

Research Article

Liposome encapsulation of antimicrobial extracts in pectin film for inhibition of food spoilage microorganisms

Orachorn Mekkerdchoo^{*}, Pasavadee Patipasena and Chaleeda Borompichaichartkul

Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

*Author to whom correspondence should be addressed, email: orachorn_william@hotmail.com

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Abstract

The purpose of this research was to determine a suitable method to encapsulate antimicrobial extracts in liposome based on pectin film to inhibit food spoilage microorganisms. Clove oil, garlic oil and pomegranate extracts were selected to be used in the experiments. Their abilities to inhibit food spoilage microorganisms against *Pseudomonas* sp. ATCC 25619, *E. coli* ATCC 25922, *E. coli* ATCC 8739, *Salmonella* Typhimurium ATCC 23564, *Salmonella* Choleraesuis ATCC 25923, *Lactobacillus* sp. TISTR 539 and *Lactobacillus* sake TISTR 890 was found to be significantly positive. For liposome preparation, critical micelle concentration (CMC) of lecithin to form liposome was 11 % w/w. Then emulsion of antimicrobial extracts was prepared using double emulsion method. Oil phase contained lecithin solution in ethanol and mixture of clove oil and garlic oil (weight ratio of clove oil to garlic oil = 1:1) while water phase contained pomegranate extract. The concentration of lecithin as varied to 12, 14 and 16 % w/w and ratio of lecithin solution to antimicrobial extracts was 1:3, 1:6 and 1:9. Then stability of the emulsions was examined. The results showed that the emulsion prepared with lecithin concentration of 12% and ratio of lecithin solution to antimicrobial extract of 1:6 gave the best stability and good inhibition. All prepared emulsions showed positive result for bacterial inhibition. Physical properties of pectin film were studied at different pectin concentrations (2.5, 3, 3.5 and 4% w/v) mixed with calcium chloride (3, 5, 7 and 10% w/v) and plasticizers (glycerol (GLY) and sorbitol (SOR) at concentration of 40, 50 and 60% of the weight of pectin into film mixture). The results showed that at pectin concentration 4% w/v with calcium chloride 3% w/v and using GLY 50% exhibited better physical properties (TS 19.09 MPa, %E 7.85%, WVP 6.49 $\mu\text{g}/\text{msPa}$) than other treatments. Then liposome with antimicrobial extracts was added into selected film condition at varied concentrations (2, 4 and 6% w/w). Antimicrobial effects of pectin film incorporated with the liposome showed no significant different ($p \leq 0.05$) in bacterial inhibition zone between liposome concentrations except those of 4 and 6 % w/w had highest inhibition zone against *Lactobacillus* sp. TISTR 539 and

Lactobacillus sake TISTR 890 significantly. The physical properties of pectin based film with antimicrobial liposome had following values; TS 18.18-15.25 MPa, %E 11.29-15.04%, WVP 8.55-11.0 $\mu\text{g}/\text{msPa}$, colour differential (ΔE) 8.57-18.81 and film opacity 2.55-6.89 $\text{Au} \times \text{nm}/\mu\text{m}$.

Keywords: liposome encapsulation, antimicrobial extract, edible film, spoilage microorganism, Thailand

Introduction

Food is a rich nutrient matrix that provides a suitable environment for proliferation of spoilage microorganisms such as *Pseudomonas sp.*, Enterobacteriaceae and lactic acid bacteria in meat and meat product [1] and common food-borne pathogens such as *Salmonella enteritidis*, *Escherichia coli* [2]. Microbial contamination reduces the shelf-life of foods and increases the risk of food borne illness, therefore adequate preservation technologies must be applied in order to preserve its safety and quality, Although traditional methods of preserving foods from the effect of microbial growth such as refrigeration that can reduce the growth of microorganisms but cannot kill microorganisms [3, 4]. The consumer demands high quality, convenient, innovative, regular and safe food products with natural flavors and taste as well as an extended shelf-life. Moreover less chemical preserved products are required [5-8] by consumers. It has driven natural antimicrobial compounds towards the investigation of replacing chemical preservatives.

Spices and herb extracts are natural compounds that have been widely studied on their nature antimicrobial activities and found that some example of they have potential for inhibition microorganism in foods [2, 4, 9-11]. Their important phytochemical in those extracts such as Eugenol from clove oil [12] or Allicin in garlic [13]. Moreover, those natural extracts are classed as generally recognized as safe (GRAS) food additives [4, 10, 13-14].

Unfortunately, most natural compounds are biologically instable because they are sensitive to the environment (water, oxygen, light) that destroy their antimicrobial activities [15-16]. Currently, novel methods have been introduced in order to improve their stabilities and their bioavailability, by using encapsulation to prevent reactivity with the environment and minimal compounds loss during storage. Therefore encapsulation provides prolonged antimicrobial activity in the final product by gradually diffusion out of bilayer membrane [17-21]. The benefit of using liposome encapsulation is both of non-polar compound (oil phase such as essential oil) and polar compound (water or ethanol phase) can be encapsulated liposome-multilayer membrane [22]. Furthermore, liposome can be constructed of natural constituents such as lecithin from egg yolk or soy beans and cholesterol so it is non-toxic.

In addition to handle and application in food industry, liposome encapsulation of antimicrobial extracts can be incorporated into pectin film for keeping structure and stability of liposome during storage. Furthermore, the use of edible films in food protection and preservation has recently increased since they offer several advantages over synthetic materials, such as being biodegradable and environmentally friendly [23]. When those film contact with food, moisture from food was induced liposome membrane to slowly release antimicrobial extracts and those extracts will be trapped between food surface and liposome membrane, thus helping maintain high concentrations that make more efficient for inhibition food spoilage and food pathogen microorganisms [5, 24-25]. Moreover, edible film has abilities to retard moisture, oxygen, aromas and solute transportation [26].

The overall objectives of the present research were to determine suitable method to encapsulate antimicrobial extracts in liposome based on pectin film to inhibit food spoilage microorganisms which have potential for food application and be able to develop guideline to apply antimicrobial extracts as natural preservative.

Materials and Methods

Organisms and cultures

Typical food spoilage microorganisms and common food-borne pathogens used in this study were *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* Typhimurium ATCC 23564, *Salmonella* Choleraesuis ATCC 25923, *Pseudomonas sp.* ATCC 25619 (Department of Medical Science, Ministry of Public Health (Nonthaburi, Thailand)) and *Lactobacillus sp.* TISTR 539, *Lactobacillus sake* TISTR 890 (Thailand Institute of Scientific and Technological Research (Pathum Thani, Thailand)). Stock cultures were standardized through 24 hours growth cycles in appropriate broth (nutrient broth (NB, Himedia, India) for *E. coli* ATCC 25922, *E. coli* ATCC 8739, *S. Typhimurium* ATCC 23564, *S. Choleraesuis* ATCC 25923, *Pseudomonas sp.* ATCC 25619 or lactobacilli MRS broth (Himedia, India) for *Lactobacillus sp.* and *L. sake* at anaerobic condition.

Determination of antimicrobial activity of clove oil, garlic oil and pomegranate extracts

Antimicrobial activity was determined by disc diffusion method [27]. The bacteria cultures from standardized culture were grown then inoculated in fresh medium (NB or MRS) at 37°C. After 24 hours of growth, each microorganism was inoculated by sterile cotton swab on the surface of nutrient agar or lactobacilli MRS agar plate. Subsequently, sterile paper discs (Antibiotica-Testblattchen paper disc, Duran, USA) (6 mm in diameter) saturated either with clove oil or garlic oil or pomegranate pericarp extracts (hexane extract, ethyl acetate extract, chloroform extract, ethanol extract and aqueous extract) were placed on surface of each inoculated plate. Standard antibiotic (Tetracycline (Becton, Dickson and Company, USA), Chloramphenicol (Benex Limited, USA)) was simultaneously used as positive control. The plate was incubated at 37°C for 24 h under aerobic conditions except *Lactobacillus sp.* and *L. sake* incubated in an atmosphere enriched in carbon dioxide. After this period, it was possible to observe inhibition zone. The antibacterial activity was evaluated by measuring the inhibition zone diameter observed. The sensitivity to the different antimicrobial solutions was classified by the diameter of the inhibition zone as: not sensitive, diameters less than 0.8 cm; sensitive, diameters 0.9–1.4 cm; very sensitive, diameters 1.5–1.9 cm; and extremely sensitive, diameters larger than 2.0 cm [28]. All analyses were done in duplicate.

