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Chitosan Active Films Containing Red Ginger Extract for Shelf-life Extention and Quality Retention of Milkfish (*Chanos chanos*)

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Abstract. Chitosan is a natural bioactive polysaccharide with intrinsic antimicrobial activity and has been recognized as a natural alternative to chemically synthesized antimicrobial polymers. This, associated with the increasing preference for biofunctional materials from renewable resources, resulted in a significant interest on the potential for application of chitosan in packaging materials. Oils extracted from plants have been the focus of numerous researches due to their potential in the food and pharmaceutical industries. An antimicrobial food packaging material was developed from chitosan with different concentrations (0.6%; 0.9%; and 1.2%) (v/v) of red ginger (Zingiber officinale var. rubrum) extract. Analysis by GCMS revealed that zingiberene (10.3%), ar-Curcumene (15.6%), α-Pinene (3.12%), β-Pinene (0.5%), trans geraniol (0.69%), 1.8 Cineole (7.52%), Endo borneol (0.46%) and β-Sesquiphellandrene (8.74%) as major components of the oil. The effect of extract's concentration to the physic-chemical properties, and microbiological and sensory quality of milkfish during 6 days of storage (4±1oC) were evaluated. Protected milkfish with film and unprotected milkfish were stored under the same conditions. The effect of glycerol as plasticizer to the tensile strength of films were observed. The films were also characterized by using of SEM analysis for surface morphological properties. The results obtained indicated that films produced from chitosan plasticized with glycerol and incorporated with red ginger extract exhibited good tensile strength and swelling behavior, also high antioxidant and antimicrobial activity. Analysis of yeast and mould counts; and total plate counts of protected milkfish samples containing red ginger were lower than unprotected samples. Therefore, the results indicate that chitosan-based films formulation incorporated with red ginger have potential as an active packaging for food safety and to extend the shelf-life of milkfish.

INTRODUCTION

Food packaging is generally proposed to prevent the degradation of food by chemical or physical contaminants, microbial contamination, and loss of aroma while retaining the quality of product during its extended shelf life. To overcome the problems, a packaging must act as a gaseous barrier to hinder the exchange of moisture, carbon dioxide, oxygen, and aromatics along with acceptable optical, physical, and mechanical properties [1]. During the last few years, there is an increased demand for healthier food products without chemical preservatives, resulting in a need to use natural additives or alternative methods to improve safety [2]. Natural materials are the eco-friendly alternative to nonrenewable petroleum-based packaging materials. These materials are desirable because of their ample availability, low cost, biodegradability, and renewability. On the other hand, poor mechanical and water vapor permeable properties are the drawback of biopolymers for packaging application [3].

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Nowadays, the research on the development of active materials with antimicrobial and antioxidant properties has increased rapidly with the purpose to protect food from microbial growth and oxidative reactions [4]. Natural materials with antimicrobial activities such as polysaccharides, proteins, and lipids, have been proven to be promising due to their environmentally friendly and biodegradability [5]. Among them, chitosan has been considered as one of the most promising candidates for future materials because of its abundance, antimicrobial, and thermoplastic property. Chitosan exhibits antimicrobial activity in vitro against a wide range of fungi, yeasts, and bacteria [2]. The satisfactory film forming property and high mechanical resistance of chitosan resulted in a significant interest on chitosan-based packaging materials [6]. The incorporation of several natural ingredients such as essential oils [7-9], plant extracts [10-12] have been promoted in the recent years to improve film properties.

Essential oils (EOs) from aromatic plants are potent antimicrobial agents. However, their volatility, low solubility in water, and susceptibility for oxidation limits their use [7]. Some essential oils such as cinnamon oil and ginger essential oil can be used to improve the properties of bio-based films. EOs could be used to plasticize binary polysaccharide network of chitosan and carboxymethyl cellulose while improving moisture permeability and maintaining antifungal activity of resulting films [13]. A variety of antimicrobial edible coatings containing ginger essential oil has been developed for some food products [14, 15]. Ginger extracts exerted significant antioxidant activity and dose-depend effect. In general, oleoresin showed higher antioxidant activity when compared to the essential oil. In terms of antimicrobial activity, ginger compounds were more effective against *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*, and less effective against *Bacillus cereus* [16].

The incorporation of ginger essential oil in films formulation is a recently adopted technique, but only one study was found on the incorporation of red ginger oleoresin in chitosan-based film [17]. In this study, the effects of red ginger oleoresin incorporation on the antibacterial and physical properties of the chitosan films were evaluated.

