2014).

Sodium alginate is a long chain polysaccharide, and the films based are usually brittle and have low moisture resistance due to their hydrophilic nature (Rhim 2004). Therefore, incorporating bivalent cation into the alginate solution creates an ion exchange that resulted in gel formation and stronger film. The interaction of alginate towards group 2A in periodic table rises in the order $Ca^{2+} < Sr^{2+} < Ba^{2+}$ (Lee & Rogers 2012). Thus, calcium ions such as calcium lactate, calcium gluconate and calcium chloride capable form a stronger alginate gel and highest solubility (Ustunol 2009). This polysaccharide is commonly incorporated with a plasticizer such as glycerol, sorbitol, propylene glycol, and other polyols, which is functioned to improve the film's flexibility (Cao et al. 2018). Several studies have also shown that plasticizer has revamped mechanical and structural properties (Karbowiak et al. 2006). Among all types of plasticizers, glycerol has been widely used since it is effective for film-forming hydrophilic polymers and preventing film brittleness (Viera et al. 2011).

Additionally, the incorporation of antimicrobials into packaging films has attracted interest due to inhibition of the growth of microorganism in food and lengthens the shelf-life, thus maintaining the standard of the food. The addition of natural substituents like lemongrass, garlic oils, rosemary, and oregano into edible film has been successfully demonstrated in many studies (Pranoto et al. 2005). Based on the literature, lemongrass oil was reported to demonstrate a relatively good antibacterial properties against *Escherichia coli*, *Enterococcus faecalis*, *Acinetobacter baumanii*, *Aeromonas veronii*, *Klebsiella pneumonia*, *Enterobacteraerogenes*, *Salmonella enterica serotype typhimurium*, *Serratia marcesens*, *Proteus vulgaris*, *Corynebacterium equii,* and *Staphylococcus aureus* (Naik et al. 2010). Furthermore, Lertsatitthanakorn et al. (2010) showed the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of lemongrass essential oils are ranged between 1.562-6.250 and >50 µL/mL against *S. aureus*, meanwhile 1.562-3.125 and 12.5-25 µL/mL against *E. coli*.

Even though there are many publications concerning edible films, there are limited studies focusing on the biological analyses against Gram-positive and Gram-negative bacteria using lemongrass oil edible films to the best of our knowledge. Therefore, this paper aims to discuss several properties of sodium alginate films containing different glycerol concentrations in terms of thickness, solubility, water vapor transmission rate, and mechanical properties. Following this, the films were incorporated with lemongrass essential oil to analyze the antibacterial properties against Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*P. aeruginosa* and *E. coli*).

Materials and Methods

Two kilos of lemongrass leaves were locally harvested from UiTM Arau plantation. Chemical substances such as sodium alginate, glycerol, calcium chloride, and anhydrous sodium sulphate were obtained from Qrec (Asia) Sdn. Bhd. and 95% ethanol was purchased from HmbG, Malaysia. Meanwhile, Mueller-Hinton agar, nutrient agar, and nutrient broth were obtained from Merck, Malaysia.

Sodium Alginate Film

Casting method is used to prepare sodium alginate film as proposed by Rhim (2004). 1.5% of sodium alginate solution is prepared by dissolving 1.5 g of sodium alginate powder into 100 mL distilled water at 70 °C. The solution was stirred continuously with mechanical stirring (300 rpm) for 30 min. After dissolution was completed, different concentrations of glycerol (plasticizer) was mixed into the solution. The film casting solution was prepared with various glycerol concentrations [0, 0.4, 0.6 to 0.8% v/v]. Subsequently, 5 mL of 2% calcium chloride solution was stirred slowly before being poured into a glass petri dish. The film was left dried in the oven, operated at 50 °C for one day, and stored in a desiccator for further use.

Physical Properties Water Solubility (WS) and Thickness

A digital thickness gauge with 0.01 mm accuracy is used to measure the film's thickness. The measurement was carried out at three points, including the upper, middle, and bottom end of the film. The mean values of the samples are recorded in triplicates.