Study of ratio of lecithin solution and antimicrobial extracts for liposome preparation by using double emulsion method and screening for the antimicrobial activity of antimicrobial extracts after liposome encapsulation by disc diffusion method

Determination of the critical micelle concentration (CMC) of lecithin solution

Soybean lecithin (phosphatidylcholine >96.4, Merck, Germany) was suspended in water by homogenization at 22,000 rpm for 5 minutes, varied concentration from 0.1-20 % w/w. Surface tension was determined by pendent drop method employing goniometer. Surface tension was calculated by the computer program. After that, a graph of surface tension of lecithin solution and log of concentration of lecithin solution was plotted to determine the CMC point.

Preparation of liposome by double emulsions method

The W1/O/W2 double emulsions were prepared by a modified two-step emulsification form [29]. Oil phase contained lecithin solution in ethanol and mixture of clove oil and garlic oil (weight ratio of clove oil to garlic oil = 1:1). The concentrations of lecithin used were 12, 14 and 16 % w/w. The weight ratios of lecithin solution to oil mixture were 1:2, 1:4 and 1:6. The water phase was solution of pomegranate extract with ethanol. The amount of the pomegranate extract in the water phase was varied in order to obtain weight ratio of lecithin solution to herb extracts (clove oil, garlic oil and pomegranate extract of equal weight) of 1:3, 1:6 and 1:9. Formation of W1/O/W2 double emulsions by lipid mixture solution (O) and aqueous solution (W1) of weight ratio 2:1 was emulsified with a homogenizer (Ystral X10/25, Ballrechten-Dottingen, Netherlands) at 22,000 rpm for 10 minutes and immediately sonicated (1 Hz, 400 w, 30 minutes) with a probe-type sonicator (Dr. Hielscher Up400s, Germany) all process were done in twice times until the mixture became uniform did not separate for at least 30 min after sonication. The primary emulsions were then mixed with aqueous solution (W2; In this study, W1 as the same solution of W2) of 3:4 weight ratio, and emulsified at 18,000 rpm for 10 minutes to form W1/O/W2 double emulsions. The emulsion was extruded three times through 100 nm polycarbonate membranes using an extruderTM (Northern Lipids, Canada) for controlled liposome size. The temperature was kept under 4 °C using a water bath during liposome preparation.

Stability of Liposome emulsion

Emulsion samples were stored in well closed bottles with nitrogen gas and stored in refrigerator at 4±1 °C. Assessment of emulsion was done during everyday storage for seven days. The percentage of separation was calculated and compared with total height of emulsion [30]. All analyses were done in triplicate.

Antimicrobial activity of liposome incorporated of antimicrobial extracts

Antimicrobial activities of liposome incorporation of antimicrobial extracts (clove oil, garlic oil and pomegranate extract) were determined by disc diffusion method [27] against microorganisms (listed in section 1) used in this study. Lecithin solutions (12, 14 and 16 % w/w) were used as negative control. The antibacterial activity was evaluated by measuring observed the inhibition zone diameter. All analyses were done in duplicate.

Study of suitable ratio of pectin and calcium chloride, plasticizer and liposome incorporated of antimicrobial extracts for preparation of pectin film

Preparation of pectin film

Pectin films preparation was modified from [31]. Pectin (2.5, 3, 3.5 and 4 % w/v, Himedia, India) were suspended in distilled water (240 mL) at 90 °C after stirring for 30 min. Then adding warm CaCl₂ solution (3, 5, 7 and 10 % w/v, Ajax Finechem, Australia) at 1 to 3 parts of pectin forming solution. After that the mixture was stirred for 10 min. The solution was degassed and sonicated twice with bath-type sonicator (Ultrasonik, Fisher scientific worldwide, Germany) for 25 min. The solution was put into 45 °C water bath for control temperature. The solutions were casted onto a acrylic plate using film coater (Film coater PI-1210, Japan) followed by drying in an oven at 40 °C until film was dried. All the films were placed on a chamber at 50% ± 5 RH for 24 hours. Determination of the mechanical properties.

*Film characterization*Film thickness

Film thickness was measured by using testing film strips (3 x 15 cm) at nine different positions using a micrometer (Dial Thickness Gauge 7301, Mitutoyo, Tokyo, Japan; 0.01 mm limit). Then all the values were averaged.

Tensile strength (TS) and percentage of elongation at break (%E)

Tensile strength (TS) and percentage of elongation at a break (%E) of the sample film (3 x 15 cm) were measured using a Intron Texture Analyzer (Intron 5565, USA). It was set at an initial distance between the grip of 5 cm and a cross-head velocity of 3 mm/s. TS of the film was calculated by dividing the maximum strength by the initial cross-sectional area. The %E was calculated by dividing the initial distance between the grip from the elongated distance until the time that the film breaks.

Water vapor permeability (WVP)

Water vapor permeability (WVP) of the film was measured according to the ASTM E96-95 (1999).

Colour differences (ΔE) and optical properties

Film colour was determined by a Minolta colorimeter CR 400 Series (Osaka, Japan) calibrated with a standard ($Y = 93.2$, $x = 0.3133$, $y = 0.3192$). The CIE Lab scale was used, lightness (L) and chromaticity parameters a^* (red–green) and b^* (yellow–blue) were measured. Test samples were performed by placing the film samples over the standard and the measurement were taken as the average of five points of each sample. Samples were analyzed in triplicates, recording five measurements for each sample. Colour differences (ΔE) were also calculated by the following equation:

$$\text{colour differences ; } \Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Where $\Delta L^* = L^* - L_0^*$; $\Delta a^* = a^* - a_0^*$; $\Delta b^* = b^* - b_0^*$; L_0^* , a_0^* , b_0^* the colour parameter values of the standard and L^* , a^* , b^* , the colour parameter values of the sample. Film opacity was determined [32]. Film sample was cut into a rectangle and placed on the internal side of a spectrophotometer cell. The absorbance (at 600 nm) was recorded for each sample using spectrophotometer (V-530PC, Japan). The opacity was expressed as absorbance units per nanometers and divided by film thickness ($\text{AU} \times \text{nm/lm}$). The measurement was repeated five times for each treatments of film, and the average value was used.

Microstructure studies by scanning electron microscopy (SEM)

Morphological investigation of the surface and cross section of the films were investigated using a scanning electron microscopy (JSM-5410 LV, Japan). The samples were sputter-coated with gold prior to examine to allow for conductivity.

Effects of the type and plasticizer concentrations on mechanical properties of pectin film

Suitable film conditions was selected in section 4.1 by judging on mechanical properties (clear, strength, flexible and low water vapor permeability (WVP) value). Selected pectin film conditions were prepared as described by 4.1. The two plasticizer are sorbitol (SOR) and glycerol (GLY) varied concentration at 40, 50 and 60 %w/w of pectin were added. All film mechanical properties were determined of the as the method described in section 4.2.

Effect of concentration of antimicrobial extracts in liposome on mechanical properties and antimicrobial activity of pectin film

Selected pectin film conditions from section 4.3 were prepared as described by section 4.1. Liposome was added by varied concentration at 2, 4 and 6 %w/w of film solution and stirring 10 minutes [33]. All film mechanical properties were determined as method listed in section 4.2 and antimicrobial activity of those films was determined by diffusion technique (disc method). Film samples were cut into circle (6 mm in diameter) from different position of film and placed on culture plate. The antibacterial activity was evaluated by measuring the observed inhibition zone diameter.