MATERIAL AND METHODS

Materials

Chitosan food grade from PT. Biotech Surindo with a degree of deacetylation (DD) = 87.2% and a viscosity of 37.10 cps, glacial acetic acid from Merck, technical glycerol from Bratachem, red ginger oleoresin from PT. Lansida, ethanol 96% from Sigma-Aldrich, and fresh milkfish (*Chanos chanos*) from Karangantu-Serang, Banten Province, Indonesia.

Methods

Preparation of Plasticized Chitosan Solution

Chitosan solution was made by dissolving chitosan in glacial acetic acid solution in different concentrations and stirred constantly using IKA overhead stirrers RW 20 at room temperature conditions for 4 minutes. Glycerol at varied concentrations (10, 25, 40 %) (glycerol/chitosan, w/w) were added to the chitosan solution, and then stirred using an IKA magnetic stirrer hot plate for 1 minute. After completion of the mixing process, the solution is filtered with Whatman filter paper to remove insoluble impurities and bubbles in the solution.

Preparation of Chitosan Edible Film

This method refers to [18]. Each of red ginger essential oil concentration of 0.6; 0.9 and 1.2 g/100 g included in plasticized chitosan, and then homogenized using a mixer for 1 min. The mixture was then molded with a size of 20 x 20 cm. Edible film drying process carried out in an oven at 70°C. Chitosan-based edible films were analyzed for physical properties, Total Phenolic Content (TPC), antioxidant analysis, and antimicrobial activity.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Gas chromatography mass spectrometry (GC-MS) was used for the analysis of the components contained in the red ginger essential oil.

Phenolic Content Analysis

Total polyphenols content was determined by the Folin-Ciocalteu method [19]. Briefly, each sample were mixed with Folin-Ciocalteu reagent then sodium carbonate was added to each sample. After 30 min, the absorbance was measured at 760 nm using spectrophotometer. The calibration curve was performed using gallic acid as a standard. The results were expressed as gallic acid equivalents.

Antioxidant Analysis

Antioxidant activity was tested as described [20]. Each sample was mixed with 1,1-diphenyl-2-picrylhydrazyl (DPPH•) ethanol solution. Absorbance was determined at 515 nm until the reaction reached a plateau. The DPPH• -scavenging activity of each sample was expressed as the inhibition percentage calculated using Equation (1).

$$DPPH \bullet \text{inhibition}(\%) = \frac{A_b - A_s}{A_b} x100 \quad (1)$$

Where A_b is the absorbance of the blank and A_s is the absorbance of the sample.

Antibacterial Analysis

Evaluation of antibacterial activity of the film was prepared by using of Total Plate Count (TPC) method. This is an in-house method based on ISO 4833.

Application of films

The milkfish were cut, wrapped with chitosan films, and kept in two different storage temperatures which were room temperature and refrigerator temperature (4 °C). The storage periods were also varied, they were 9 hours for the storage at room temperature, and 6 days for refrigerator temperature storage. Organoleptic study and microbiological test were held to the milkfish samples during the storage periods. There were 22 people involved in this study to evaluate the changes in color, freshness, and texture of the milkfish. The affectivity of the film as an active packaging was confirmed by using of Total Plate Count (TPC) method for its antibacterial and antifungal activity.

RESULTS AND DISCUSSION

GC-MS Study

Chemical components resulting from GCMS analysis of 33 components, the complete data can be seen in Fig. 1 and Table 1.