23 cm^2 dimension of the film was weighed and immersed in 30 mL distilled water and left for an hour at room temperature. Then, the pieces of film were filtered and dried in the oven at 60 °C until constant weight. The test was conducted in triplicate, and the percentage of solubility is calculated as follow (Romero-Bestida et al. 2005):

$$Solubility\ (\%) = \frac{Initial\ dry\ weight - Final\ dry\ wieght}{Initial\ dry\ weight} \times 100 \quad (1)$$

Water Vapor Transmission Rate (WVTR)

The determination of WVTR was carried out according to the ASTM standard E96-80 using the desiccant method. The films were dried in oven at 50 ºC for 24 h and cut into 2 x 3 cm^2 before being glued on the test tube's mouth with silica gels. The test tube and dried film were pre-weighed and left in a desiccator with a saturated calcium chloride solution at room temperature with a relative humidity of 75.7%. The films were then weighed every 24 h for seven days (168 h). The mean value of the triplicates was calculated as in (2).

$$WVTR = \frac{(\frac{G}{t})}{A} \quad (2)$$

where G/t is the slope of the straight line, g/h; A is the test area, m^2, $WVTR$ is the rate of water vapor transmission, $g.m^2/h$.

MECHANICAL PROPERTIES

Characterization of film mechanical properties involved the determination of tensile strength (MPa), percentage of elongation at break (%E), and Young's modulus using a tensile machine (Instron) followed with ASTM D-882 (1981b). The films were cut into 75 10 mm^2 and clamped at pneumatic grips (25 psi); stretch at 0.5 mm/s with an initial 100 mm grip.

EXTRACTION AND ANALYSIS LEMONGRASS ESSENTIAL OIL (LEO)

The chemical constituents of lemongrass essential oil (LEO) was extracted and analyzed from the leaves by using steam distillation method and analyzed using GC-MS (Beetle 2017). The lemongrass leaves were reported to produce a higher yield of essential oil compared to the lemongrass stalks. GC-MS from Agilent 7890B with HP-5MS capillary column of 30 m long, film thickness of 0.50 µm and 0.25 mm internal diameter (ID) was used. The injector temperature (split mode 1:50) and helium as carrier gas were set at 250 °C, and the column with a flow rate of 0.575 L/min. Temperature for the oven was set initially at 50 °C (1 min) before being increased up to 300 °C (60 °C min^{-1}) and kept for the final 5 min. The chemical constituents of LEO were identified by comparing retention times with appropriate standards and the computer mass spectra libraries of Wiley 7 and GC/MS Libraries.

ANTIBACTERIAL FILMS WITH LEO ADDITIVES

The film was prepared using a casting method as suggested by Pranoto et al. (2004). 1.5% w/v of sodium alginate was prepared by dissolving 1.5 g of sodium alginate powder in 100 mL distilled water at 70 °C under constant stirring at 300 rpm. Then, the best concentration of glycerol, which is 0.4% was added into the mixture. Diluted LEO (50 concentration with 95% ethanol), was then mixed into the edible film solution at 0% (control) and 0.4% v/v solution. 5 mL of 2% calcium chloride solution has slowly added solution as a crosslinking agent and cast into a glass petri dish and left in the oven at 50 ºC for 24 h.

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

The functional group of sodium alginate film without LEO (control) and antibacterial films with 0.4% LEO were identified by using FTIR spectroscopy, where the wavenumber over the range 4000-400 cm^{-1}.

ANTIBACTERIAL TEST

Antimicrobial activity was analyzed using disc diffusion method on Mueller-Hinton agar (MHA). The medium was prepared as suggested by the manufacturer's instruction (Merck). Particularly, the sodium alginate edible film was tested on common foodborne bacteria which are *S. aureus*, *B. subtilis* (Gram-positive) and *P. aeruginosa*, *E. coli* (Gram-negative). Tetracyline and ampicillin were set as positive control while ethanol was set as negative control.