Results and Discussion

Antimicrobial activity of clove oil, garlic oil and pomegranate extracts

Antimicrobial activities of antimicrobial extracts are presented in Table 1. Clove oil had significantly largest inhibition zone of every tested microorganisms especially *Lactobacillus sake* (4.75 cm). Moreover, it also had activity against tested microorganisms equally or more than standard antibiotic especially against to *Salmonella* Typhimurium, followed by pomegranate extracted by ethanol and garlic oil. The active compound of clove oil is eugenol which is around 93-95% [12]. Its mechanism is distortion of the lipid-protein interaction of bacterial cell membrane and rendering them more permeable or act on bacterial enzyme such as ATPase [10]. Active compounds screening of ethanolic pomegranate extracts are sterol, flavonoid, triterpene, phenol and tannin, they have antimicrobial activity to inhibit *E.coli* O157:H7 [34]. The active compound of garlic oil is allicin (diallyl thiosulfinate), the main antimicrobial effect of allicin is its chemical reaction with thiol groups of various enzymes such as alcohol dehydrogenase, thioredoxin reductase and RNA polymerase in bacteria [35].

Table 1. Inhibition zone of clove oil, garlic oil and pomegranate extracts from ethanol by disc diffusion method. (6 mm in disc diameter).

Microbial strain	Diameter of Inhibition zone ^{1,2}				
	Clove oil	Garlic oil	Pomegranate extracts from ethanol	Tetracycline	Chloramphenical
<i>E. coli</i> ATCC 25922	2.25±0.07 ^b	0.95±0.07 ^d	1.48±0.39 ^c	2.2±0.12 ^b	2.5±0.06 ^a
<i>E. coli</i> ATCC 8739	2.05±0.07 ^b	1.1±0.05 ^d	1.48±0.18 ^c	2.0±0.07 ^b	2.5±0.11 ^a
<i>Salmonella</i> Typhimurium	3.0±0.21 ^a	1.5±0.12 ^d	1.33±0.24 ^c	2.2±0.07 ^c	2.6±0.05 ^b
<i>Salmonella</i> Choleraesuis	2.75±0.35 ^b	1.20±0.14 ^c	1.73±0.6 ^d	2.4±0.13 ^c	2.9±0.09 ^a
<i>Pseudomonas</i> sp.	2.33±0.21 ^a	1.1±0.14 ^c	1.8±0.08 ^b	N/A	N/A
<i>Lactobacillus</i> sp.	3.38±0.17 ^a	1.46±0.07 ^c	1.56±0.05 ^b	N/A	N/A
<i>Lactobacillus</i> sake	4.75±0.21 ^a	1.50±0.15 ^c	2.13±0.08 ^b	N/A	N/A

- : no inhibition; N/A: Not detected

¹ The average of diameter of Inhibition zone ± standard error

² Different letters within the same row and for the same microbial strain significant different ($p \leq 0.05$)

Study of ratio of lecithin solution and antimicrobial extracts for liposome preparation by using double emulsion method and screening for the antimicrobial activity of antimicrobial extracts after liposome encapsulation by disc diffusion method.

Critical micelle concentration (CMC) of lecithin solution

Critical micelle concentration (CMC) is minimum concentration of an emulsifier (amphiphilic component) in solution at which micelles in the solution are formed. CMC is a key parameter for the optimization of emulsifier in liposome formulations. For liposome preparation, emulsifier concentration for liposome preparation must be more than minimum concentration of emulsifier that can encapsulate antimicrobial extracts. Figure 1 shows a plot of lecithin solution and log of concentration of lecithin solution. In the plot, CMC was determined around 11 % w/w. Therefore, selected concentration of lecithin solution for preparation of liposome incorporated with antimicrobial extracts were 12, 14 and 16 % w/w for further study.

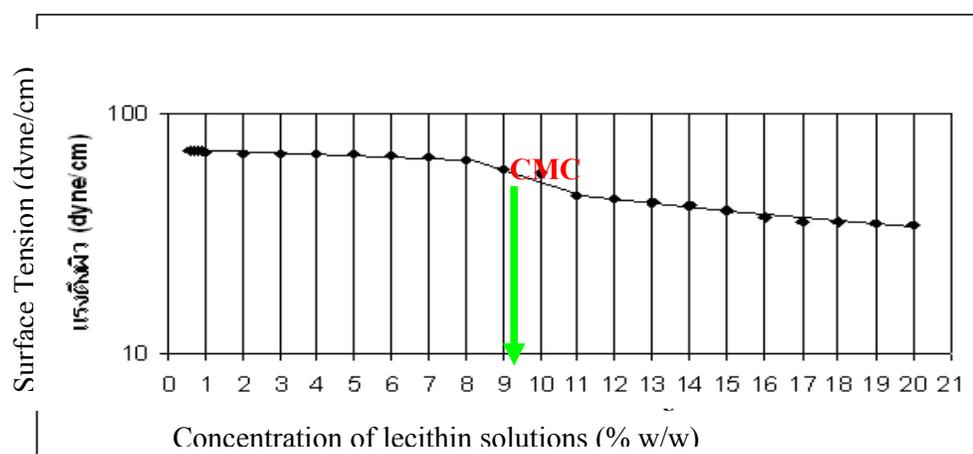


Figure 1. Surface tension and log of concentration of lecithin solution between 0.1- 20% w/w.

Storage stability and antimicrobial activity of liposome incorporated of antimicrobial extracts of liposome emulsion

After extrusion through membrane, liposome emulsion was stored at 4°C to study the storage stability. It was noticed that the separation of emulsion could be divided into (1) aqueous-emulsion (2) oil-emulsion (3) aqueous-oil-emulsion. The separation was much evidence when storage time was increased. On other hands when increasing concentration of lecithin solution, the separation was slow. However, when concentration of antimicrobial extracts was increased, that induced the separation.

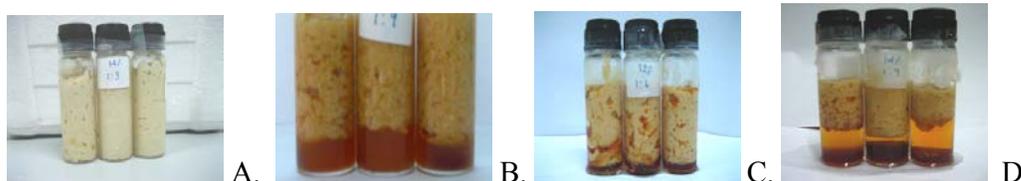


Figure 2. Type separation of emulsion; emulsion cream (A), aqueous separate and emulsion cream (B), oil separate and emulsion cream (C) and aqueous separate, oil separate and emulsion cream (D).

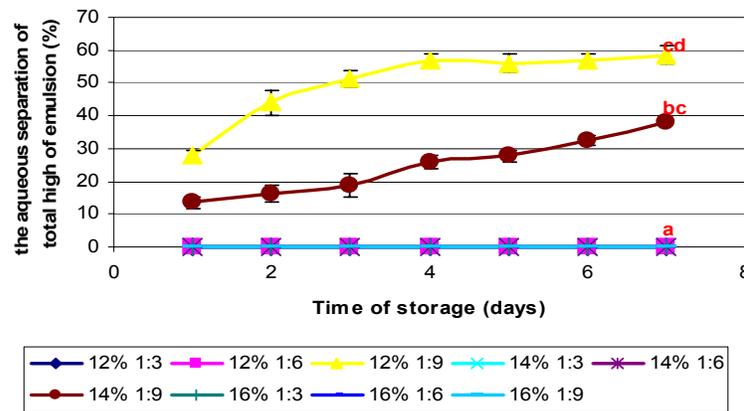


Figure 3. Percentage of aqueous separation which different concentration of lecithin solutions and different ratio of lecithin solutions to antimicrobial extracts, when stored at 4°C for seven days.