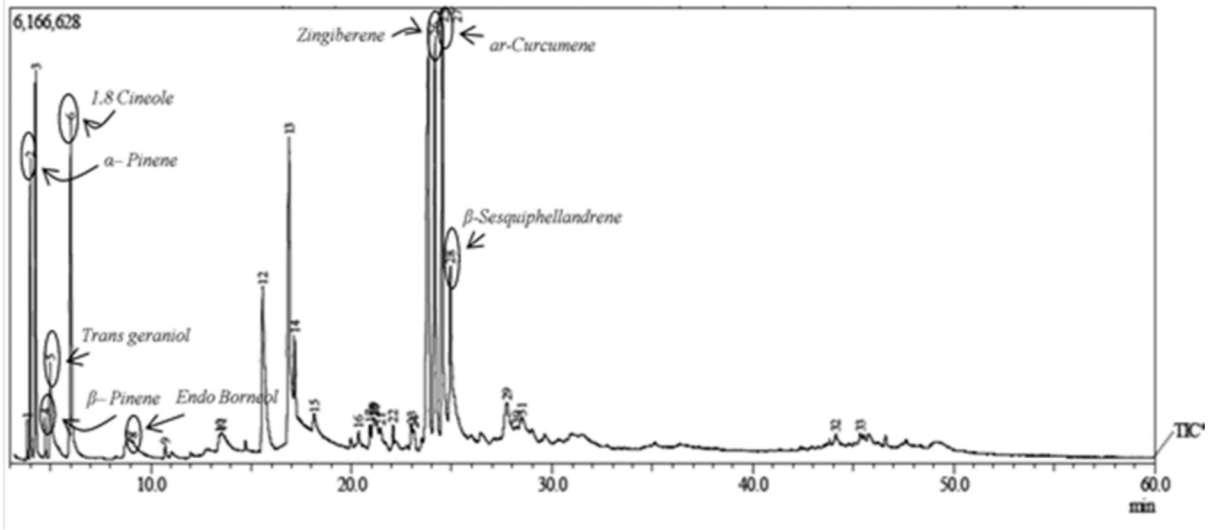

FIGURE 1. GC MS analysis of red ginger essential oil

The main components of the essential ginger oil that can be analysed were zingiberene (10,3%); ar-Curcumene (15,6%); α-Pinene (3,12%); β-Pinene (0,5%); trans geraniol (0,69%); 1,8 Cineole (7.52%); Endo borneol (0,46%) and β-Sesquiphellandrene (8,74%). These components are phenolic and terpenoid that can be used as antioxidants and antimicrobials.

TABLE 1. Components of the Red Ginger Essential Oil

Peak	% Area	Name
1	0.37	Tricyclo 2.2.1.02,6 heptane, 1,7,7-trimethyl-
2	3.12	alpha.-Pinene
3	7.38	Camphene
4	0.5	beta.-Pinene
5	3.17	trans-Geraniol
6	7.52	1,8-Cineole
7	0.92	Linalool
8	0.46	endo-Borneol
9	0.31	Bicyclo 2.2.1 heptan-2-one, 1,7,7-trimethyl-
10	0.34	Myrcenol
11	0.79	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl- (
12	7.14	Citral (isomer 1) cis, trans-
13	9.66	2,6-Octadienal, 3,7-dimethyl-
14	2.73	bornyl acetate
15	0.69	trans-Geraniol
16	0.35	alpha.-Copaene
17	0.55	2,4-Diisopropenyl-1-methyl-1-vinyl-cyclohexane
18	0.44	Geranyl acetate
19	0.46	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
20	1.21	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (Z)-
21	0.82	Zingiberene
22	0.43	(-)-Isoledene
23	0.55	Alloaromadendrene
24	0.57	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (Z)-
25	15.60	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (CAS) ar-Curcumene
26	10.3	Zingiberene
27	11.28	beta.-Bisabolene
28	8.74	beta.-sesquiphellandrene
29	2.10	Aromadendrene
30	0.30	Caryophyllene
31	0.46	beta.-Eudesmol
32	0.46	1-Pentene, 3,3,4-Trimethyl-5-Phenyl-
33	0.28	6,10,14-Hexadecatrienoic acid, 3,7,11,15-tetramethyl-, methyl ester, [R-(E,E)]-

Research reported by Z. Kamaliroosta, et al [21] shows that the chemical content in the essential oil of the red ginger is almost identical to the results of this study, which differ only the concentration of each component. The same result was reported by H. Purnomo, et al [22], the essential oil of ginger produced has several components in common with this study as zingiberene, ß-sesquiphellandrene, and geraniol. The composition and concentration of the active components contained in the essential oil of ginger depends on the varieties of ginger, origin and cultivation, moisture, and extraction methods [23]. Table 1 shows the complete results of the essential oil analysis of the red ginger using GCMS.

Tensile Properties

Tensile strength is recognized as the maximum stress supported by film before break and percent elongation at break is a mechanical property that provides information about deformation of a material prior to breakage. If the material is proposed for some food packaging applications, certain deformation is mandatory before fracturing [24]. This research was held in different concentration of chitosan and acetic acid solutions. The first composition of film solution is 1% of chitosan dissolved in 2% of acetic acid solution, and the second one is 2% of chitosan in 1% of acetic acid solution. Glyserol, as a plastisizer, was also added into the film solution as much as 0.1 mL.

