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ANTIBACTERIAL PROPERTIES OF CHITOSAN EDIBLE FILMS INCORPORATED WITH MUSK LIME EXTRACTS FOR THE PRESERVATION OF SQUIDS

(Sifat Antibakteria Filem Kitosan Boleh Dimakan Yang Ditambah Dengan Ekstrak Limau Kasturi Bagi Pengawetan Sotong)

Choong Kah Wai[1], Tee Yee Bond[2], Nyam Kar Lin[1], Pui Liew Phing[1]*

[1]*Department of Food Science and Nutrition, Faculty of Applied Sciences,*
UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia
[2]*Centre for Pre-University Studies,*
Stella Maris International School,
Lorong Setiabistari 2, Medan Damansara, 50490 Kuala Lumpur, Malaysia.

[*]*Corresponding author: puilp@ucsiuniversity.edu.my*

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Abstract

The chitosan (CH) films incorporated with different concentrations (1% to 5%) of musk lime extracts (MLE) were investigated for physical, mechanical, chemical, and antibacterial properties. Storage test on squids' shelf-life was also conducted. The addition of MLE into CH film had increased the inhibition zones against gram-negative bacteria, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*. It also reduced the moisture content, water solubility, tensile strength and elongation at break. Films with higher concentration of MLE had higher intensity in opacity and appeared more greenish. From the Fourier transforms infrared (FTIR) spectra, the addition of 4% and 5% of MLE in CH films shifted the $-CH_3$ stretching at peak 2921 cm^{-1} and the signal of the hydrophilic representative zones (-OH and –NH stretch, amide II and carboxyl group) were slightly reduced at 3200 cm^{-1}, 1570 cm^{-1} and 1400 cm^{-1}, respectively. The FTIR results did not suggest any cross-linkage between the MLE and CH. At 4 °C storage condition, wrapping the squids with MLE incorporated films had prolonged their shelf-life from 5 days to 15 days compared to wrapping with neat CH film.

Keywords: edible film, shelf-life, antibacterial properties, chitosan, musk lime

Abstrak

Filem kitosan (CH) yang ditambah dengan ekstrak limau kasturi (MLE) dalam kepekatan yang berbeza (1% hingga 5%) telah disiasat untuk sifat fizikal, mekanikal, kimia, dan antibakteria. Ujian hayat makanan ke atas sotong juga dijalankan. Penambahan MLE ke dalam filem CH telah meningkatkan zon perencatan terhadap bakteria gram-negatif, *Pseudomonas aeruginosa* dan *Vibrio parahaemolyticus*. Ia juga mengurangkan kandungan kelembapan, keterlarutan air, kekuatan tegangan dan pemanjangan. Filem dengan kepekatan MLE yang lebih tinggi mempunyai intensiti yang lebih tinggi dalam kelegapan dan kelihatan lebih hijau. Spektrum inframerah transformasi Fourier (FTIR) menunjukan bahawa penambahan 4% dan 5% MLE menyebabkan peralihan regangan $-CH_3$ di puncak 2921 cm^{-1} dan isyarat wakil zon hidrofilik (-OH dan -NH, amida II dan kumpulan karboksil) dikurangkan sedikit masing-masing pada 3200 cm^{-1}, 1570 cm^{-1} dan 1400 cm^{-1}. Keputusan FTIR tidak menunjukkan sebarang hubungan silang antara MLE dan CH. Pada suhu penyimpanan 4 °C, pembungkusan sotong dengan filem yang ditambah dengan MLE didapati menambah jangka hayat sotong dari 5 hari kepada 15 hari, jika dibandingan dengan filem CH yang dibalut dengan kemas.

Kata kunci: filem yang boleh dimakan, jangka hayat, sifat antibakteria, kitosan, limau kasturi

Introduction

Food packaging had been designed as a medium of transportation and containment for food products since the 19th centuries [1]. There is an increase in demand for eco-friendlier and safer packaging materials, mainly for those free from chemical preservatives and packaging with natural antimicrobial compounds [2]. Several technologies were utilized in packaging to prevent food from microbial and chemical hazards. These include active, intelligent and edible packaging catered to various of food products such as oxygen scavengers for high lipid food, gas-sensing devices as indicator for the maturity of fruits and quince seed film with thymol and oregano essential oil to pack fish products [3, 4].

Among the technologies, edible packaging is one of the most practical and useful packaging technologies [5]. There is a great potential for edible spices packaging to be applied on meat products by improving their flavor, nutritional value and shelf-life [6]. Some studies had showed the use of chitosan film as an effective medium to deliver bioactive compounds into food components [7, 8]. Compared to the traditional method of marinating with spices, the delivery of bioactive components was not as efficient because of the direct interaction and thus, oxidation of bioactive components with food samples [9]. Muppalla et al. [10] reported that the addition of clove essential oil (2%) into carboxymethyl cellulose films and wrapped onto chicken meat could extend its shelf-life from 4 days to 12 days. Emiroğlu et al. [11] reported that the wrapping of beef patties with soy edible film incorporated with 5% of thyme and oregano essential oil reduced the growth of coliforms such as *Escherichia coli* and *Pseudomonas spp*.

Musk lime is one of the most frequently used spices and fruits in Southeast Asia [12]. Its pulp was generally used as flavouring in food and beverages for steaks, desserts and drinks [13]. Although the seeds and peels contained nutritious components such as unsaturated fatty acid and other bioactive compound, they were not fully utilized by the industries [14, 15]. To our best knowledge, there had not been any studies done on the use of extracts from the musk lime (seeds, peels and pulps) in edible films.