Different letters within different line and for the same day, only 7th days significant different ($p \leq 0.05$)

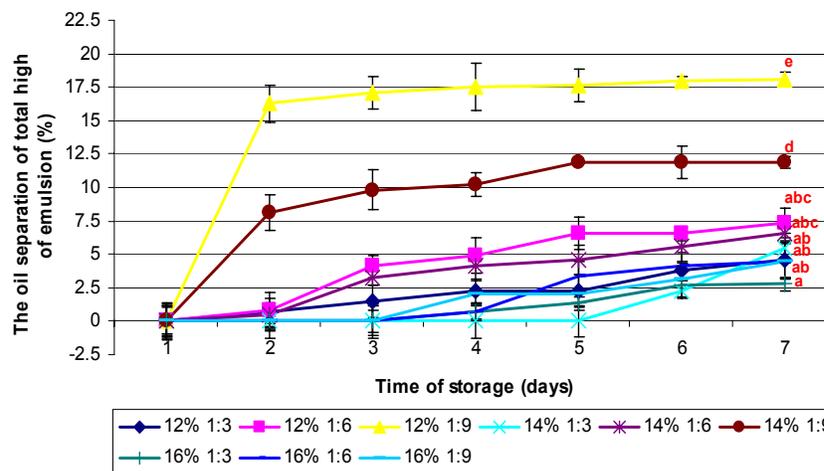


Figure 4. Percentage of oil separation which different concentration of lecithin solutions and different ratio of lecithin solutions to antimicrobial extracts, when stored at 4°C for seven days.

Different letters within different line and for the same day, only 7th days significant different ($p \leq 0.05$)

During extrusion, heat and pressure occurred at the membrane surface that practically made liposome broken and release antimicrobial extracts therefore emulsions will be separate after extrusion [36].

The result showed the separation of emulsion most induced (fast and most separation) when concentration of antimicrobial extracts were highest while concentration of lecithin solution was lowest. During the storage, liposome will become more heterogeneous after that liposome may fuse or aggregate, thus liposome became bigger. Moreover, the lipids used in liposome formation can become oxidized or hydrolyzed, resulting in bilayer permeability changes that induced to leakage of liposome. From Fig. 3-4, ratio of lecithin solution to antimicrobial extracts at 1:3 was good form of emulsion.

Table 2. Inhibition zone of prepared emulsions incorporated of antimicrobial extracts by disc diffusion method.

Concentration of lecithin solutions (% w/w)	Weight ratio of lecithin solutions to antimicrobial extracts	Diameter of Inhibition zone (cm)					
		<i>E. coli</i> ATCC 25922	<i>E. coli</i> ATCC 8739	<i>Salmonella</i> Typhimurium	<i>Pseudomonas</i> sp.	<i>Lactobacillus</i> sp.	<i>Lactobacillus</i> sake
12	1 : 3	2.1±0.07 ^{bc}	1.8±0.15 ^{ns}	2.2±0.05 ^b	2.3±0.11 ^{bc}	1.9±0.05 ^a	1.9±0.05 ^c
	1 : 6	2.3±0.08 ^{cd}	1.8±0.07 ^{ns}	2.5±0.12 ^c	2.9±0.08 ^d	2.3±0.15 ^c	2.4±0.05 ^d
	1 : 9	1.9±0.2 ^b	1.9±0.2 ^{ns}	2.5±0.09 ^c	1.5±0.07 ^a	2.05±0.07 ^b	1.58±0.12 ^{bc}
14	1 : 3	2.3±0.13 ^{cd}	1.9±0.11 ^{ns}	1.7±0.07 ^a	2.0±0.05 ^{ab}	2.0±0.11 ^{ab}	1.7±0.09 ^{bc}
	1 : 6	2.2±0.07 ^{cd}	1.6±0.2 ^{ns}	2.2±0.2 ^b	3.0±0.13 ^d	2.2±0.14 ^c	2.1±0.17 ^c
	1 : 9	2.2±0.2 ^{cd}	1.7±0.1 ^{ns}	2.2±0.14 ^b	1.8±0.2 ^{ab}	1.8±0.1 ^a	1.5±0.05 ^b
16	1 : 3	2.7±0.12 ^e	1.9±0.23 ^{ns}	2.8±0.14 ^d	2.8±0.15 ^{cd}	2.2±0.08 ^c	1.17±0.08 ^a
	1 : 6	2.7±0.18 ^e	1.8±0.18 ^{ns}	2.8±0.16 ^d	3±0.17 ^d	2.7±0.12 ^d	1.5±0.06 ^b
	1 : 9	2.4±0.2 ^{cd}	1.6±0.25 ^{ns}	2.6±0.13 ^{cd}	2.7±0.14 ^{cd}	2.7±0.21 ^d	1.33±0.11 ^{ab}

Different letters within the same column and for the same microbial strain significant different ($p \leq 0.05$)

The antimicrobial properties of liposome emulsion are shown in Table 2. All emulsions showed positive result for bacterial inhibition as the inhibition zones more than 0.8 cm that means microorganisms were sensitive to antimicrobial substrates. The highest antimicrobial activity of emulsions was ratio of lecithin solution to antimicrobial extracts 1:6 and inhibition power of bacteria will be increasing when the ratio of antimicrobial extracts were increasing. Except ratio 1:9 as this ratio experience more separation than other emulsions therefore less of antimicrobial extracts were encapsulated. However, when concentration of lecithin solution was increased, inhibition power will be increasing because emulsions had more.

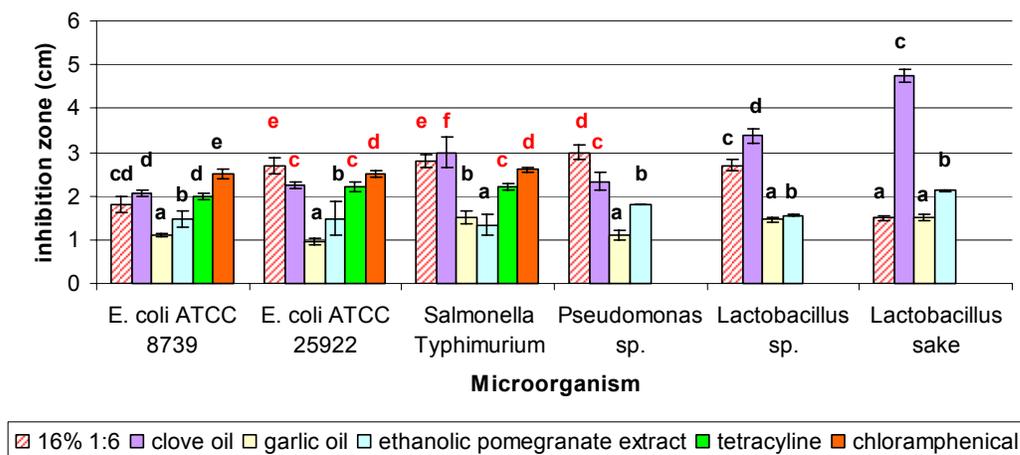


Figure 5. Inhibition zone of prepared emulsions at concentration of lecithin solution 16 %w/w and ratio of lecithin solution to antimicrobial extracts 1:6 and natural antimicrobial extracts without separation.

Means within the same column with same letter are not significantly different ($p \leq 0.05$)

When compared inhibition zone between prepared emulsion (concentration of lecithin solution 16 %w/w and ratio of lecithin solution to antimicrobial extracts 1:6) and natural antimicrobial extracts without encapsulated (Figure 5). Result shows that emulsions enhanced antimicrobial

activities after encapsulation more than using single antimicrobial extract without encapsulation. Moreover, when compared with antibiotic, emulsions had activity to against tested microorganisms over standard antibiotic especially *E.coli* ATCC 25922, *Salmonella* Typhimurium significantly. Therefore, the combination of three antimicrobial extracts together was more effective to inhibit bacteria. Liolios et al. (2009) studied liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and antimicrobial activity was founded to increase after encapsulation. Liposome can interact with bacteria cells in many ways for example inter-membrane transfer, contact release, absorption, fusion and phagocytosis. The mechanism of interaction depends on cell type (cell wall/membrane composition), as well as the liposome membrane physicochemical characteristics. The use of liposome improves the cellular transport and releases the active component in side cell that the dramatically increased antimicrobial activity, after liposome encapsulation [37].