Figure 2 shows the effect of chitosan concentration to the tensile of the films resulted in this study. Film containing 1% of chitosan concentration in 2% of acetic acid solution results better physical property compared with film containing 2% of chitosan in 1% of acetic acid solution. This behavior is possibly due to the high viscosity of film solution containing 2% of chitosan which promote low dispersion of chitosan molecules and results the decreasing of tensile strength.

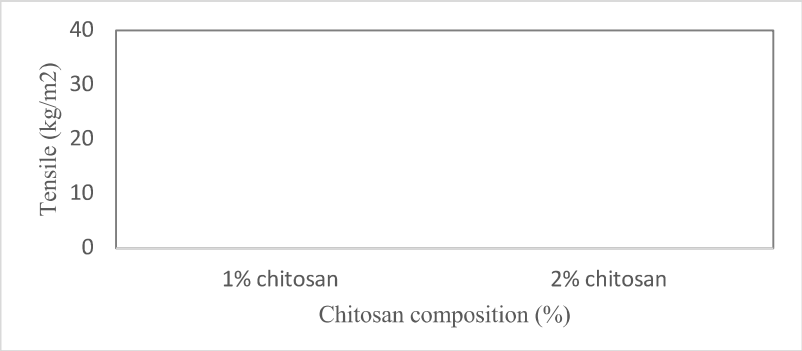

FIGURE 2. Effect of chitosan concentration to the tensile strength of chitosan films

The tensile strength of films prepared from 1% of chitosan which dissolved in 2% of acetic acid solution with different concentrations of plasticizer were studied and described in Fig. 3.

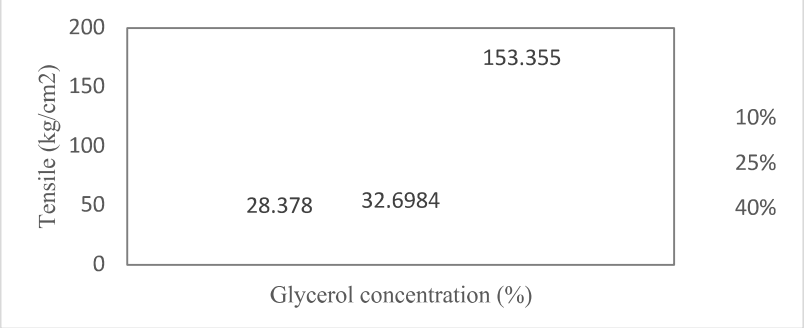

FIGURE 3. Effect of glycerol concentration to the tensile strength of chitosan films

Figure 3 shows that the addition of glycerol caused an increase in tensile strength. Glycerol is a non-volatile plasticizer [25]. The improvement of mechanical property was correlated to the films structure and to the interactions between chitosan and glycerol. Different effects of glycerol to the mechanical properties of resulted films were found from some studies. H. Liu, et al, 2013 observed glycerol plasticized (high-amylose) starch–chitosan films and found that the addition of glycerol at 5% (w/w) and higher concentrations resulted in decrease in tensile strength, increase in elongation at break due to plasticization [26]. Another article reported the preparation of plasticized alginate from sodium alginate of high molar mass which has been processed with glycerol [25]. This paper explained that the addition of glycerol resulted in a decrease in tensile strength and Young's modulus and an increase in elongation at break.

The tensile strength of all the films developed with the incorporation of the red ginger oleoresin were evaluated, and the results are shown in Fig. 4.

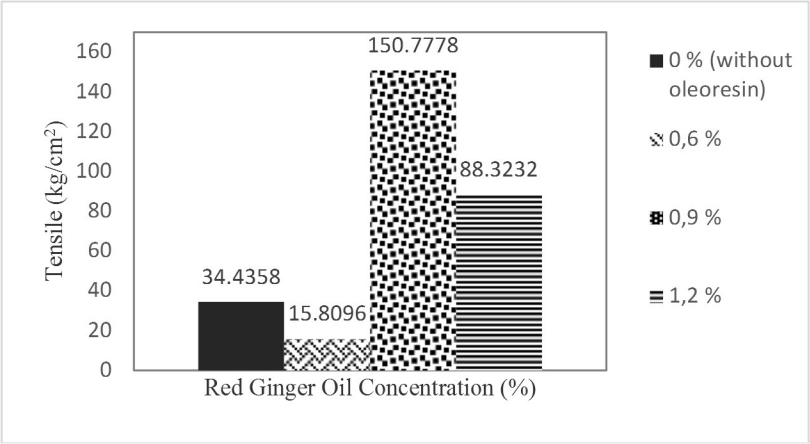

FIGURE 4. Effect of Red Ginger Oleoresin to the tensile strength of chitosan films