Squids had relatively short shelf-life due to high contents of amino acids [16]. Even in ice storage at 0 °C, the squids were reported for rejection and spoilage at around 10 days to 14 days [17, 18]. The present work developed chitosan edible films with the addition of various concentrations musk lime extracts (seeds, peels and pulps). They were studied in terms of physical, mechanical, chemical and antibacterial properties. Then, an optimized film was used to wrap around the squids and a storage test was done. This was with the aim to possibly increase the shelf-life of the squids.

Materials and Methods

Materials

Musk and squids (*Loligo spp*.) were purchased from local hypermarket, namely Pasar Segar, Pandan Jaya, Selangor, Malaysia. Chitosan powder and Tween-80 emulsifier were of food grade, while chemicals and reagent-grade were of analytical grade and purchased from Chemolab Sdn. Bhd., Selangor, Malaysia.

Preparation and extraction of spices

The musk limes were washed with distilled water and the stems were removed from fruits. They were sliced and hand-squeezed to partially remove the juice. The peels, seeds and pulps were placed on aluminium foils and subjected to overnight heating at 50 °C with a convection oven. The dried spices (peels, seeds and pulps) were ground into fine powder particles using a grinder (Sharp, Japan). The extraction of MLE was conducted as described by Weerakkody et al. [19] with modifications. About 100 g of ground spices was added into 1000 mL of 95% ethanol in a 2 L beaker. The solution was stirred overnight at 25 °C using a magnetic stirrer (Labmart, Korea) with the top of the beaker covered. Then, the solution was filtered with Whatman No.3 filter paper with the aid of a vacuum filter (Millipore, USA). A rotary evaporator (Büchi, Switzerland) was used to evaporate the filtrates at 45 °C in vacuum until there was no more condensation of wastes in the waste collector. The extracts were kept at 4 °C in sterile universal bottles until further use.

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Preparation of film

The film samples were prepared according to Remya et al. [8] with modifications. Chitosan (CH) solution (1.5%, w/v) was prepared by dissolving 1.5 g of CH powder in 100 mL 1% acetic acid by stirring overnight. The CH solution was filtered using vacuum filter with Whatman No.3 filter paper to remove suspended particles. About 25 mL of the CH filtrate was poured into a 50 mL beaker. CH film without MLE will be the control in the characterization tests. For CH films with MLE, they were prepared with the addition of 1.0, 2.0, 3.0, 4.0 and 5.0% v/v of the CH solution, respectively. Glycerol as plasticizer (0.5% v/v of CH solution) and Tween-80 as emulsifier (0.05% v/v of CH solution) were also then added. The solution was homogenized using a homogenizer (Daihan Scientific, Korea) at 9,000 rpm for 3 minutes. 25 mL of the homogenous solution was casted onto a 90 mm x 15 mm Petri dish and dried in a convection oven at 40 °C for 24 hours. The dried film was manually peeled using a spatula and stored in a desiccator until further analyses. The samples were labelled as 'CH + 0% MLE' to 'CH + 5% MLE'.

Physical properties of films: Moisture content

The sample was cut into 2 x 2 cm and stored in a desiccator for a day. It was weighed for initial mass and subjected to convection oven heating at 105 °C for 6 hours until a constant weight was achieved (final weight) [20]. The moisture content was reported in average of triplicate with standard deviation. It was calculated by:

$$Moisture\ content = \frac{M_i - M_f}{M_i} \times 100\% \tag{1}$$

where M_i stands for initial mass sample and M_f stands for final mass of dried sample.

Water solubility

The water solubility test was determined according to Zaman et al. [21]. After the determination of moisture content, the dried film was immersed in excess distilled water (50 mL) for 24 hours. Then, the sample was taken out and blotted dry with filter paper, followed by weighing (final weight). The water solubility was reported in average of triplicate with standard deviation. It was calculated by:

$$Water\ solubility = \frac{M_f - M_i}{M_f} \times 100 \tag{2}$$

where M_i stands for initial mass dried sample and M_f stands for final mass of immersed sample.

Color

The sample's color was determined using a colorimeter33 (Hunterlab, USA) as conducted in the work of Zaman et al. [21]. A circular sample with 64 mm diameter was cut and placed in ColorFlex sample cup after standardization with white and black plates. The color of the film was expressed as L*(lightness-darkness), a* (red-green) and b* (yellow-blue) values. The results were reported in average of triplicate with standard deviation. The following equation was used to calculate the index of ΔE to identify the total color differences between each sample:

$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 (b^* - b)^2} \tag{3}$$

where L*, a* and b*are the color values of the musk lime film samples and L, a and b are the color parameters of thewhite standard tile.

Mechanical properties of films

The thickness of film was measured using a micrometer. For each sample, three measurements were taken from different location on the film selected randomly and reported as average readings with standard deviations. Tensile strength and elongation at break were determined according to Remya et al. [8] with slight modifications using a tensile testing machine (Lloyd Instrument, UK). The films were cut into 20 x 50 mm rectangular strip and held parallel by the grip with a separation of 30 mm. The speed that pulled apart was set to be 20 mm/min. The results were reported in triplicates with standard deviations.

Chemical properties of films

FTIR spectra of ML films and CH films were analyzed using FTIR spectroscopy (Thermo Fisher Scientific, Nicolet iS5FT-IR spectrometer, Waltham, MA, USA) according to Hoque et al. [22]. All spectra were collected nder 20 scans in transmittance mode from 4000 cm^{-1} to 600 cm^{-1} at a resolution of 4 cm^{-1} against background spectrum recorded from empty cell at 25 °C.