From the experimental, the results showed that the emulsion prepared with lecithin concentration of 12 %w/w and ratio of lecithin solution to antimicrobial extract of 1:6 gave the best stability, good inhibition and less using of lecithin.

Study of suitable ratio of pectin and calcium chloride, plasticizer and liposome incorporated of antimicrobial extracts for preparation of pectin film.

Physical properties of pectin film

Pectin film has film thickness varied from 0.02-0.045 mm. The increasing of thickness due concentration of pectin and calcium chloride were increased as film thickness depend on solute solid in film forming solution [38]. TS is a measure of film strength [39], TS of films varied from 4.93 to 46.875 MPa. When pectin concentration increased enhanced TS while CaCl₂ concentration increased reduced TS significantly because pectin is gelling agent [40] so when pectin increased, interaction between intermolecular chains will be increased and film is stronger. SEM (Figure 7) showed more cracks while CaCl₂ increased because it created many junction zones and pinholes that made film strength reduction. In addition, excess calcium salt left in the films would interfere with the measurement of cross-linked calcium and may also alter the mechanical properties of the film [41]. %E is a measure of film stretch ability prior to breakage that showed how much film flexibility. %E was varied from 3.74% to 12.99 %, when concentration of pectin decreased while concentration of CaCl₂ increased that increased %E significantly because CaCl₂ is elastically active chain [42]. Moreover, when considering film's morphology, it was found that film structure was less dense when CaCl₂ increased. Generally, the strength and flexibility of films are negatively correlated [31]. WVP is a measure of ease of moisture to penetrate and pass through a material. WVP varied from 1.256 to 11.458 μg/m s Pa. WVP values significant increased when Pectin and CaCl₂ increased because pectin film is hydrophilic film, what could be related with a higher number of free hydroxyl group that enhanced interactions with water favoring water vapor transmission through the films [43]. Color of the film may influence the consumer acceptability of a product [44] that was observed by reading ΔE. Pectin films appeared clear and transparent. Experiments showed that ΔE varied from 0.017 to 0.045 did not different significantly when pectin and CaCl₂ increased, pectin films had the greatest L*-value but lowest a*- and b*-values. It is generally know that ΔE values less than 3.0 cannot be detected by human eyes [45]. When compared ΔE values with another film that was founded pectin film had ΔE lower than those of egg albumin films (1.7-2.3) [46], HPMC mixture films (chitosan/ hydroxypropyl methylcellulose; 0.52-1.18) [47]. film opacity varied from 0.921 to 2.99 Au × nm/μm, film opacity values significant decreased when CaCl₂ increased. The obtained results indicated that films based on pectin showed closed to values to

the other polyol-plasticized films and some commonly used synthetic films such as low-density polyethylene (3.05 A600/mm) and oriented polypropylene (1.67 A600/mm) [48].

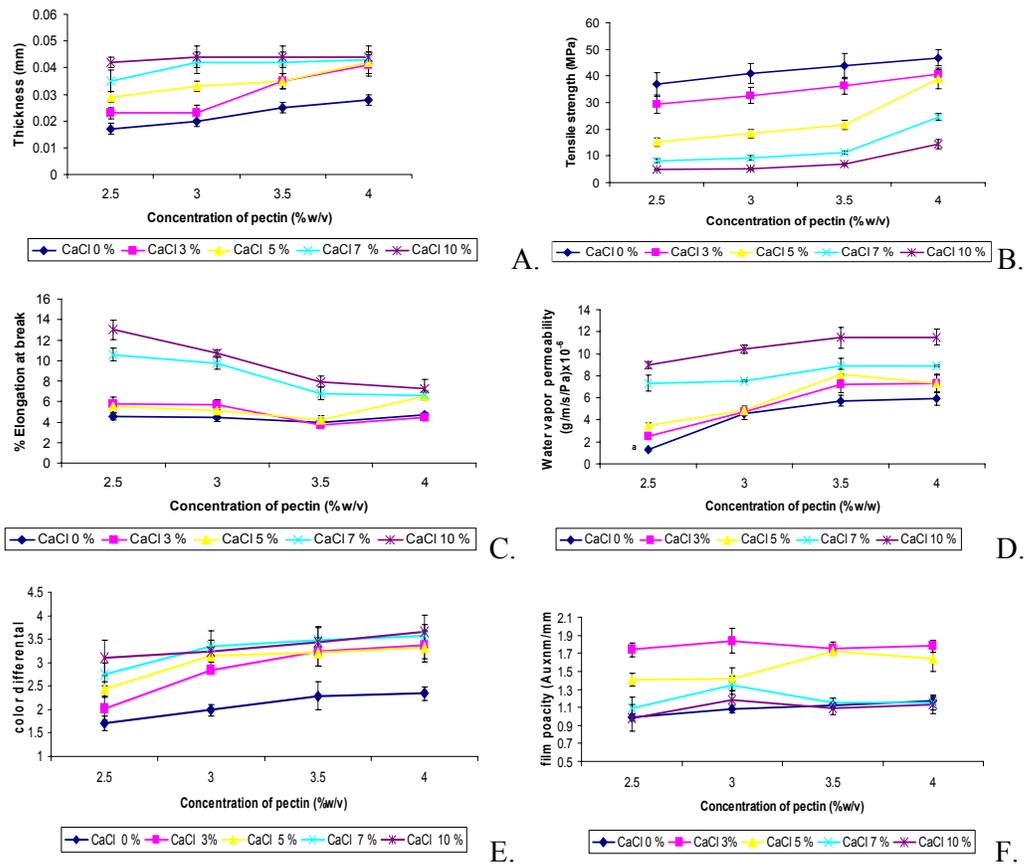


Figure 6. Physical properties of pectin films: thickness (A), TS (B), %E (C), WVP (D), ΔE (E) and film opacity (F).

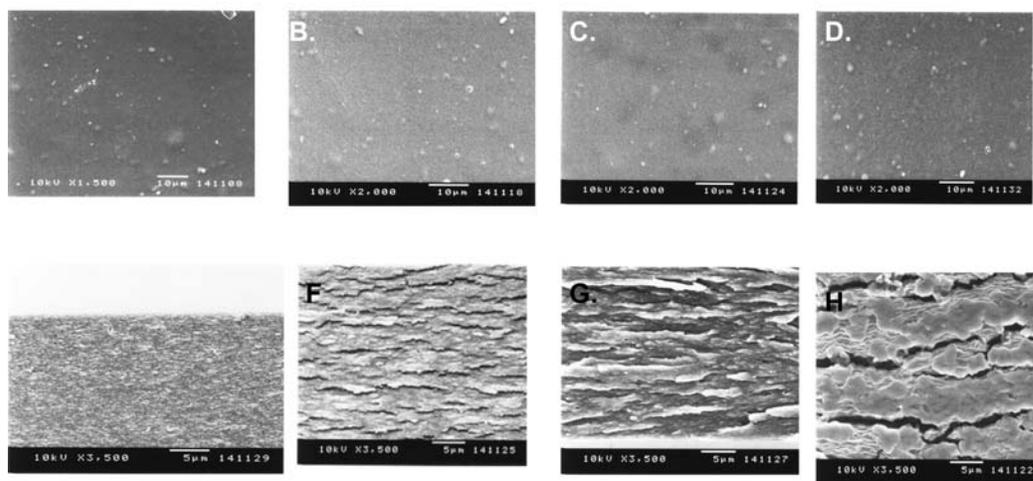


Figure 7. The surface (A,B,C, D) and cross-section (E,F,G,H) of pectin films at concentration of pectin 3.5%(w/v) with CaCl₂ 3,5,7,10%(w/v).

