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Effect of *Antigonon leptopus* extract in corn starch-glycerol based film as colourimetric indicator film for monitoring fish freshness

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Abstract

A colourimetric indicator can be used to test the freshness of fish in a rapid, straightforward and non-destructive manner. Anthocyanin, a natural dye found in *Antigonon leptopus* flowers, has a sensitive colour reactivity to a wide range of pH levels and can be made into colourimetric indicator films on a laboratory scale, making it a viable replacement for artificial dyes. The study's purpose was to see how the concentration of *A. leptopus* extract affected the colourimetric assessment of fish freshness. Ethanol was used to extract anthocyanin from *A. leptopus*. FTIR and pH-respond spectroscopies were used to characterise the extracts. Colourimetric indicator films were developed to assess fish freshness by incorporating *A. leptopus* extract into a corn starch-glycerol matrix via the solution casting method, with concentrations of 10%, 30% and 50%, respectively. The effect of *A. leptopus* extract on visual aspects, thickness, morphology, FTIR spectra, UV-Vis spectra and colour responses was analysed. Microbial analysis, TVB-N contents, firmness, and pH of the fish samples were analysed after storage. The colour of the original films became darker as the extracted content increased. There was no significant ($p>0.05$) effect of *A. leptopus* extract on film thickness. SEM micrographs revealed that the composite films had homogeneous and whitish granules on the surface and that increasing the *A. leptopus* extract concentration caused the films to become rougher. FTIR and UV-Vis spectroscopies showed successful binding between *A. leptopus* extracts and corn starch-glycerol film. With increasing pH, the colour change of the films exposed to pH = 3-11 solutions was no significant difference due to improper storage. An increase in the microbial population, TVB-N content and pH was observed over the storage period as a result of fish deterioration. Colour changes were also identified in the film which became darker. Overall, colourimetric indicator film with 50% *A. leptopus* extract was found to be the optimal formulation since it had the highest values in ΔE^* during progressive spoilage of fish.

1. Introduction

Intelligent packaging films are packaging systems that can track the condition of the packaged food when it is stored or delivered in order to improve food safety and quality (Fang *et al*., 2017), which attached a colourimetric indicator film. When the pH of the food inside the package changes, the colourimetric indicator changes colour so that consumers can immediately discern between fresh and damaged food without having to open the packaging, potentially improving food quality and reducing food waste (Sani *et al*., 2021). Many researchers have used pH-sensitive dyes such as bromothymol blue, dimethylphenol blue, bromocresol green, bromocresol violet, phenol red, methyl red, and others in food packing materials to check the freshness of food. Anthocyanins have a colour reaction that is sensitive to a wide pH range (Choi *et al*., 2017). The sensitivity of anthocyanins differs depending on the plant source (Rawdkuen *et al*., 2020). For example, in their

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analysis, anthocyanin derived from butterfly pea had the strongest pH sensitivity whereas red dragon fruit and roselle had low pH sensitivity. In this study, the anthocyanins derived from a new source, *A. leptopus* were used to develop colourimetric indicator films to monitor the freshness of fish. *Antigonon leptopus* is native to Mexico, it is widely naturalized and cultivated as ornamental in warm, tropical climates around the world, such as Africa, India, Australia, North, Central and South America and numerous islands in the Pacific Ocean (Burke and Ditommaso, 2011). *Antigonon leptopus* is used to make honey as a nectar source (Burke and DiTommaso, 2011). In the West Indies and Central America, it's also used in traditional medicine. *Antigonon leptopus* contains anthocyanin, which can be extracted in 15 mg by solvent extraction (Youssef *et al*., 2021). However, less information about anthocyanin from *A. leptopus* was found in the literature.