PREPARATION OF INOCULUM

S. aureus, B. subtilis (Gram positive) and *P. aeruginosa, E. coli* (Gram negative) bacteria were cultured on a nutrient agar plate and incubated for 24 h. Then, 4 to 5 colonies from each pure bacterial culture transferred into 9 mL of nutrient broth and incubated for 18-24 h at 37 °C and adjusted to meet 0.5 MacFarland standards at 600 nm using UV Spectrophotometer.

SPREAD PLATE TECHNIQUE

100 µL of bacteria suspension was pipetted at the center of the surface of MHA agar plate. Hockey stick was used to spread the bacteria uniformly over the agar surface and incubated for another 24 h at 37 °C.

ANTIMICROBIAL ACTIVITY ON ANTIBACTERIAL FILM

The 6 mm diameter disk was soaked into a casting solution of sodium alginate 0.4% film sample and placed on MHA plates. For positive control, 6 mm of Whatman filter paper disc was soaked into tetracycline and ampicillin. Each MHA disk was subjected to three parameters: Negative control (ethanol), positive control (antibiotic), and film. Then, the disks were pressed down gently to assure thorough contact between the film and agar surface. All plates were then incubated in an inverted position at 37 ºC for 24 h. After 24 or/until 48 h, the inhibition zones were measured using a ruler and compared to the acceptable inhibitory zone diameter (mm) limit of control strains (Li et al. 2006).

RESULTS AND DISCUSSION

PHYSICAL PROPERTIES OF SODIUM ALGINATE FILMS

Films thickness was gradually increased from 0.02 to 0.08 mm in glycerol concentration as shown in Table 1. This is due to glycerol easily dissolved in water and increased the viscosity of casting solution by binding with the water; thus, the thickness of edible films increased (Sitompul & Zubaidah 2017). Besides, these films' thicknesses meet with the Japanese Industrial Standard (JIS), in which the maximum standard thickness of edible film shall be below 0.25 mm. In addition, the water solubility of films was tremendously increased from 40.15 to 84.67% as the concentration of glycerol is increased as shown in Table 1. The higher solubility of film in water was due to glycerol's hygroscopic properties, which encourage the moisture adsorption. This indicates these edible films can be degraded naturally when dumped to the environment. However, films with low solubility in water or water resistance are favorable to maintain product quality and improve food materials' shelf life. Alternatively, it is undeniable that films with higher solubility in water are beneficial because they can be used in 'ready to eat' products (Pitak & Rakshit 2011).

Water-vapor transmission rate (WVTR) is a crucial barrier property in packaging material. The WVTR was measured gravimetrically and was expressed as an amount of water vapor in one gram able to pass through a material, usually within 24 h (Kopacic et al. 2018). The range WVTR is from 0.1167 to 0.1667 g/h.m^2 as shown in Table 1. To sum up, the results acquired in this study show that glycerol concentration greatly influenced the barrier properties of sodium alginate-based films. The high WVTR value corresponds to the less barrier against moisture. Therefore, film with 0.4% v/v of glycerol is the most favorable as it has less WVTR value among other sodium alginate films. Figure 1 illustrates the edible sodium alginate-based film with the physical appearance of transparent, soft, and flexible for labeled B, C and D; control film labeled A shows translucent, hard, and brittle.

TABLE 1. Thickness, water solubility, water vapor transmission rate for sodium alginate-based films

Glycerol concentration % (v/v)	Thickness (mm)	WS (%)	WVTR (g/h.m^2)
0.0000	0.020 ± 0.001	40.1535 ± 1.4468	0.1167
0.4000	0.047 ± 0.005	63.1912 ± 3.3585	0.1333
0.6000	0.050 ± 0.000	77.2568 ± 0.0461	0.1500
0.8000	0.080 ± 0.000	84.6699 ± 2.4651	0.1667