The results showed that pectin concentration 3% w/v with calcium chloride 3% w/v and pectin concentration 4% w/v without or 3% w/v calcium chloride exhibited better physical comparing to other film mixtures.

Effects of the type and plasticizer concentrations on mechanical properties of pectin film

Selected pectin films from section 3.1 were incorporated with sorbitol (SOR) and gercerol (GLY) at varied concentration at 40, 50 and 60 %w/w of pectin. The mechanical properties of films are showed in Figure 8. The results indicated that these properties varied with the natural of plasticizers used. The thickness of films varied from 0.03-0.048 mm. Glycerol-plasticized films was thinner than sorbitol-plasticized film significantly because glycerol has lower molecular weight (92.09) than sorbitol (182.2). Therefore, the amount of glycerol is always less than sorbitol at same level of ratio to pectin [49]. Moreover, film thickness depend on solute solid in film forming solution so all solid in film were pectin, CaCl_2 and plasticizer [38] so plasticized film were thicker than non- plasticized film significantly, glycerol-plasticized films more thin than sorbitol due to their lowest solid content [49]. Both of glycerol and sorbitol had water sorption properties that made film thicker [50]. TS varied from 14.29 to 26.44 MPa and decreased significant as concentration of plasticizer increased, TS in films plasticized by sorbitol was significantly larger than in plasticized by glycerol at the same concentration due to plasticizer molecules bind with water molecules and shield active centres along polymer chains, thereby decreasing intermolecular interaction [50] and plasticizers in this studied is polyol that have hydroxyl group which is open chain that make competition between plasticizer and pectin for calcium. Since polyol plasticizers and calcium from a complex, plasticizer interfered with the formation of junction zones. Therefore, junction zones are not so dense and reduced polymer-polymer interaction that effected to TS decreasing [51] and films plasticized with glycerol, which as a molecular weight (MW) of 92, had about twice higher mol content than that of sorbitol (MW 182) plasticized films [52] and plasticizer with lower MW more film plasticization than higher MW [49]. %E varied from 5.6 to 12.69% and decreased significant as concentration of plasticizer increased, glycerol-plasticized films had significantly larger %E than in plasticized by glycerol at the same concentration because plasticizer weakened intermolecular forces between adjacent polymer chains and increased free volume and chain mobility that made %E was increased [52]. The reason for glycerol-plasticized films had larger %E is glycerol hydroscopic character that tends to provide additional water into the film matrix and the matrix of the film become less dense and under stress movement of polymer chains were facilitated so increased free volume and chain mobility[53]. WVP varied from 4.58 to 12.3 $\mu\text{g}/\text{m s Pa}$. The result showed that WVP increased as plasticizer concentration increased, the films plasticized by glycerol had significantly lager WVP values than film plasticized by sorbitol at same concentration. The addition of plasticizer reduced the cohesive forces between intermolecular chain and increased free volume and segmental motions enhance water molecules diffused more easily and higher WVP resulted [46]. glycerol had significantly lager WVP values than film plasticized by sorbitol due to glycerol attributed higher hygroscopicity relative therefore possess a higher capacity to absorb water from environment than sorbitol [53]. Moreover, film morphology (SEM, Figure 9) of sorbitol-plasticized films showed more compact and denser structure than glycerol-plasticized films so sorbitol significantly decreased film WVP compared with glycerol [52]. Experiments showed that ΔE varied from 1.59 to 2.76 and did not different significantly between film plasticized by sorbitol or glycerol but ΔE were reduced when compared with un-plasticizer films because added plasticizer had clear, glossy and white that can be reduced ΔE values. Film opacity varied from 1.22 to 1.73 $\text{Au} \times \text{nm}/\mu\text{m}$ and did not different significantly between film plasticized by sorbitol or glycerol but film opacity values significant deceased when compared with non-plasticizer films.

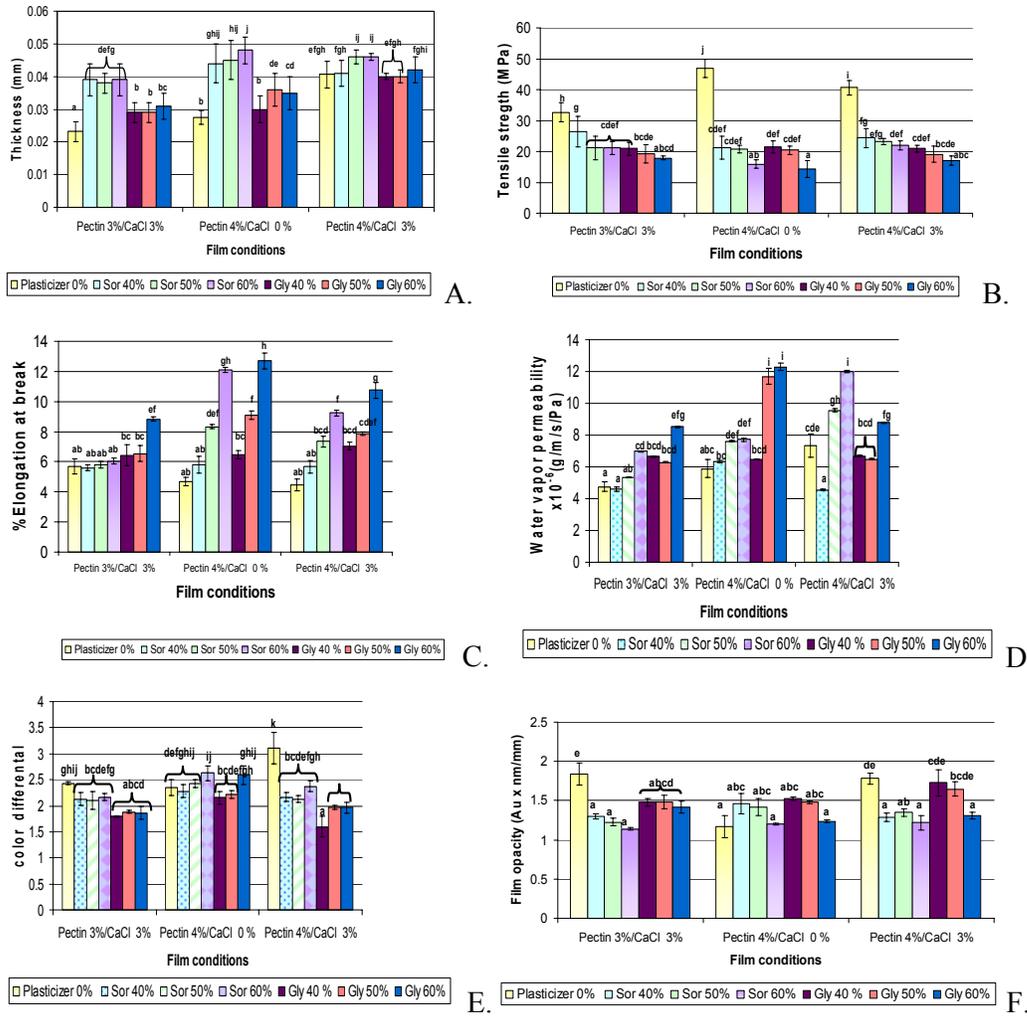


Figure 8. Physical properties of pectin plasticizer films: thickness (A), TS (B), %E (C), WVP (D), ΔE (E) and film opacity (F).