Monitoring fish freshness is of great importance for consumers, retailers, and industries. There are many common methods used to investigate the freshness of fish (Huang *et al*., 2019). However, most of these methods generally require long analysis time and professional operators. Normal food packaging is unable to monitor the state of the packaged food or the food environment inside the package, as well as give the user accurate information (Merz *et al*., 2020). The use of intelligent food packaging, such as colourimetric indicator films, to check the freshness of fish has just been launched, although this is not a variable study. Chemical dyes are commonly used in new concepts of intelligent food packaging, such as pH-sensitive colourimetric indicators, but they are potentially toxic and harmful to humans. Natural colours are non-toxic, they should be utilised to substitute chemical dyes. Anthocyanin from *A. leptopus* could be used to replace artificial dyes because it is a natural dye with a colour reaction that is sensitive to a wide pH range (Pereira *et al*., 2015). Furthermore, employing anthocyanin from *A. leptopus* can increase its value in the food packaging industry. The purpose of the study was to determine the effect of *A. leptopus* extract concentration as a colourimetric indicator in assessing fish freshness.

2. Materials and methods

2.1 Materials

Antigonon leptopus flowers were obtained from Seberang Takir, Kuala Nerus, Terengganu in August 2021. All samples were harvested and the chosen parts, which were the proper flowers, stored at -18^{o}C. *Rastrelliger kanagurta* were purchased in August 2021 at Jeti Pulau Kambing, Kuala Terengganu, Terengganu, kept in ice (0°C) during transportation to the laboratory, and stored in -18°C.

2.2 Extraction and quantification of Antigonon leptopus extract

Antigonon leptopus flowers were dried at 50°C for 24 hrs (Memmert drying cabinet, United Kingdom). Forty grams of dried *A. leptopus* were blended with ethanol:1 M HCl mixture solution (85:15, v/v) at a solid: liquid ratio of 1:30 (Wu *et al*., 2019). The mixture was then stirred continuously for 24 hrs in the dark. The mixture was then filtered, and centrifuged at 4000 rpm for 20 mins. The supernatant was neutralised to pH 7.0 with NaOH (1 M), then concentrated at 40°C in the dark in a rotary evaporator. The concentrated solution was tested for its absorbance at the maximum wavelength (λmax) using a spectrophotometer (IRTracer-100 Spectrophotometer, Shimadzu, Japan) in the range of 400-4000cm^{-1}. The colour changes of the extract were analysed after mixing in different pH solutions (pH 3-10).

2.3 Preparation of the colourimetric indicator films

Approximately 4 g of commercial corn starch powder and 1.5 g of glycerol were dissolved in distilled water to form corn starch-glycerol solution (Prietto *et al*., 2017). The solution was heated to 80°C and stirred at 8 rpm for 2 hrs. After 2 hrs, the solution was cooled until it reached 40°C. A certain content of extract (0.1 g, 0.3 g, or 0.5 g) was then added into the corn starch-glycerol solution with continuous stirring for 30 min to obtain extract/corn starch-glycerol mixtures with extract concentrations of 10%, 30%, and 50% (w/w, based on corn starch-glycerol), respectively. The mixture was homogenized at 8000 rpm for 20 mins. The mixture was then poured on a clean mould and dried for 48 hrs.

2.4 Thickness and morphology of colourimetric indicator films

The thickness of the films was measured with a digimatic calliper (0.001mm, Mitutoyo), and the average of five different point positions measured in each film was calculated (Merz *et al*., 2020). A scanning electron microscope (SEM) (Hitachi tabletop TM1000, Hitachi High-Technologies Corp., Tokyo, Japan) was used to examine the surface section of films. The photos were taken at a 10kV acceleration voltage with magnifications varying from 300 to 1000× (Loo and Sarbon, 2020).

2.5 Colour responses analysis of colourimetric indicator films

The films were immersed in 1M HCl (pH 3.0, 4.0, 5.0 and 6.0) and 1M NaOH (pH 7.0, 8.0, 9.0 and 10.0) solutions (Pereira *et al*., 2015). Then, the pH indicator films were kept at room temperature for 10 mins prior to analysis using a chromameter (Chroma meter CR-400, Konica Minolta, Japan).