The highest tensile strength was found in film with the incorporation of 0.9% red ginger oleoresin, achieving up to 150.7778 kg/cm2. The value of tensile strength for chitosan film decreased at the concentration level of 1.2 % red ginger oleoresin considerably due to the development of a heterogeneous film structure and to the weakening of glycerol-chitosan interactions by effect of the oil presence. However, films formed from chitosan incorporated with 0,9 and 1,2% red ginger oleoresin exhibited higher tensile strength than films formed from chitosan without oleoresin or chitosan with 0,6% oleoresin.

Some articles have studied the effect of essential oils to the mechanical properties of bioactive films especially tensile strength and found different results depend on types of polymers and types of plants as the source of the oil. X. Song, et al, 2017 reported that the incorporation of lemon essential oil provoked a decrease in water content, transparency, whiteness index, water vapor permeability, solubility and tensile strength properties of corn and wheat starch films [27]. According to the article reported by [28], the incorporation of *Origanumvulgare L.* essential oil in the biocomposite films based on fish gelatin and chitosan caused a significant decrease ($p < 0.05$) in tensile strength and elastic modulus, although no significant change was observed in elongation at break.

Film Thickness

This research resulted various thickness of chitosan film products. According to Fig. 5, chitosan film solutions with different concentrations of red ginger oleoresin resulted different thickness of product.

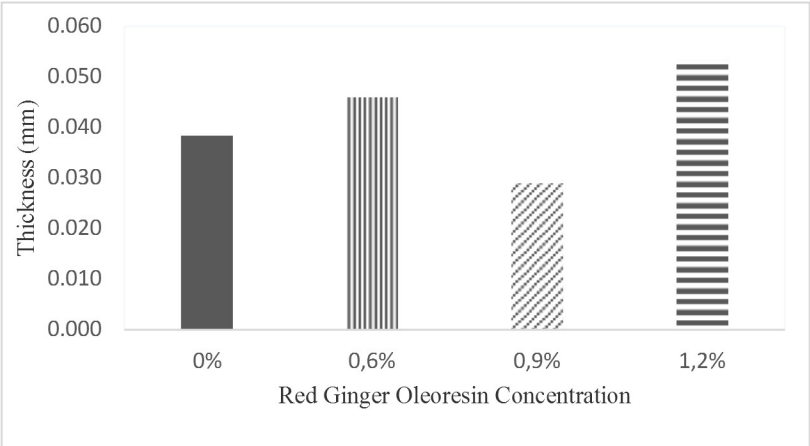

FIGURE 5. Effect of Red Ginger Oleoresin concentration to the thickness of chitosan films

Based on Fig. 5, films with higher concentration of oleoresin resulted from this study were generally thicker compared with films containing lower concentration of oleoresin and film without the incorporation of oleoresin. An exception data was obtained when using 0.9% concentration of the red ginger oleoresin, where the thickness was at the lowest value. Similar result was obtained by [14] who reported that the incorporation of ginger essential oil into gelatin-based films increased the thickness. In another article reported that the incorporation of essential oil did not make any change in thickness. The value of film thickness can be used for the tensile strength, ductility (percent elongation), water vapor permeability, and transparency calculations [11].

Swelling Study

Swelling ratio of films were determined by using gravimetric method [29]. The films were immersed in distilled water for 3 h, and then the swollen films were filtered using stainless steel sieves (± 200 mesh) and hanged for approximately 1 h until no more water drop off from the films. The swelling ratio of films were calculated using Equation (2) [30].

$$Swelling\ ratio\ (\%) = \frac{Ws-Wd}{Wd} x100 \quad (2)$$

Where *Ws* is the weight of the film in a state of swelling (g) and *Wd* is the weight of dry film (g).

Figure 6 shows that the swelling ratio of resulted film increases with the addition of red ginger oleoresin. The increase of oleoresin concentration causes higher viscosity of film solution and results the reduction of the pore size after drying process. Small pore size effects difficulty for water or any other aqueous solutions to enter the film matrix, and as a result, smaller swelling ratio can be obtained in the addition of the oleoresin. The smallest swelling ratio resulted from this research was 332,1% which was proceed from 1 % chitosan dissolved in 2% acetic acid, added with 0,4 % of glycerol and 1,2 % concentration of red ginger oleoresin.