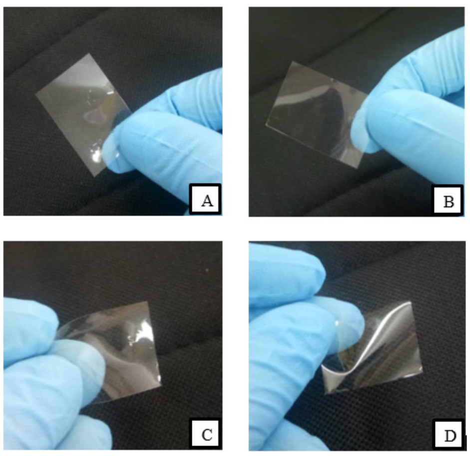

FIGURE 1. The appearance of sodium alginate edible films with 0% (A), 0.4% (B), 0.6% (C) and 0.8% (D) of glycerol concentration

MECHANICAL PROPERTIES OF SODIUM ALGINATE FILMS

Sodium alginate which is a polysaccharide has made interaction with other additives including glycerol plays an important role in tensile strength (TS), Young's Modulus (YM) and elongation at break (EB) in the films. TS is a measurement of the maximum tensile force that film can hold, YM represents the resistance of a material to deform under load, while EB is the ability of the film to stretch up until its rupture. Figure 2 illustrates TS decreased significantly from 10.6291 to 4.5513 MPa as glycerol added from 0.4% v/v to 0.8% v/v respectively. The TS values have met the minimum standards of JIS, which is 3.92 MPa. The decreased TS value is due to glycerol weakening the intermolecular forces between the chain-to-chain interactions and increasing the sodium alginate solution's free volume. Thus, the mechanical resistance of films is reduced and films become softer (Guilbert et al. 1996; Sothornvit & Krochta 2005). TS has similar trends with YM in which the value is significantly decreased from 42.6693 to 10.9573 MPa for the concentration of glycerol from 0.4 to 0.8% v/v, respectively. Meanwhile, this edible film has a stretchy characteristic, thus EB value reportedly increased dramatically from 36.7845 to 77.9713%. According to JIS, the EB values in this study are acceptable; the elongation value of a good film must exceed 50% but not less than 10%. In particular, EB value was considered as good mechanical results compared to others research. These results can be explained by reducing internal hydrogen bonding between polymer chains by the glycerol, enhancing the flexibility and extensibility of films (Maizura et al. 2007).