Antibacterial properties of films

The antibacterial properties of samples were evaluated according to Velu et al. [23] with slight modifications. An overnight pre-cultured of *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* was separately suspended into 10 mL of nutrient broth and 10 mL of 3% NaCl nutrient broth respectively. The turbidity of bacterial suspensions was adjusted to 0.5 Mcfarland standard. A bacterial suspension (100 µL) was inoculated on Mueller-Hilton Agar (MHA) and swabbed evenly on MHA plate by sterile swabs under aseptic condition.

The edible film discs (6.5 mm) were punched and placed onto the pre-inoculated MHA plate using sterile forcep and gently pressed on it. Streptomycin (10 mg/mL) and ampicillin (20 mg/mL) was used as positive control for *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*, respectively. Negative control was prepared by impregnating 20 µL of 95% ethanol on 6.5 mm sterile paper disc (Whatman filter paper No.3) and dried for 20 minutes in laminar flow. The plates were incubated for 24 hours at 37 °C. The total diameter of inhibition zone was measured by a ruler and reported in millimeter (mm). The results were reported in triplicates with standard deviations.

Storage test of squids

The pre-treatment of squids was carried out according to Kumar et al. [24]. The squids were washed with potable water to remove debris or dirt. Then, the squid pens, viscera, tentacles, eyes and beaks were removed. The squid mantles were used for the present work. They were cut for 10 g into pieces with 3 cm x 3 cm and kept at 4°C in chiller prior to experiment.

The pH test was carried out according to Tantasuttikul et al. [25] with slight modifications. The buffer solutions of pH 4, 7 and 10 were calibrated before conducting the test. The squid mantles were placed into the stomacher bag and mixed with 90 mL sterile distilled water and homogenized by a stomacher (LGC Scientific Sdn Bhd, Malaysia) for two minutes. The film was peeled and not homogenized with the squid mantles. The pH reading was taken using a digital pH meter (EUTech Instruments, Singapore) and performed in duplicates and reported in average with standard deviation.

The total plate count was carried out according to Remya et al. [8]. 10 g of squid mantles were mixed with 90 mL of 0.1% sterile peptone water in stomacher bag and homogenized under a stomacher for 2 minutes. The film was peeled and not homogenized with the squid mantles. A suitable of serial dilution ranges was prepared in each of universal bottles to enumerate the colonies in the range of 30 to 300. Each serial dilution was inoculated for 100 µL on Plate Count Agar using spread plate technique and incubated at 37 °C for 24 hours.

The colonies counts were converted into logarithms from the number of colony forming units (cfu/g). The control (without film wrapping) and 4% musk lime film wraps were conducted at each interval. Measurements in duplicates were taken at intervals of 0, 3, 6, 9, 12, 14 and 16 days.

Statistical analysis

One-way ANOVA with the Tukey test was carried out to determine the significant differences among the films with different MLE concentrations. All the significant differences were set as 95% interval ($p < 0.05$). Paired-t test was conducted to analyze the significant difference in terms of total plate count and pH reading between two different batches of squids (without wrapping and with MLE film wrapping).

Results and Discussion

Physical properties of films

The moisture contents of the film samples were shown in Table 1. The gradual addition of (MLE) had led to a significant decrease of moisture content. The addition of MLE could have reduced the water evaporation rate from the film. The addition of MLE had occupied the spaces in between of chitosan polymer network [26]. As a result, the initial polymer network volume was altered and this caused lesser water was entrapped in between the network [27].

Table 1. Moisture content and water solubility on different concentration of musk lime extracts incorporated with chitosan film

% MLE in Chitosan Film	Moisture Content (%)	Water Solubility (%)
0	24.70 ± 1.80^a	58.60 ± 2.16^a
1	24.41 ± 1.32^a	31.80 ± 3.50^b
2	19.22 ± 0.56^b	31.25 ± 1.14^b
3	18.07 ± 1.47^b	23.11 ± 1.65^c
4	17.94 ± 2.04^b	12.67 ± 2.46^d
5	15.27 ± 1.13^c	9.15 ± 0.96^e

MLE = Musk lime extracts, CH = Chitosan. Values were expressed as triplicate in mean ± standard deviation. a–e different superscripts within the same column indicate significant differences between formulations ($p < 0.05$).

Water solubility was studied to test the samples' resistance against water [28]. As reported in Table 1, the water solubility significantly decreased as the concentration of MLE increased. This seemed to reduce the hydrophilicity of the CH film. The water solubility of a material is related to its water binding capacity [29]. The hydrophilicity of film was reduced due to the addition of hydrophobic components in MLE which includes the fatty acids and polyphenolic compounds [15]. The antioxidant extracts could have limited the polysaccharide-water interactions by reducing its availability to form hydrogen bonds with water molecules [30].

The color parameters of all the film samples were shown in Table 2. The ΔE showed a gradual increasing trend from the increasing addition of MLE into films. This denoted the degree color changes. From the L*, a* and b* parameters, the increasing concentration of MLE in the samples lowered the transparency, reddish and yellowish attributes. Concurrently, it increased the opacity, greenness and blueness of the films as shown in Figure 1. The MLE extracted in the present work was dark green in color. The color attributes from MLE were from pigments such as flavonoids (hesperidin and narginin), carotenoids, chlorophyll and others [31].

Table 2. Parameter of color in different concentration of musk lime extracts incorporated with chitosan film

% MLE in Chitosan Film	L*	a*	b*	ΔE
0	31.11 ± 0.24^a	-0.78 ± 0.07^a	4.71 ± 0.04^a	62.80 ± 0.24^a
1	41.38 ± 0.01^b	7.38 ± 0.01^b	36.01 ± 0.04^b	63.30 ± 0.01^b
2	33.92 ± 0.11^c	12.33 ± 0.02^c	24.44 ± 0.07^c	65.51 ± 0.11^c
3	28.67 ± 0.01^d	9.93 ± 0.01^d	17.77 ± 0.01^d	67.04 ± 0.01^d
4	24.82 ± 0.02^e	4.56 ± 0.06^e	5.18 ± 0.07^e	69.05 ± 0.02^e
5	21.93 ± 0.03^f	1.51 ± 0.02^f	0.89 ± 0.01^f	71.88 ± 0.02^f

MLE = Musk lime extracts, CH = Chitosan. Values were expressed as triplicate in mean ± standard deviation. a–d different superscripts within the same column indicate significant differences between formulations ($p < 0.05$).