2.6 Application of the colourimetric indicator films for monitoring fish freshness

The fish samples were placed inside a container with a colourimetric indicator film attached to the bottom. The containers were stored at 4°C for 14 days. The change in film colour was visually monitored, and the colour was measured using a chromameter.

2.7 Firmness measurement

Texture Analyzer TA-XT plus (Stable Micro Systems Ltd., UK) equipped with a 36-mm diameter cylindrical probe (P/36R) and controlled by Texture Exponent 32 software was used to measure whole fish firmness by compression on the left side, approximately 2 cm from the operculum and over the lateral line. All samples were thawed at room temperature for 3 hrs prior to the texture analysis. For the determinations, the mackerel fish were compressed by 30% of their height (1 mm/s crosshead speed, 30 kg load cell) and the maximum force (N) was recorded (Wan Mohamad Din *et al*., 2020).

2.8 Microbial analysis of fish samples

Approximately 10 g of fish sample were taken under aseptic conditions and mixed with 90 mL of peptone water in stomacher bags to prepare a 10 to 1 dilution, then homogenised for 30 s at 25°C in a stomacher (BagMixer 400, Interscience, France). A 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilution series was prepared. One millilitre of 10^{-3}, 10^{-4}, and 10^{-5} dilution were pipetted onto agar. An L-spreader was used to spread the inoculum in an aseptic manner. After 24 hrs of incubation at 37°C, colony numbers were counted and reported as log CFU/g (Mohd Zin *et al*., 2021).

2.9 pH measurement

pH measurements of fish samples were performed using a pH meter. pH values were obtained using 5 g of minced fish samples with 5 mL of distilled water. The pH meter was calibrated using pH buffer solutions at pH values of 10.00±0.01, 7.00±0.01, and 4.01±0.01 at 25°C (Chong *et al*., 2020).

2.10 Determination of functional groups

The functional groups of *A. leptopus* flowers were determined using FTIR Transmission (Nicolet iS10, Thermo Scientific, US) in which 4 mg of *A. leptopus* flowers powder was mixed with 200 mg of KBr (1:50). The mixture was homogenized using agate mortar and pestle and then it was pressed into pellet (1-2 mm thick films) with a 15-ton hydraulic press. The FTIR spectra were obtained from wave numbers of 600 to 4000 cm^{-1} during 64 scans with 2 cm^{-1} resolution. The resulting spectrum represented the molecular absorption and transmission which then created a molecular fingerprint of the sample (Chew *et al*., 2020).

2.11 Total volatile basic nitrogen contents

Total volatile basic nitrogen (TVB-N) contents were determined by steam distillation based on the method described by Berizi *et al*. (2018) with a slight modification. Approximately 10 g of minced fish was blended with 50 mL distilled water and impregnated further for 30 mins with shaking every 10 mins. After that, the mixture was filtered. Approximately 5 mL of filtrate was mixed with 5 mL magnesium oxide (0.1% w/v). Steam distillation was performed using a Kjeldahl distillation unit (Vapodest 30, Gerhardt, France). The volatile base was distilled into 10 mL of 20 g/L boric acid containing methyl red and bromocresol green indicators. Then, the collected distillate was titrated with 0.01 mol/L HCl. The results are expressed as mg/100 g. The content of TVB-N was calculated using the following equation:

TVB-N content (mg/100 g) = V1-V2c×14m×5/100×100

Where V1 is the titration volume for the tested sample (ml), V2 is the titration 145 volume of blank (ml), and c is the actual concentration of HCl (mol/l), m is the 146 weight of minced pork sample (g).

2.12 Statistical analysis

All data were analysed by Minitab software and were described as the mean ± standard deviation (SD). The results were computed using one-way analysis of variance (ANOVA) and Fischer's Least Significant Difference (LSD) test, with $p < 0.05$ considered significant.