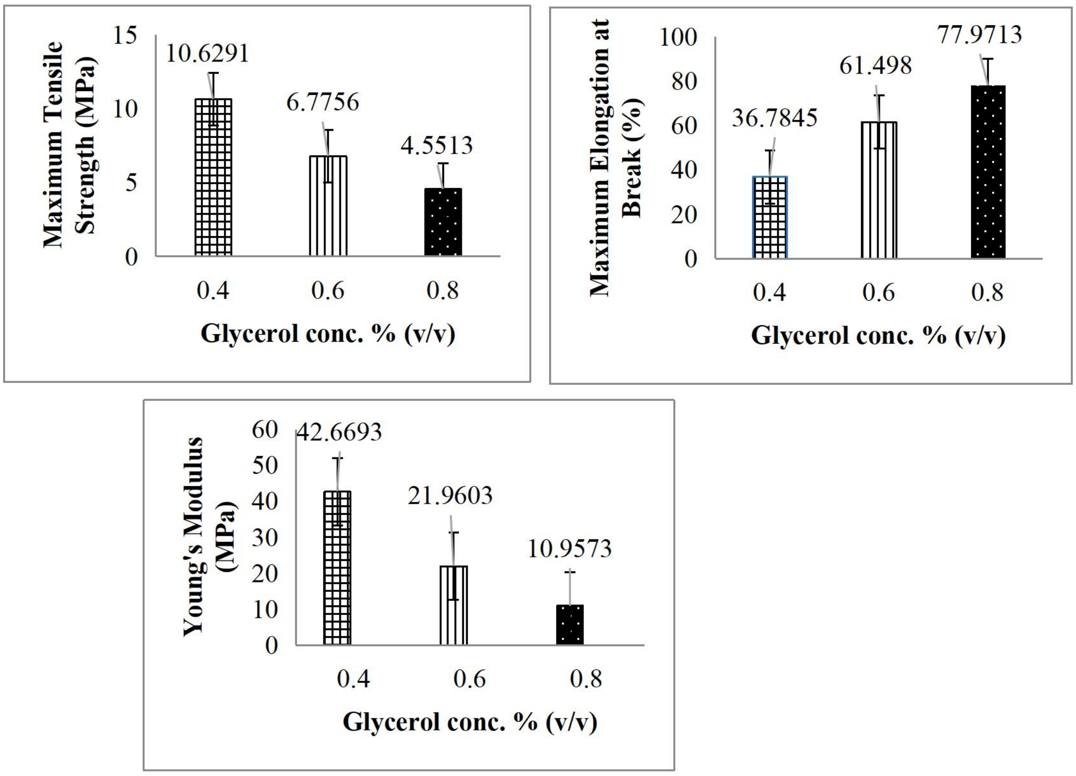

FIGURE 2. The data of tensile strength, Young's modulus and elongation at break of the sodium alginate-based films incorporated with 0.4, 0.6 and 0.8% v/v concentration of glycerol

COMPOSITION AND ANALYSIS OF THE LEMONGRASS ESSENTIAL OIL (LEO)

LEO extracted from lemongrass appeared to be yellowish and exuded a fresh, sweet, grassy, and citrus-like aroma. The yield of oil was determined to be 1.21 g LEO/1.16 kg lemongrass leaves. Table 2 represents chemical constituents analyzed by the GC-MS instrument. The lemongrass essential oil is high in citral and other monoterpene compounds which include α-citral (26.63%), β-citral (19.43%), and β-Pinene (7.77%). Besides, β-myrcene (2.44%), verbenol (1.98%), linalool (1.38%), and geranyl acetate (1.98%) were identified as minor constituents that naturally occur as monoterpene alcohol. Our primary compound of LEO or *Cymbopogon* species findings bears a close resemblance to most studies (Ali et al. 2017; Boukhatem et al. 2014; Chowdhury et al. 2010). These results offer compelling evidence for citral as essential components in which it exhibits strong antifungal, antibacterial and antiparasitic properties (Zeng et al. 2015). Moreover, the chemical compound called citral will be determined the quality of LEO itself, which natural mixture of two monoterpene aldehydes, geranial (trans-isomer) and neral (cis-isomer) (Desai et al. 2014). This confirms that germanials and nerals are essential to act as natural antibacterial agents in sodium alginate-based film.

TABLE 2. Chemical constituents present in LEO

Retention time (min)	Percentage (%)	Identified compound	Common name
2.957	2.440	-myrcene	-myrcene
3.009	7.770	-Pinene	-Pinene
3.454	1.380	1,6-octadien-3-ol,3,7-dimethyl-	Linalool
3.746	3.150	Bicyclo [3.1.1] hept-3-en-2-ol,4,6,6-trimethyl-	Verbenol
4.409	19.430	2,6-Octadienal, 3,7-dimethyl-, (Z)	-citral
4.700	26.630	2,6-Octadienal, 3,7-dimethyl-, (E)	-citral
5.415	1.980	2,6-octadien-1-ol,3,7-dimethyl-,acetate,(E)	Geranyl acetate

Besides, the functional groups sodium alginate film with LEO (0.4% v/v) and without LEO (control) were identified using FTIR. Figure 3 shows spectra of a similar transmittance peaks pattern for with and without LEO. This result indicates that the incorporations of LEO in the sodium alginate film have no interaction with the functional group. A similar result was reviewed by Maizura et al. (2007), where FTIR spectra of starch-alginate films with various concentrations of lemongrass oil presented a similar pattern with the control film. The most significant difference can be seen at the strong and broad OH stretching of the hydroxyl group at around 3260.07 cm^{-1}. The hydroxyl group was contributed by the water that was involved in the preparation of the films in the control film. However, the film with LEO additives seems to have weak OH centered stretching at 3402.60 cm^{-1}, which may be due to the hydrophobic characteristic contributed by the oil (Kok & Wong 2018). The bands within ~1599 and ~1408 cm^{-1} were associated with asymmetric and symmetric stretching vibrations of carboxylate salt ions. In addition, the weak signal was observed on both spectra at ~2932 cm^{-1} showing the presence of C-H stretching vibration.