Figure 1. Appearances of chitosan-based films incorporated with different concentrations (0%, 3% and 5% from left to right) of musk lime extracts

Mechanical properties of films

The thicknesses, tensile strength (TS) and elongation at break (EB) of all the samples were reported in Table 3. The addition of MLE in the CH film significantly reduced the TS and EB, with 'CH + 5% MLE' reported the lowest TS and EB at 3.03 MPa and 15.02%. The decrease of EB may be due to the decrease of moisture content in the films. Hosseini et al. [32] reported that higher moisture contents in the films resulted in higher elasticity and extensibility. From the present work, the CH film with increased MLE concentrations significantly reduced its moisture content.

There were studies which reported similar influence in the tensile properties in films from the addition of various extracts. Rubilar et al. [27] reported a decrease in the chitosan film's TS (43 MPa to 22.56 MPa) and EB (28% to 10.9%) with the increasing concentration of grape seed extracts. Bourtoom [33] reported that the addition of 50% palm oil in rich starch-chitosan film reduced its TS (12.91 MPa to 9.91 MPa) and EB (24.28% to 18.99%). The addition of 4% oleic acid in chitosan film also significantly reduced the TS (17 MPa to 11 MPa) and EB (12% to 7.4%).

Table 3. Thickness and mechanical properties on different concentration of musk lime extracts incorporated with chitosan film

% MLE in Chitosan Film	Thickness (mm)	Tensile Strength (MPa)	Elongation at Break (%)
0	0.057 ± 0.011^{a}	11.36 ± 0.27^{a}	30.27 ± 1.81^{a}
1	0.117 ± 0.026^{b}	7.51 ± 1.23^{b}	22.13 ± 1.87^{b}
2	0.126 ± 0.194^{b}	6.42 ± 0.47^{bc}	19.47 ± 1.29^{b}
3	0.151 ± 0.024^{b}	4.52 ± 0.51^{c}	19.03 ± 0.40^{b}
4	0.181 ± 0.013^{c}	4.14 ± 0.41^{c}	19.33 ± 1.20^{bc}
5	0.219 ± 0.025^{d}	3.03 ± 0.17^{c}	15.02 ± 0.65^{c}

MLE = Musk lime extracts, CH = Chitosan. Values were expressed as triplicate in mean ± standard deviation. a–d different superscripts within the same column indicate significant differences between formulations ($p < 0.05$)

Chemical properties of films

The Fourier-transform infrared spectroscopy (FTIR) study was done to analyze the interaction between the chitosan-based film and MLE. All distinctive peaks from all film samples were shown in Figure 2. In general, all chitosan films showed similar pattern with main absorption peaks at around 3280 cm^{-1}, 2900 cm^{-1}, 1570 cm^{-1}, 1400 cm^{-1}, 1150 cm^{-1} and 1000 cm^{-1}. These wavenumbers were found in several studies on pure chitosan film [34-36].