3. Results and discussion

3.1 Characterization of extract from Antigonon leptopus

3.1.1 Colour variation in Antigonon leptopus extract solutions

Figure 1 illustrates the colour variation of extract solution from *A. leptopus* in different pH solutions. The colour of the solution changed from reddish brown to yellow when the pH was raised from 3 to 10. Anthocyanins may have been present in the extract solution when the colour changed. Since anthocyanin's chemical features make it unstable and cause it to decay quickly. Aliaño-González *et al*. (2020) claimed that the

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colour change of anthocyanin solutions at different pH levels was mostly caused by structural modification. In acidic conditions, anthocyanin is present in the form of flavylium cation, and the colour of the solution is red. Flavylium cation is the predominant equilibrium form of anthocyanin in acidic conditions (Huang *et al*., 2021). Anthocyanins can be present in 4 structures under acidic conditions which include flavonoid cations (orange/red), chalcones (yellow), carbinol pseudo-base (colourless), and blue quinoidal base (blue/violet). With the increase in pH value, red flavonoid cations are reduced and colourless carbinol bases are produced. It is connected to the nucleophilic reaction between flavonoid cations and water occurring. Deprotonation of flavonoid cations causes the formation of an acidic hydroxyl group (Huang *et al*., 2021).

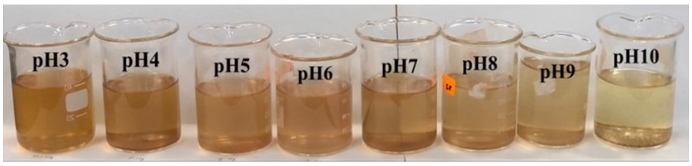

Figure 1. Colour variation of extract solution from *A. leptopus.*

3.1.2 FTIR spectra of extract solutions from Antigonon leptopus

Figure 2 depicts the FTIR spectra of *A. leptopus* extract solutions. The functional groups were separated based on their bonding site, and the presence of a diverse range of functional groups of bioactive compounds was confirmed by the peaks obtained when the extract was passed through the FTIR region (Table 1). Skoog *et al*. (2017) reported that the different functional groups will absorb radiation of a different characteristic frequency of the infrared spectrum. Ghassempour (2008) reported that the wave number that lies between 3700 cm^{-1} to 3100 cm^{-1} indicates the vibration characteristics of hydroxyl groups so the broad peak at 3348.42 cm^{-1} indicated the hydroxyl group (O-H) and the peak and wide band that corresponded to the vibration amplitude of an O-H group is highly intense. Figure 2 also shows absorption bands for saturated hydrocarbon groups, 2924.09 cm^{-1} for the methyl group ($C\text{-}H_3$) and 2854.65 cm^{-1} for the methylene group ($C\text{-}H_2$) (Vasincu *et al*., 2014). Moreover, the bands at 1643.35 cm^{-1} can be assigned to the stretching vibration of C=O groups (Favaro *et al*., 2018) or benzene skeleton vibration (Fei *et al*., 2020). Other significant

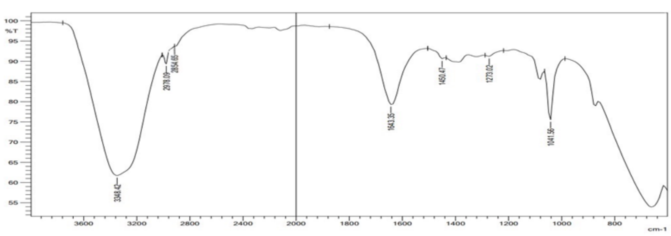

Figure 2. FTIR spectra of extract from *A. leptopus.*

Table 1. Functional group assignment of *A. leptopus* extract.