ANTIBACTERIAL ACTIVITY

Tetracycline was used as positive control against *E. coli, P. aeruginosa,* and *B. subtilis*, while antibiotic ampicillin was used as positive control for *S. aureus.* Meanwhile, 95% ethanol used in 50% v/v LEO preparation was used as negative control. The susceptibility level of bacteria towards edible films and control were indicated by ‘susceptibility’, ‘intermediate’, and ‘resistant’ based on standard inhibition value. Table 3 represents the inhibition zone for selected bacteria against sodium alginate incorporated with LEO, positive control and negative control. Figure 4 shows the inhibition zone of *P. aeruginosa, E. coli, S. aureus,* and *B. subtilis* on the films.

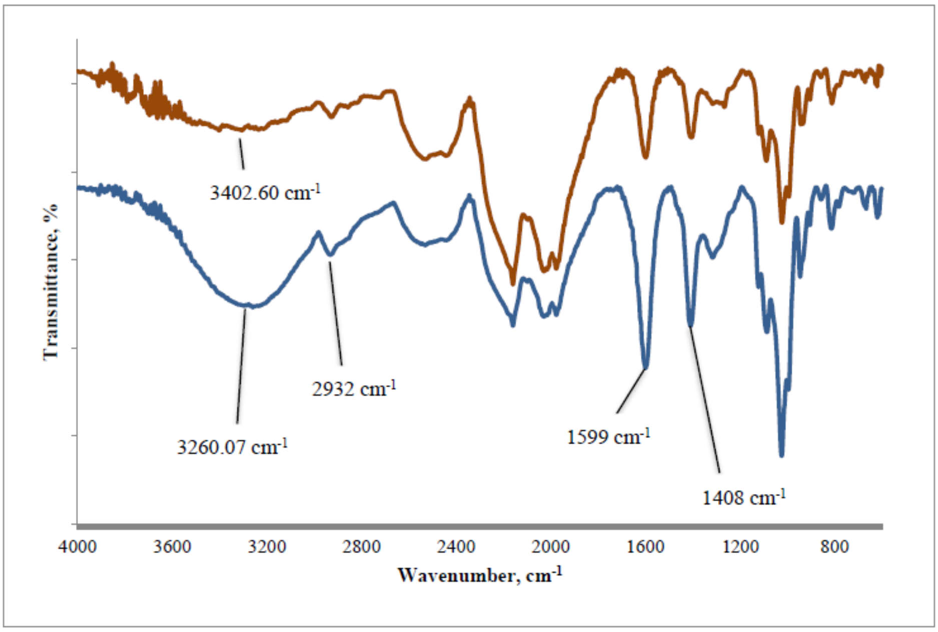

FIGURE 3. FTIR spectra of sodium alginate films with LEO (upper) and without LEO (bottom)

TABLE 3. Zone of bacterial inhibition

Disc	Diameter of inhibitory zone (mm)							
	Escherichia coli		*Pseudomonas aeruginosa*		*Bacillius subtilis*		*Staphylococcus aureus*	
	Diameter	(R/I/S)	Diameter	(R/I/S)	Diameter	(R/I/S)	Diameter	(R/I/S)
Film	21.0 ± 1.41	S	13.5 ± 0.71	R	18.0 ± 2.83	S	16.0 ± 0.00	I
Ethanol	7.0 ± 0.00	R	8.0 ± 1.41	R	0.0	R	0.0	R
Tetracycline	23.0 ± 1.41	S	20.5 ± 2.12	S	29.5 ± 0.71	S		-
Ampicillin	-	-	-	-		-	16.0 ± 0.00	I

*(R/I/S): Represent the interpretation of inhibitory activity towards bacteria either it is resistant/intermediate/susceptible

Table 3 shows the most susceptible strains towards sodium alginate incorporated with LEO is *E. coli* (21.0 ± 1.41 mm) and followed by Gram-positive *B. subtilis* (18.0 ± 2.83 mm). However, the *S. aureus* is intermediated susceptible (16.0 mm) and followed by the resistant *P. aeruginosa* at 13.5 ± 0.71 mm.