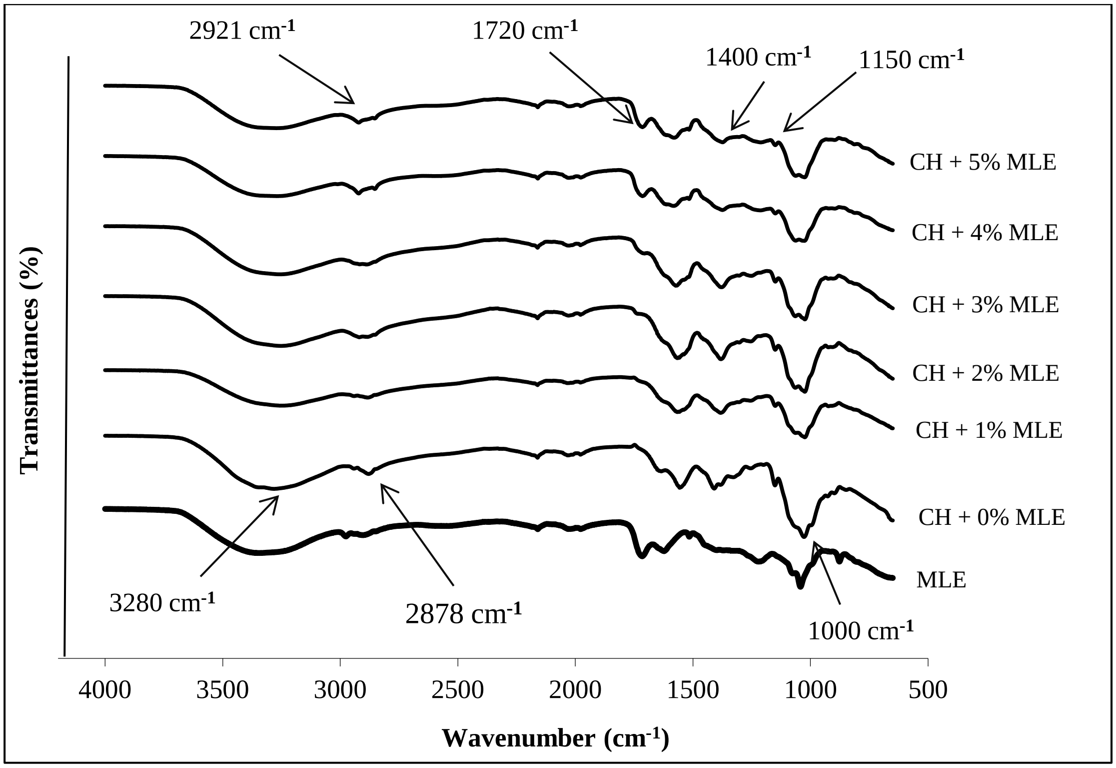

Figure 2. FTIR spectra of all types of films and musk lime extracts

The absorption peaks in the area around 3200 cm^{-1} to 3300 cm^{-1} belonged to the stretching of O-H and N-H bonds [37]. Absorption area of 2800 cm^{-1} to 2900 cm^{-1} corresponded to $–CH_3$ stretching, while 1550 to 1590 cm^{-1} and 1400 cm^{-1} were denoted as amide II (C-N and –NH stretching) and carboxylic group (–COO-), respectively [35]. 1000 cm^{-1} and 1150 cm^{-1} was expected to be carbonyl group (C-O) [34]. Interestingly, the absorption peak at 1720 cm^{-1} became more obvious from 3% to 5% of MLE in the samples. This absorption peak was similarly found in the spectrum of MLE. It could be from the polyphenolic compounds which consisted of C=O bonding [26, 38].

Compared to 'CH + 0% MLE' sample, there was a shift of absorption peaks from wavenumber 2878 cm^{-1} to 2921 cm^{-1} in 'CH + 4% MLE' and 'CH + 5% MLE' samples. This showed a hydrophobicity interaction within the CH film samples [38]. As there was no improvement in tensile strengths from the addition of MLE into the CH films, thus, it is indicative that there was no cross-linkages between them. This phenomenon was similarly reported by Yuan et al. [39] in the development of chitosan film added with carvacrol and pomegranate peel extracts. Reportedly, it was due to the insufficient of interactions between the chitosan and the polyphenolic compound.

Antibacterial properties of films

The antibacterial activities of chitosan films with different concentrations of MLE were shown in Table 4. While MLE and chitosan respectively exhibited inhibition towards *Pseudomonas aeruginosa* (PA) and *Vibrio parahaemolyticus* (VP), the increasing addition of MLE into the chitosan-based films further improved them with the highest inhibition zone against PA and VP at 8.32 mm and 9.88 mm, respectively. Note that there was no significant difference (p > 0.05) of inhibition activities between the addition of 4% and 5% of MLE into the films. Thus, 'CH + 4% MLE' was considered as the optimum sample for further storage test with squids.

Table 4. Antibacterial activity of musk lime extracts incorporated with chitosan film on main strains of bacteria found in squid

Total Diameter Inhibition Zone (mm) Including Film (6.5mm)		
Type of Films	***Pseudomonas aeruginosa***	***Vibrio parahaemolyticus***
CH + 0% MLE	6.78 ± 0.24^{a}	6.97 ± 0.15^{a}
CH + 1% MLE	7.03 ± 0.02^{a}	7.03 ± 0.14^{a}
CH + 2% MLE	7.13 ± 0.17^{ab}	7.13 ± 0.27^{a}
CH + 3% MLE	7.33 ± 0.19^{b}	9.00 ± 0.24^{b}
CH + 4% MLE	8.07 ± 0.26^{c}	9.28 ± 0.62^{c}
CH + 5% MLE	8.32 ± 0.22^{c}	9.88 ± 0.82^{c}

MLE = Musk lime extracts, CH = Chitosan. Values were expressed as triplicate in mean ± standard deviation. a–c different superscripts within the same column indicate significant differences between formulations ($p < 0.05$).

The active sites of chitosan molecules had diffused and slowed the microbes. It was evidenced by Allwin et al. [40] which obtained 14 mm of inhibition zone for PA due to its active amino residues. Chaiyakosa et al. [41] reported the use of chitosan to reduce VP in shrimp. The antibacterial effect of chitosan could be due to the interruption of cell wall by electrostatic force generated from cationic group of protonated amino group (NH_3^+) with negative residue of bacteria cell wall.

The low pH or acidity of MLE used in the films may have inhibited the growth of PA and VP because they grow optimally at neutral pH [42]. It was reported that the citric acid extracted from musk limes fruits inhibited the growth of Vibrio *spp*. [43]. The flavonoid in citrus fruits such as naringin in musk the limes was proven to inhibit PA [44-45].

Storage test of squids

The trends of squids' pH without wrapping (control) and with 'CH + 4% MLE' wrapping throughout 16 days were shown in Figure 3. The present work recorded the squid's initial pH as 6.6, which was in par with other works [46]. Without the film, the squid's pH gradually increased to 7.5, which is just slightly above the neutral pH- a favorable pH for common microbes to develop. In contrary with the wrapping, the squid's pH was in the acidic range with pH 3 at Day 3 and 5.6 at Day 16. Pairing with the TPC results from the storage test, it was notable that the acidic condition contributed by the MLE in the wrapping had slowed down the growth of microbes. The gradual increase in pH value with storage time was due to the presence of cathepsin and alkalinizing bacteria from the squids [47]. This caused the accumulation of alkaline compounds such as ammonia and trimethylamine.

The trends of the TPC of squid samples without wrapping (control) and with 'CH + 4% MLE' wrapping throughout 16 days were shown in Figure 4. The initial TPC of both samples were at log 4.55, which was in par with that reported by other works [48, 49]. While both samples gradually increased in the TPC with time, it was markedly reduced with the wrapping of 'CH + 4% MLE', denoting a hindrance in the growth of microbes.