Means within the same column with same letter are not significantly different ($p \leq 0.05$)

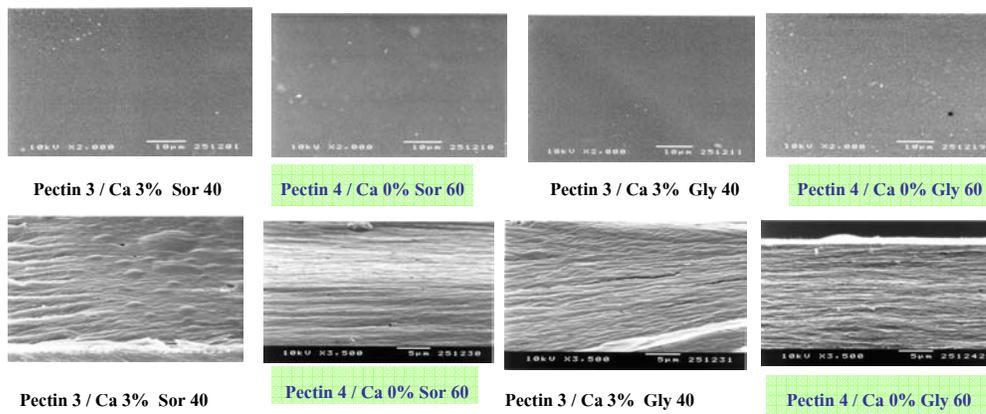


Figure 9. The surface and cross-section of pectin films plasticized by sorbitol and glycerol.

From experiment that showed sorbitol-plasticized films had thickness and TS values more than glycerol-plasticized while film plasticized by glycerol had %E and WVP values more than film plasticized by sorbitol at same concentration. The results showed that at pectin concentration 4% w/v with calcium chloride 3% w/v and using GLY 50% exhibited better physical properties than other treatments.

Effect of concentration of antimicrobial extracts in liposome on mechanical properties and antimicrobial activity of pectin film

Mechanical properties

Liposome was added into selected film condition at varied concentration (2, 4 and 6% w/w). It was showed the effect of different concentrations of liposome incorporated into pectin film and the resultant change in the properties. The mechanical properties of films are showed in Figure 10. The thicknesses of films varied from 0.052 to 0.053 mm. and did not different significantly between liposome concentrations but thickness values significant increased when compared with control films. This increase might be due to hydrophilic group of pectin interaction favoured by liposome (hydrophilic group outside) in film-forming solution and tent to form larger clusters in the film matrix that might be exhibited higher thickness [53]. TS varied from 15.521 to 18.18 MPa. Incorporation of liposome that encapsulated antimicrobial extracts had effect to TS of film, as seen in the reduced TS values when compared with control film but concentration of liposome did not different significantly for TS values. It is the reason of reduced TS values because of the presence oils in liposome probably interferes with ionic interactions facilitated by Ca ions that caused a greater reduction of TS [54] or liposome may weaken the strong interactions between pectin molecules [53]. Generally, incorporation of additives other that make lower TS values [55]. On the other hand, incorporation of liposome was creased %E when concentration of liposome increased almost two times that showed liposome had plasticizer properties. However, addition of liposome higher than 4%w/w reduced %E value. This is due to non-homogenous distribution of liposome that made aggregates within the final film structure [56]. WVP varied from 8.55 to 11 $\mu\text{g}/\text{m s Pa}$. Incorporation of liposome affected the WVP of pectin films. WVP value tended to increase as higher concentration of liposome was incorporated. It is probably occurred due to antimicrobial extracts might contribute to extend intermolecular interactions of the structural matrix in pectin film. Therefore, it enhanced moisture passing through the film [54]. In addition, WVP depend on hydrophilic part and ration of hydrophilic: hydrophobic in film [57] therefore in film that corporate liposome which had continuous phase was water (hydrophilic) so that increased hydrophilic part of film that result to WVP values were increased. When considered film morphology (SEM, Figure 11) of liposome incorporated in pectin film that was found film structure had less dense and porous structure so WVP values were increased. However, pectin film with liposome still had lower WVP values when compared with other films such as whey protein isolated film (116 $\text{g}/\text{m s Pa}$) [58], gelatin film (16.9 $\text{g}/\text{m s Pa}$) [59], chitosan film (117 $\text{g}/\text{m s Pa}$) [60] and pomace extract in low methoxyl film (73.2 $\text{g}/\text{m s Pa}$) [52]. Addition of liposome affected the appearance of edible film in both colour and film opacity. ΔE varied from 8.57 to 18.81 and increased significantly as liposome adds more higher. The colour tended to yellowish as indicated by the increase of b value and L values decreased. The b value produced by the incorporation of liposome that had garlic oil and pomegranate extracts encapsulated and L values decreased so it indicates that the colour of the edible film tends to darken. Film opacity varied from 2.55 to 6.89 $\text{Au} \times \text{nm}/\mu\text{m}$. Film opacity values significant increased when liposome concentration increased. Mostly, film transparency was reduced as the antimicrobial agents were incorporated [54, 61].

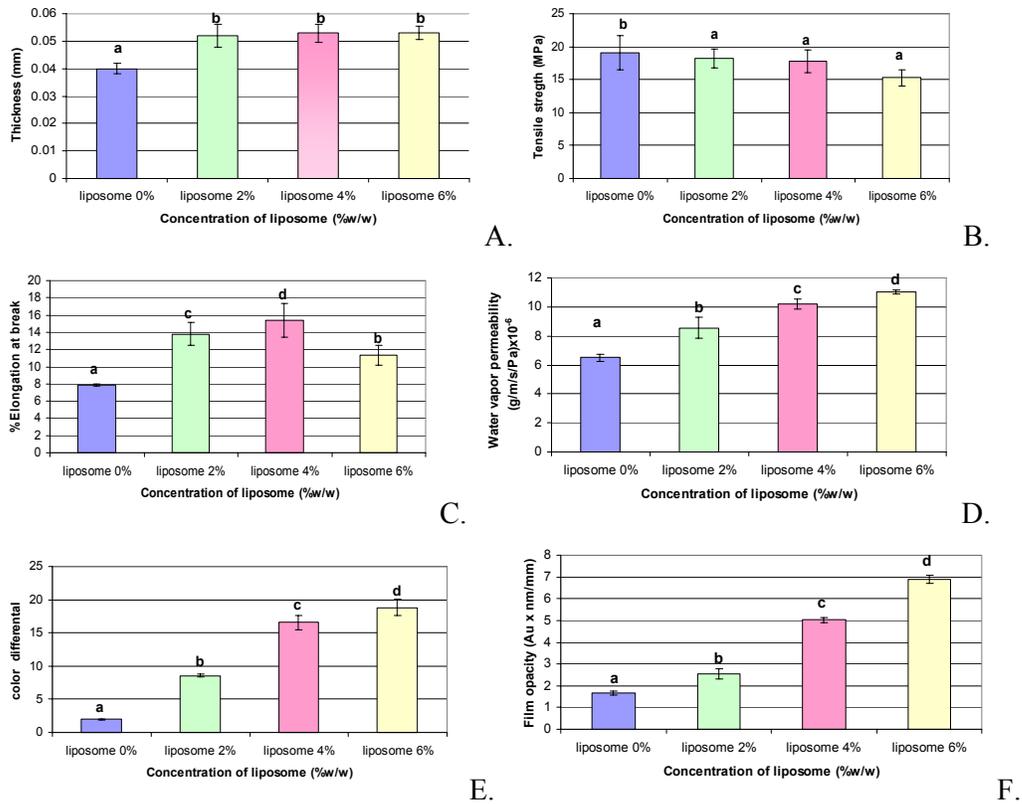


Figure 10. Physical properties of pectin films incorporated of liposome: thickness (A), TS (B), %E (C), WVP (D), ΔE (E) and film opacity (F). Means within the same column with same letter are not significantly different ($p \leq 0.05$).