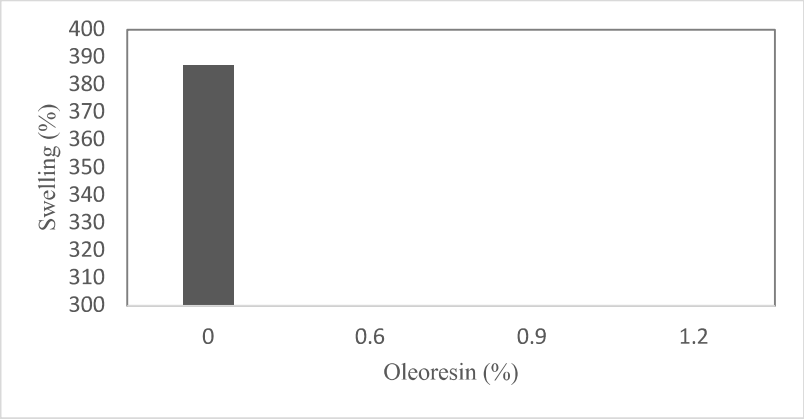

FIGURE 6. Effect of Red Ginger Oleoresin concentration (%) to the swelling properties of films (%)

SEM Study

Scanning Electron Microscopy (SEM) analysis was held to evaluate the effect of oleoresin addition to the morphology of chitosan-based films. Figure 7 shows the different between film without oleoresin and film with the addition of red ginger oleoresin. The film with oleoresin seems to be smoother and the porosity is evenly distributed. The presence of oleoresin in the material can fill the film matrix and improve its texture so that it becomes smoother and elastic compared to film without oleoresin in it.

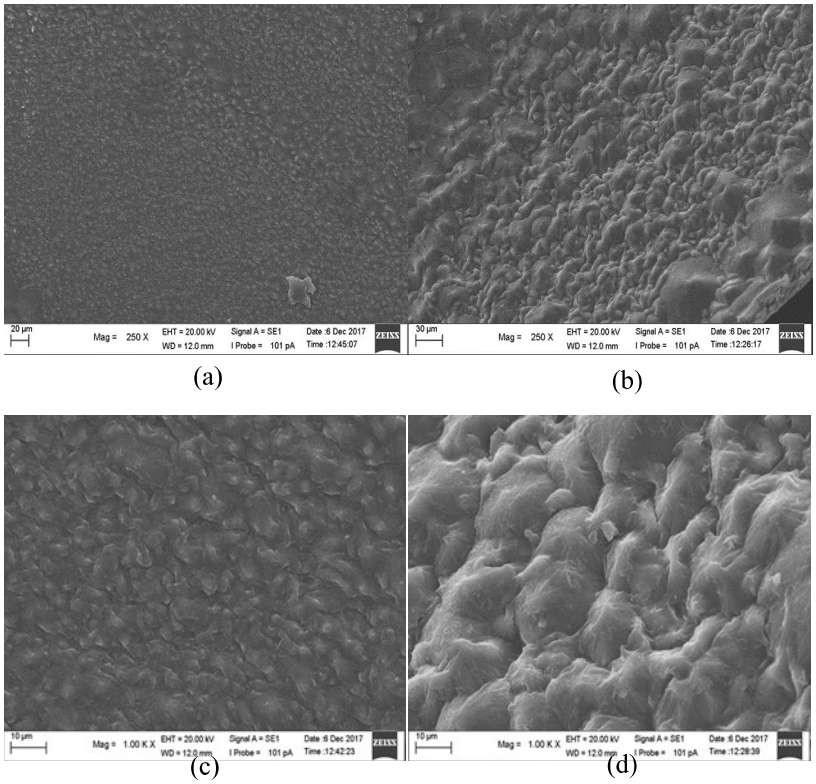

FIGURE 7. Surface morphology of the prepared chitosan film formulations: a) film with oleoresin 250x of magnification, b) film without oleoresin 250x magnification, c) film with oleoresin 1000x of magnification, d) film with oleoresin 1000x of magnification

Total Phenolic Content (TPC) Analysis

Total Phenolic Content (TPC) analysis was held by Folin-Ciaoceltau method using gallic acid standard. Gallic acid is a phenolic compound which are very often to be used as standard for Total Phenolic Content analysis. The absorbance of the reaction mixture was measured by using a spectrophotometer UV-VIS at wavelength of 760 nm after the incubation at temperature of 40°C. Total phenolic content was expressed as milligram of gallic acid equivalent per liter of red ginger oil. The results are shown in Table 2.