The maximum acceptable microbial counts limit was at log 6.0 [48]. A food is considered unsafe for consumption when the TPC is more than log 6.0. Based on this limit as shown in Figure 4, the shelf-life of the squid was extended from five days to fifteen days when it is wrapped with the film sample. This marked extension was due to the antibacterial properties of the film as discussed above.

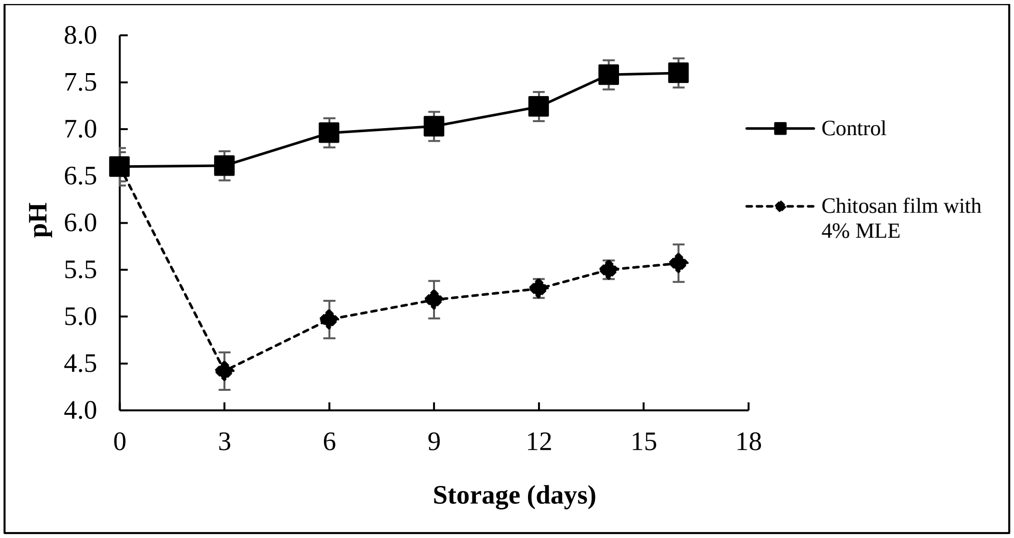

Figure 3. The changes of squids' pH with and without 'CH + 4% MLE' wrapping throughout the storage period

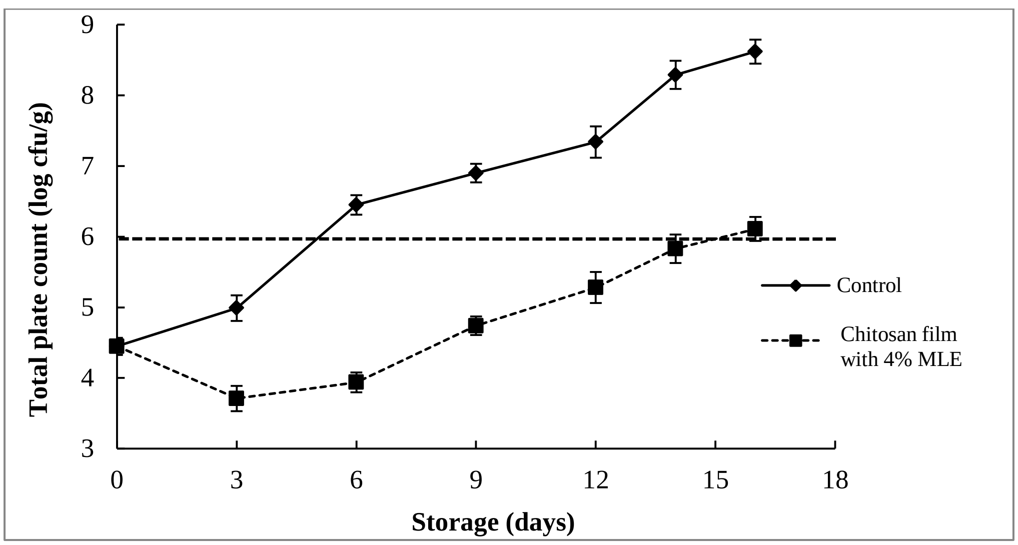

Figure 4. Changes of TPC in log (CFU/mL) with and without 'CH + 4% MLE' wrapping throughout the storage period

Conclusion

The present work had developed a chitosan-based edible film, incorporated with musk lime extracts. From the tests done, it is conclusive that the higher concentration of musk lime extract in chitosan film would result in increased antibacterial properties, thickness and total color changes. Concurrently, it reduced the moisture content, water solubility and tensile properties. At optimized concentration of 4% of MLE added in the chitosan-based film, it effectively extended the shelf-life of squid in the 4°C storage. In a nutshell, MLE has the potential to be used as a safe alternative additive into film materials for shelf-life extension of food its antibacterial properties. It is suggested that other physical analysis of the film such as water vapor permeability, oxygen permeability and scanning electron microscope to be explored in future research.