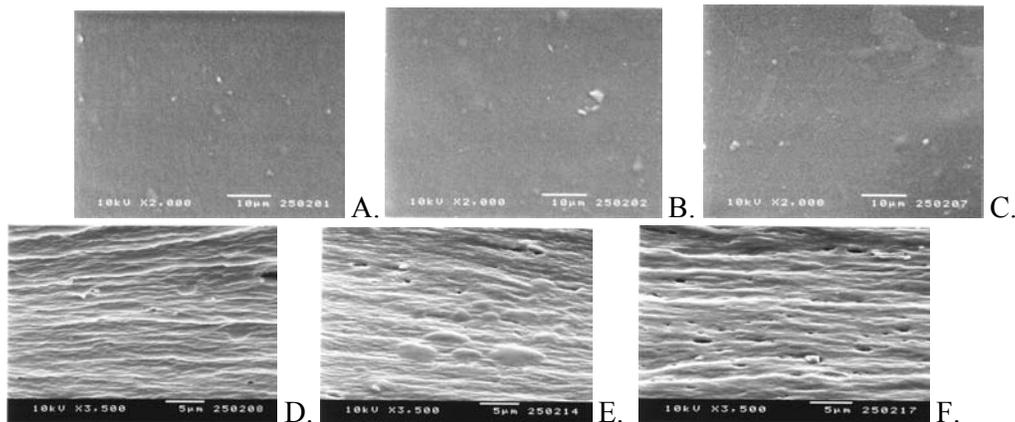


Figure 11. The surface (A,B,C) and cross-section (D,E,F) of pectin films incorporated liposome at varied concentration 2, 4 and 6% w/w respectively.

The result showed that TS of the film decreased while the %E, WVP, ΔE and film opacity increased when compared to the control, the pectin film incorporated with 4% w/w liposome had highest %E and the other of physical properties were acceptable.

Antimicrobial activity

The results of the antimicrobial assessment of pectin film incorporated with antimicrobial liposome bacteria are presented in Figure 12. The bacteria selected here are commonly associated with food spoilage and food borne pathogen. The result showed antimicrobial effects of pectin film incorporated with the liposome had no significant difference. Except those of 4 and 6 % w/w had highest inhibition zone against *Lactobacillus sp.* and *Lactobacillus sake* significantly.

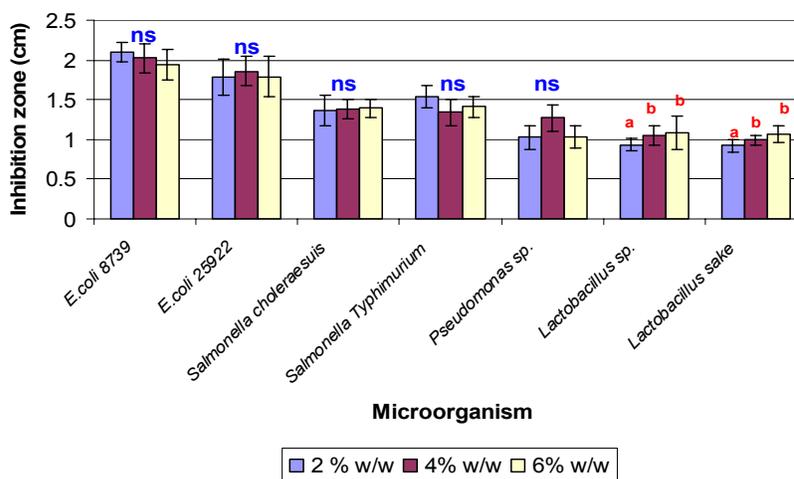


Figure 12. Inhibition zone of pectin film incorporated with the liposome by disc diffusion method

Means within the same column with same letter are not significantly different ($p \leq 0.05$)

Inhibition zone of pectin film incorporated with the liposome had larger than 0.8 cm that mean microorganism is sensitive to antimicrobial extracts [28]. This films showed most inhibition in *E. coli* and secondly in *Salmonella sp.* significantly. However, increasing level of antimicrobial liposome at higher concentration did not reveal significantly an inhibitory of *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella Typhimurium* ATCC 23564, *Salmonella Choleraesuis* ATCC 25923, *Pseudomonas sp.* ATCC 25619. It was generally caused by the maximum capability of polymer to carry active agents beside the occurrence of functional groups interaction phenomenon that made active compound can not be released out of polymer [62]. Generally, antimicrobial activity always is higher when antimicrobial concentration increased [63]. These results prove that liposome could be immobilized in the pectin film and subsequently released, thereby inhibiting target microorganisms.

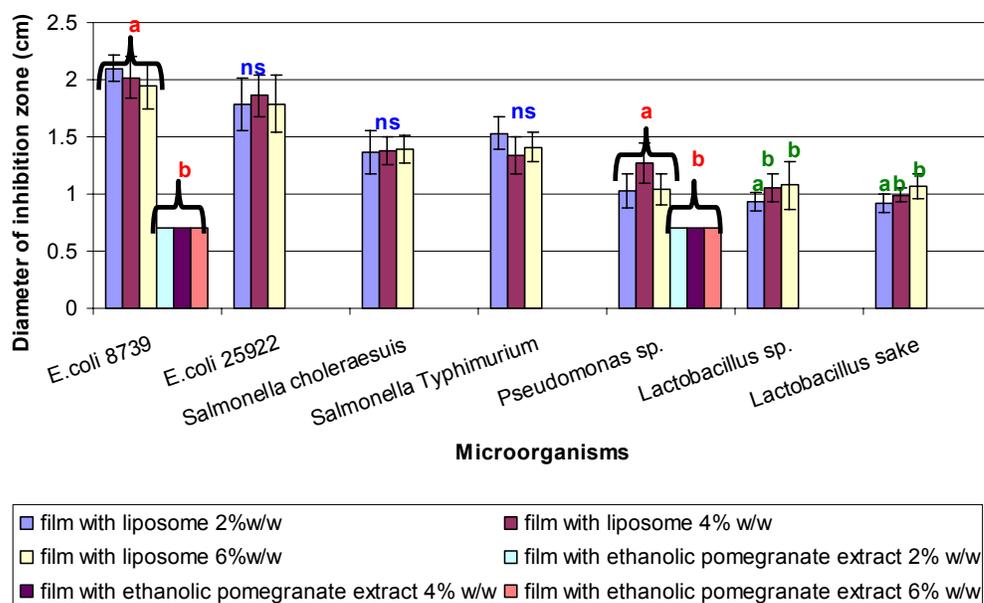


Figure 13. Inhibition zone of prepared of pectin film incorporated with the liposome and film incorporated pomegranate extracted from ethanol at same concentration.

Means within the same column with same letter are not significantly different ($p \leq 0.05$).

When compared of pectin film incorporated with the liposome and film incorporated with clove oil or garlic oil or pomegranate extracts at same concentration showed in Figure 13. It was found that film incorporated with clove oil or garlic oil, the inhibition zone of was not observed with tested bacteria. However, incorporation of pomegranate extracts revealed a weak inhibitory effect, indicated by minimal growth underneath film discs was 0.7 cm that less than 0.8 cm. It means microorganisms were not sensitive to antimicrobial film [28]. Therefore, film incorporated with antimicrobial extracts without encapsulation had antimicrobial activity less than antimicrobial extracts encapsulated before incorporate in film. This due to lost of antimicrobial extracts between film preparation or drying [64] especially in edible films are enriched with essential oils, the drying temperatures usually were employed to form the edible coating are high enough to volatilize a high percentage of the active aromatic components in essential oil [65].

Conclusion

From the experiment it was found that pectin concentration 4% w/v with calcium chloride 3% w/v and using GLY 50% was good film to use for incorporating liposome. The incorporation of antimicrobial liposome into pectin film led to moderate changes in pectin film physical properties. When testing on antimicrobial activity, it was founded that microorganism is sensitive to extremely sensitive to pectin film incorporated with the liposome. Pectin film incorporated with 4% w/w liposome was selected due to it high antimicrobial activity that gave large inhibition zone significantly to lactic acid bacteria and the other of physical properties were acceptable. Therefore, an antibacterial pectin film incorporated with the liposome is promising and has good potential in many food applications.

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