No.	Wavelength (cm^{-1})	Description
1	3348.42	Hydroxyl group (O-H)
2	2924.09	Methyl group ($C\text{-}H_3$)
3	2854.65	Methylene group ($C\text{-}H_2$)
4	1643.35	C=O Stretching
5	1450.47	C-N Vibration
6	1273.02	C=C bond
7	1041.56	Stretching vibration of C–O–C esters

bands at 1450.47 cm^{-1} and 1273.02 cm^{-1} can be referred to as C–N vibration (Favaro *et al*., 2018) and the C=C bond that arises from the pyran ring (Musa *et al*., 2019), respectively.

3.2 Physicochemical characterization of colourimetric indicator films

3.2.1 Visual aspects, thickness and morphology

Figure 3 displays the visual appearance of colourimetric indicator films containing 10%, 30% and 50% extract, respectively. The brown colour of the films is due to the addition of extract to the film formulation. The visual colour difference was confirmed in the image demonstrated in Figure 3. A similar observation of an increase in concentration of anthocyanin, the films becoming darker was also reported by Li *et al*. (2021), who extracted anthocyanin from purple tomato to create a colourimetric indicator for application in intelligent packaging. The results of colour parameters, as presented in Table 2, also verified this observation. Table 2 illustrates the colourimetric parameters (L*, a*, b* and ΔE) of colourimetric indicator films containing 10%, 30% and 50% extract. The amount of extract concentration placed into the colourimetric indicator films determined the degree of colour shift. The L* and ΔE* values fell with the addition of extract, however, the a* and b* values increased, showing a propensity toward redness and blueness. The increase in extract level causing a reduction in the L* value of colourimetric indicator films is assigned to the higher indicator dye content in the films. Nogueira *et al*. (2019), also noticed a reduction in the brightness of arrowroot starch films with blackberry powder. Moreover, Lee and Coates (2003), reported that the ΔE* had more than 2 proves that there will be a visually perceptible difference to the

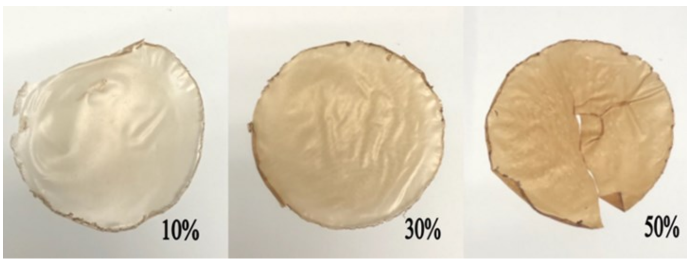

Figure 3. Visual appearance of colourimetric indicator films containing 10%, 30% and 50% of *A. leptopus* extract.

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Table 2. Colourimetric parameters (CIELab*) of colourimetric indicator films containing 10%, 30% and 50% of *A. leptopus* extract.

A. leptopus extract (%)	L*	a*	b*	ΔE*
10	86.77±0.58[c]	2.74±0.27[a]	12.19±1.11[a]	87.67±0.45[c]
30	77.74±2.69[b]	5.62±1.50[b]	19.38±2.03[b]	80.37±2.03[b]
50	68.59±5.87[a]	9.49±1.72[c]	25.90±2.23[c]	74.63±3.60[a]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different between sample by Fischer's Least Significant Difference (LSD) test, with $p < 0.05$ considered significant.

human eye between the films. Anthocyanin film with 10% has the highest ΔE* value (87.67±0.45) followed by 30% anthocyanin (80.37±2.03) and 50% anthocyanin (74.63±3.60). This could be explained by the addition of *A. leptopus* extract into the film's formulation which will change the colour appearance.