E. coli was reported to possess a high antimicrobial activity on sago starch-alginate film incorporated with LEO. There is almost no difference in the dispersion and dissolving of oil in the film with or without glycerol. The solubility of oil in the film has made uniform dispersion (Maizura et al. 2007).

In contrast, *P. aeruginosa* was resistant to LEO-alginate film as the inhibition zone was less than 14 mm. Our results align with Naik et al. (2010) where *P. aeruginosa* is resistant to all LEO concentrations, including its pure oil. This is a good agreement with Burt (2004), as Gram-negative is less sensitive towards

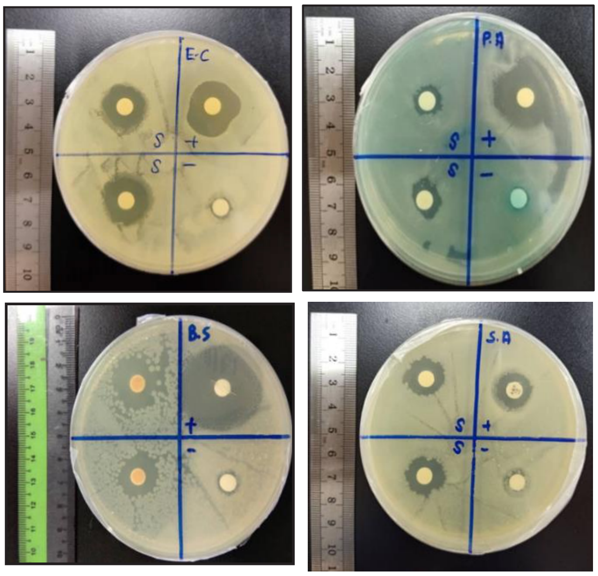

FIGURE 4. Inhibition zone of *E. coli, B. subtilis*, *P. aeruginosa,* and *S. aureus* on sodium alginate incorporated with LEO

antibacterial agents. These bacteria consist of additional outer membranes that limit the diffusion of hydrophobic compounds into the cell through lipopolysaccharides covering.

In this study, both Gram-positive bacteria (*B. subtilis* and *P. aeruginosa*) show a good potential for antimicrobial activity, which are susceptible and intermediate susceptible, respectively. The Gram-positive bacteria were expected to be susceptible as their cell has lipophilic ends that accelerate the diffusion of hydrophobic active compounds in essential oils into the cell (Cox et al. 2000). The slight lower of inhibition might contribute by partial loss of volatile phenolic compound during film preparation.

The variations in antimicrobial inhibition by Gram-negative and Gram-positive bacteria were suggested because of the amount of citral active compound present in essential oil. Different species contributed a different composition of citral. These citral compounds will penetrate into the cell membrane's phospholipid layer and subsequently accumulate and inhibit the bacteria by disrupting their membrane integrity (Abe et al. 2003). Further observation carried out by Gupta et al. (2016) confirmed with our findings, the highest inhibition of LEO was achieved by *P. aeruginosa*, followed by *B. subtilis, E. coli* and least inhibition on *S. aureus*.

CONCLUSION

To conclude, it was found the properties of sodium alginate films are influenced by the amount of glycerol added. The films with the additional 0.4% glycerol showed the lowest WVTR (0.1333 g/h.m^2), solubility (63.19%), the highest TS (10.63 MPa), and acceptable EB value (36.78%). Significantly, the shelf life of the food could be enhanced with the films incorporated with LEO. This is due to high citral compounds present, which are α-citral (26.63%) and β-citral (19.43%). The sodium alginate/LEO films prepared exhibited the highest inhibition against *E. coli*, followed by *B. subtilis, S. aureus,* and *P. aeruginosa*. The films were more effective against Gram-positive bacteria compared to Gram-negative bacteria. For further study, the effectiveness and freshness of selected foods can be carried out due to the film structure could be affected by polymer-water interactions under high moisture conditions.

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