TABLE 2. TPC analysis of chitosan resulted films

Oil Composition (%)	Replication	TPC Eq Gallic Acid (mg/L)	Average TPC Eq (mg/L)
0	1	182.00	184.10
	2	186,20	
0.6	1	270.40	271.75
	2	273.10	
0.9	1	656.30	619.50
	2	582.70	
1.2	1	608.70	608.70
	2	608.70	

This research was performed with the concentration of chitosan was 1%, acetic acid was 2%, and glycerol as plasticizer was 0.1 mL. The red ginger oil added in the mixture were varied at concentration (0 %; 0.6 %; 0.9 % and 1.2 %) (v/v). The data proofs that the addition of red ginger oil gives the positive effect to the Total Phenolic Content (TPC) of film solution mixture. The highest value of Total Phenolic Content (TPC) was obtained at the using of 0.9

% concentration of oil, which was achieving 619,50 mg/L (mg of gallic acid equivalent per litre of red ginger oil).The values of Total Phenolic Content were from calibration curve of Equation (3).

$$y = 0.00389503x + 7.95865e\text{-}004 = 0.99985 \quad (3)$$

Where x is the absorbance and y is the concentration of gallic acid.

Antioxidant Activity

Antioxidant activity of chitosan film solutions enriched with red ginger oil was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) photometric assay. Samples at different concentrations of the red ginger oil were added to ethanolic solution of DPPH. The reaction mixture was mixed and kept at the room temperature in the dark. The absorbance of the mixture was measured using a spectrophotometer UV-VIS at wavelength of 515 nm. In this research, the antioxidant activity could not be measured by using DPPH method. The absorbance value of the four samples of film solutions at different concentrations of the red ginger oil could not be seen. The solutions might be too murky due to the precipitate inside the solution mixtures.

Antimicrobial Activity

Antimicrobial activity of all films formulations was analysed by using Total Plate Count (TPC) and Total Mould and Yeast Count (TMYC) method. The total plate count is the enumeration of aerobic, mesophillic organisms that grow in aerobic conditions under moderate temperatures of 20-45°C. This includes all aerobic bacteria, yeast, molds and fungi that grows in the specific agar. This count includes all pathogens and non-pathogens and is used to determine the hygienic status of food produced. Table 3 shows the result of Total Plate Count (TPC) analysis of films containing different concentration of the red ginger oil. Total Plate Count analysis according to Table 3 explains that there was an increase in antimicrobial activity with the increasing of the red ginger oleoresin concentration contained in the films. Similar results were obtained by some authors who had used essential oils to enrich chitosan based active packagings [31-33].

TABLE 3. Total Plate Count Results Analysis

Concentration of oleoresin in the film (%)	Colony per gram of sample	Average colony per gram of sample
0,6	0×10^2 2×10^2 10×10^2	$4,0 \times 10^2$
0,9	2×10^2 0×10^2 2×10^2	$1,2 \times 10^2$
1,2	2×10^2 4×10^2 4×10^2	$3,2 \times 10^2$

Total Mould and Yeast Count (TMYC) also provided in this research to study the antimicrobial activity of resulted films against mould and yeast. The test was confirmed that films produced from chitosan incorporated with red ginger oleoresin are effective to fight against mould and yeast, and it was convinced by the fact that there was no colony of mould and yeast found on the film during the test procedures.

Film Application Study

Fresh milkfish *(Chanos chanos)* was used for film application study. Figure 8 shows the milkfish samples used in this study which were unwrapped and wrapped with chitosan-based films.

FIGURE 8. Fresh milkfish treatment before the storage conditions

The organoleptic evaluation was performed and focused on the eye, gill, and the existence of mucus on the body. The data were collected from 22 people and reported through Table 4, while Fig. 8 presents the average scores of all the treatments.