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References

1. Robertson, G. L. (2005). Food packaging: principles and practice. CRC press.
2. Giatrakou, V. I. and Savvaidis, I. N. (2012). Bioactive packaging technologies with chitosan as a natural preservative agent for extended shelf-life food products. In Modified atmosphere and active packaging technologies. CRC Press, Florida: pp. 708-753.
3. Jouki, M., Yazdi, F. T., Mortazavi, S. A., Koocheki, A. and Khazaei, N. (2014). Effect of quince seed mucilage edible films incorporated with oregano or thyme essential oil on shelf life extension of refrigerated rainbow trout fillets. *International Journal of Food Microbiology*, 174: 88 - 97.
4. Restuccia, D., Spizzirri, U. G., Parisi, O. I., Cirillo, G., Curcio, M., Iemma, F., Puoci, F., Vinci, G. and Picci, N. (2010). New EU regulation aspects and global market of active and intelligent packaging for food industry applications. *Food Control*, 21(11): 1425 - 1435.
5. Embuscado, M. E. and Huber, K. C. (2008). Edible films and coatings: Why, what, and how. Springer, New York: pp.1 - 25.
6. Saremnezhad, S., Azizi, M. H., Barzegar, M., Abbasi, S. and Ahmadi, E. (2011). Properties of a new edible film made of faba bean protein isolate. *Journal of Agricultural Science and Technology*, 13(2): 181 - 192.
7. Mastromatteo, M., Danza, A., Conte, A., Muratore, G. and Del Nobile, M. A. (2010). Shelf life of ready to use peeled shrimps as affected by thymol essential oil and modified atmosphere packaging. *International Journal of Food Microbiology*, 144(2): 250 - 256.
8. Remya, S., Mohan, C. O., Bindu, J., Sivaraman, G. K., Venkateshwarlu, G. and Ravishankar, C. N. (2016). Effect of chitosan based active packaging film on the keeping quality of chilled stored barracuda fish. *Journal of Food Science and Technology*, 53(1): 685 - 693.
9. Quintavalla, S. and Vicini, L. (2002). Antimicrobial food packaging in meat industry. *Meat Science*, 62(3): 373 - 380.
10. Muppalla, S. R., Kanatt, S. R., Chawla, S. P. and Sharma, A. (2014). Carboxymethyl cellulose–polyvinyl alcohol films with clove oil for active packaging of ground chicken meat. *Food Packaging and Shelf Life*, 2(2): 51 - 58.
11. Emiroğlu, Z. K., Yemiş, G. P., Coşkun, B. K. and Candoğan, K. (2010). Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties. *Meat Science*, 86 (2): 283 - 288.
12. Lim, T. K. (2012). Citrus x microcarpa. Springer, Netherlands: pp. 865 - 870.
13. Beh, L. K., Zakaria, Z., Beh, B. K., Ho, W. Y., Yeap, S. K. and Alitheen, N. B. M. (2012). Comparison of total phenolic content and antioxidant activities of freeze-dried commercial and fresh fruit juices. *Journal of Medicinal Plants Research*, 6(48): 5857 - 5862.
14. Chen, H. C., Peng, L. W., Sheu, M. J., Lin, L. Y., Chiang, H. M., Wu, C. T., Wu, C.S. and Chen, Y. C. (2013). Effects of hot water treatment on the essential oils of calamondin. *Journal of Food and Drug Analysis*, 21(4): 363 - 368.
15. Manaf, Y. N., Osman, A., Lai, O. M., Long, K. and Ghazali, H. M. (2008). Characterisation of musk lime (*Citrusmicrocarpa*) seed oil. *Journal of the Science of Food and Agriculture*, 88(4): 676 - 683.
16. Paarup, T., Sanchez, J. A., Moral, A., Christensen, H., Bisgaard, M. and Gram, L. (2002). Sensory, chemical and bacteriological changes during storage of iced squid (*Todaropsis eblanae*). *Journal of Applied Microbiology*, 92(5): 941 - 950.
17. Lapa-Guimarães, J., de Felício, P. E. and Guzmán, E. S. C. (2005). Chemical and microbial analyses of squid muscle (*Loligo Plei*) during storage in ice. *Food Chemistry*, 91(3): 477 - 483.
18. Vaz-Pires, P., Seixas, P., Mota, M., Lapa-Guimarães, J., Pickova, J., Lindo, A. and Silva, T. (2008). Sensory, microbiological, physical and chemical properties of cuttlefish (*Sepia Officinalis*) and broadtail shortfin squid (*Illex coindetii*) stored in ice. *LWT-Food Science and Technology*, 41(9): 1655 - 1664.
19. Weerakkody, N. S., Caffin, N., Turner, M. S. and Dykes, G. A. (2010). In vitro antimicrobial activity of less-utilized spice and herb extracts against selected food-borne bacteria. *Food Control*, 21(10): 1408 - 1414.

20. Association of Analytical Chemists (1995). Official methods of analysis of AOAC international.
21. Zaman, N. B. K., Lin, N. K. and Phing, P. L. (2018). Chitosan film incorporated with *Garcinia Atroviridis* for the packaging of indian mackerel (*Rastrelliger kanagurta*). *Ciência e Agrotecnologia*, 42(6): 666 - 675.
22. Hoque, M. S., Benjakul, S. and Prodpran, T. (2011). Properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin incorporated with cinnamon, clove and star anise extracts. *Food Hydrocolloids*, 25(5): 1085 - 1097.
23. Velu, S., Abu Bakar, F., Mahyudin, N. A., Saari, N. and Zaman, M. Z. (2014). In vitro antimicrobial activity of musk lime, key lime and lemon extracts against food related pathogenic and spoilage bacteria. *International Food Research Journal*, 21(1): 379 - 386.
24. Kumar, V., Sukumar, D. and Muruganantham, M. (2015). Microbial quality of frozen squid (*Sepioteuthis lessoniana*, Lesson 1830) treated with food grade commercial chemicals. *Indian Journal of Animal Research*, 49(5): 736 - 741.
25. Tantasuttikul, A., Kijroongrojana, K. and Benjakul, S. (2011). Quality indices of squid (*Photololigo duvaucelii*) and cuttlefish (*Sepia aculeata*) stored in ice. *Journal of Aquatic Food Product Technology*, 20 (2): 129 - 147.
26. Siripatrawan, U. and Harte, B. R. (2010). Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids*, 24(8): 770 - 775.
27. Rubilar, J. F., Cruz, R. M., Silva, H. D., Vicente, A. A., Khmelinskii, I. and Vieira, M. C. (2013). Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. *Journal of Food Engineering*, 115(4): 466 - 474.
28. Cano, A., Cháfer, M., Chiralt, A. and González-Martínez, C. (2016). Physical and antimicrobial properties of starch-PVA blend films as affected by the incorporation of natural antimicrobial agents. *Foods*, 5 (1): 3.
29. Ojagh, S. M., Rezaei, M., Razavi, S. H. and Hosseini, S. M. H. (2010). Effect of chitosan coatings enriched with cinnamon oil on the quality of refrigerated rainbow trout. *Food Chemistry*, 120(1): 193 - 198.
30. Gómez-Guillén, M. C., Ihl, M., Bifani, V., Silva, A. M. D. O. E. and Montero, P. (2007). Edible films made from tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (*Ugni molinae Turcz*). *Food Hydrocolloids*, 21(7): 1133 - 1143.
31. Asif, M. (2014). Brief information related to potential therapeutic tendencies of citrus plants. *International Journal of Biological Research*, 2(2): 116 - 120.
32. Hosseini, M. H., Razavi, S. H. and Mousavi, M. A. (2009). Antimicrobial, physical and mechanical properties of chitosan-based films incorporated with thyme, clove and cinnamon essential oils. *Journal of Food Processing and Preservation*, 33(6): 727 - 743.
33. Bourtoom, T. (2008). Edible films and coatings: characteristics and properties. *International Food Research Journal*, 15(3): 237 - 248.
34. Pelissari, F. M., Grossmann, M. V., Yamashita, F. and Pineda, E. A. G. (2009). Antimicrobial, mechanical, and barrier properties of cassava starch− chitosan films incorporated with oregano essential oil. *Journal of Agricultural and Food Chemistry*, 57(16): 7499 - 7504.
35. Pranoto, Y., Rakshit, S. K. and Salokhe, V. M. (2005). Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT-Food Science and Technology*, 38(8): 859 - 865.
36. Xuan, X. T., Fan, Y. F., Ling, J. G., Hu, Y. Q., Liu, D. H., Chen, S. G., Ye, X.Q. and Ding, T. (2017). Preservation of squid by slightly acidic electrolyzed water ice. *Food Control*, 73: 1483 - 1489.
37. Xu, Y. X., Kim, K. M., Hanna, M. A. and Nag, D. (2005). Chitosan–starch composite film: Preparation and characterization. *Industrial Crops and Products*, 21(2): 185 - 192.
38. Tongnuanchan, P., Benjakul, S. and Prodpran, T. (2012). Properties and antioxidant activity of fish skin gelatin film incorporated with citrus essential oils. *Food Chemistry*, 134(3): 1571 - 1579.
39. Yuan, G., Lv, H., Yang, B., Chen, X. and Sun, H. (2015). Physical properties, antioxidant and antimicrobial activity of chitosan films containing carvacrol and pomegranate peel extract. *Molecules*, 20(6): 11034 - 11045.
40. Allwin, S. J., Jeyasanta, K. I. and Patterson, J. (2015). Extraction of chitosan from white shrimp (*Litopenaeus vannamei*) processing waste and examination of its bioactive potentials. *Advances in Biological Research*, 9(6): 389 - 396.
41. Chaiyakosa, S., Charernjiratragul, W., Umsakul, K. and Vuddhakul, V. (2007). Comparing the efficiency of chitosan with chlorine for reducing *Vibrio parahaemolyticus* in Shrimp. *Food control*, 18(9): 1031 - 1035.
42. Kirbaşlar, F. G., Tavman, A., Dülger, B. and Türker, G. (2009). Antimicrobial activity of turkish citrus peel oils. *Pakistan Journal of Botany*, 41(6): 3207-3212.

43. Musa, N., Wei, L. S., Seng, C. T., Wee, W. and Leong, L. K. (2008). Potential of edible plants as remedies of systemic bacterial disease infection in cultured fish. *Global Journal of Pharmacology*, 2(2): 31 - 36.
44. Wang, Y. C., Chuang, Y. C. and Hsu, H. W. (2008). The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. *Food Chemistry*, 106(1): 277 - 284.
45. Vandeputte, O. M., Kiendrebeogo, M., Rasamiravaka, T., Stevigny, C., Duez, P., Rajaonson, S., Diallo, B., Mol, A., Baucher, M. and El Jaziri, M. (2011). The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. *Microbiology*, 157(7): 2120 - 2132.
46. Márquez-Ríos, E., Morán-Palacio, E. F., Lugo-Sánchez, M. E., Ocano-Higuera, V. M. and Pacheco-Aguilar, R. (2007). Postmortem biochemical behavior of giant squid (*Dosidicus gigas*) mantle muscle stored in ice and its relation with quality parameters. *Journal of Food Science*, 72(7): 356 - 362.
47. Wang, J. J., Lin, T., Li, J. B., Liao, C., Pan, Y. J. and Zhao, Y. (2014). Effect of acidic electrolyzed water ice on quality of shrimp in dark condition. *Food Control*, 35(1): 207 - 212.
48. Tomac, A. and Yeannes, M. I. (2012). Gamma radiation effect on quality changes in vacuum-packed squid (*Illex argentinus*) mantle rings during refrigerated (4 - 5° C) storage. *International Journal of Food Science and Technology*, 47(7): 1550 -1557.
49. Zaragozá, P., Fuentes, A., Ruiz-Rico, M., Vivancos, J. L., Fernández-Segovia, I., Ros-Lis, J. V., Barat, J.M. and Martínez-Máñez, R. (2015). Development of a colorimetric sensor array for squid spoilage assessment. *Food Chemistry*, 175: 315 - 321.