TABLE 4. Organoleptic Study Before and After The Storage

Sample Conditions	Average Score					
	Before Store			After Storage		
	eye	gill	mucus	eye	gill	mucus
Unwrapped, kept in room temperature	7,86	7,05	8,77	3,77	5,32	6,27
Unwrapped, kept in refrigerator	7,86	6,41	8,59	3,09	5,00	6,00
Wrapped, kept in room temperature	8,27	8,14	8,41	6,14	7,32	7,68
Wrapped, kept in refrigerator	7,68	8,68	8,27	8,14	6,68	8,05

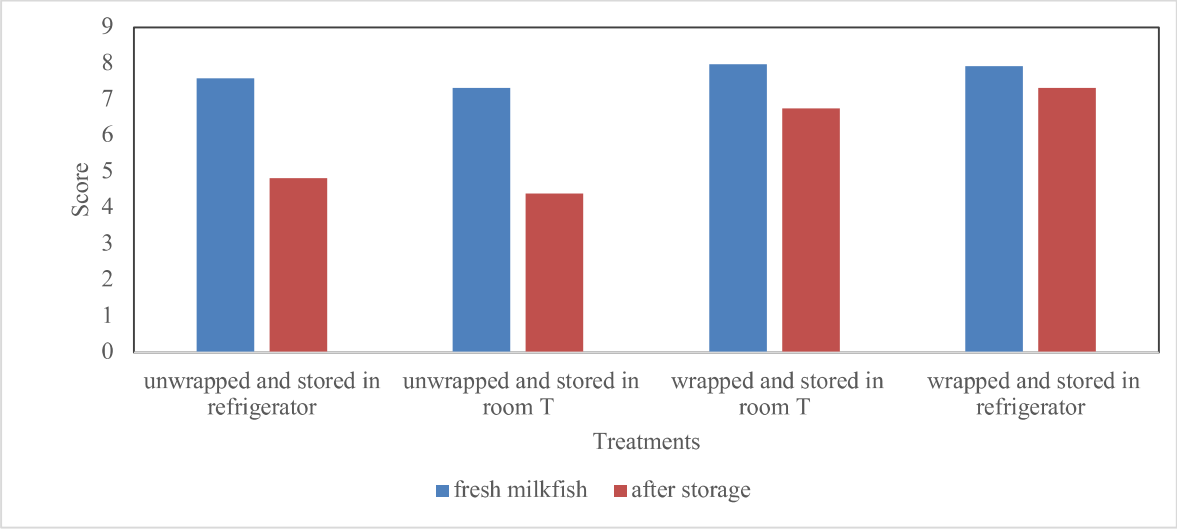

FIGURE 9. Organoleptic Study

Table 4 and Fig. 9 shows that the milkfish with film protection during the storage gave better appearance compared with the milkfish without film. After 9 h of storage at room temperature, the sample protected with film resulted higher average score than the sample without protection. The similar data was also obtained after 6 d of storage in refrigerator (4^0C). This fact proofs that chitosan based active packaging is able to extend shelf life of milkfish *(Chanos chanos*). Chitosan and red ginger oil used as materials to produce film in this study have the active substances that quite effective to inhibit the microorganisms' growth.

To confirm the resulted data from organoleptic study, the measurement of microorganism growth was also performed in this research. Table 5 presents the amount of bacteria and fungi in the fresh milkfish and in the sample after a period of storage time.

TABLE 5. Amount of Bacteria and Fungi

Storage Conditions	Bacterial count		Fungal Count	
	fresh milkfish	stored milkfish	fresh milkfish	stored milkfish
wrapped	56.10^4	23.10^5	17,7.10^1	28,7.10^1
unwrapped	65.10^3	17,7.10^5	17.10^1	65.10^1

From Table 5, it can be seen that milkfish wrapped in film has better bacteria and fungus resistance compared to sample which is not wrapped in plastic film. Bacteria and fungi cannot grow fast during storage time because it is inhibited by the active substance contained in the film layer. Chitosan is an active polymer containing antimicrobial ability, and with the addition of red ginger oil can increase its antimicrobial activity.

CONCLUSIONS

Edible active films based on chitosan, glycerol and red ginger oleoresin were successfully developed. As the content of red ginger oleoresin increased, the films showed an increase in the phenolic content but could not be detected on the antioxidant activity. The incorporation of red ginger oleoresin in glycerol plasticized chitosan films improved the thickness, tensile strength, and enhanced microbial activity of the films. Analysis of yeast and mould counts and total plate counts of protected milkfish samples containing red ginger were lower than unprotected samples. Therefore, the results indicate that chitosan-based films formulation incorporated with red ginger have potential as an active packaging for food safety and to extend the shelf-life of milkfish.

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