The thickness of the developed films varied between 0.096 mm (10%) and 0.108 mm (50%) (Table 3). The effect of the *A. leptopus* extract concentration on the film's thickness was not obvious. There is no significant difference observed between the thickness of colourimetric indicator films when analysed using one-way ANOVA between the percentage of *A. leptopus* extract addition. Similar results are reported by Prietto *et al*. (2017), who reported that the difference between various concentrations of anthocyanin did not affect the thickness of the indicator film in black bean seed coat and red cabbage. However, Nogueira *et al*. (2019) had different results, they found that the thickness of arrowroot starch films increased as the blackberry powder concentrations increased. Farias *et al*. (2012) also discovered that the thickness of cassava starch films increases when the plasticizer and acerola pulp concentrations rise. This difference was produced by a higher concentration of extract-integrated solids in the same mass of film-forming solution per unit area in the carrier plate.

Table 3. Thickness of colourimetric indicator films containing 10%, 30% and 50% *A. leptopus* extract.

A. leptopus extract (%)	Thickness (mm)
10	0.096±0.011[a]
30	0.101±0.011[a]
50	0.108±0.011[a]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different between sample by Fischer's Least Significant Difference (LSD) test, with $p < 0.05$ considered significant.

The surface morphology of samples with 10%, 30% and 50% anthocyanin and the images are presented in Figure 4. The surface morphology of the corn starch-glycerol-*A. leptopus* extract films appeared almost homogeneous and had whitish granules on the surface. This study displays the distinct patterns that correspond to withering ghost starch granules (Medina-Jaramillo *et al*., 2017). Corn starch-glycerol with 50% anthocyanin shows a rougher surface structure compared to 10% and 30% anthocyanin. It has been shown that various concentrations of anthocyanin alter the film's morphology. A similar result was observed by Hamzah *et al*. (2021), anthocyanin concentration increased the sago starch films becoming rougher surfaces. Mary *et al*. (2020) reported that the rough film surface indicated the anthocyanins were mixed homogeneously and a strong reaction between starch and anthocyanin existed.

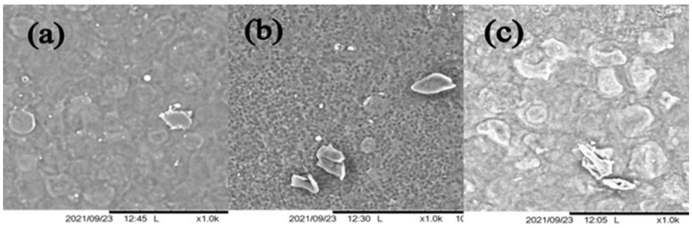

Figure 4. Surface morphology of colourimetric indicator films containing (a) 10%, (b) 30% and (c) 50% of *A. leptopus* extract.

3.3 Functional groups analysis

Figure 5 presents the FTIR spectrum for corn starch-glycerol films with (a) 10% *A. leptopus* extract (b) 30% *A. leptopus* extract (c) 50% *A. leptopus* extract. The 10%, 30% and 50% *A. leptopus* extract films showed similar results, which were depicted at 3300 cm^{-1}, 2924.09 cm^{-1}, 1643.35 cm^{-1}, 1242.16 cm^{-1} and 1010.70 cm^{-1}. The data shows that increasing *A. leptopus* extract concentration from 10% to 50% resulted in the decrease of the peak of O-H group in films from 3294.42 cm^{-1} to 3286.70 cm^{-1}. The slight reduction in wavenumber was probably due to an increase in the concentration of *A. leptopus* extract, which increases the interaction between anthocyanin polyphenols and corn starch hydroxyl groups (Merz *et al*., 2020). Corn starch was the major component in the films. The glycerol contained in the films consisted of C-H stretching vibration that shows at 2924.09 cm^{-1} in both colourimetric indicator films (Bilanovic *et al*., 2016). Liu *et al*. (2017) reported that the C=O from the amylose and amylopectin contribute to the film to withstand force and support the structure of the films. The addition of *A. leptopus* extract in the film mixture induced chemical and physical interactions with corn starch and glycerol, which involved the addition of a pyran ring detected at 1242.16 cm^{-1} and shifted to a

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higher wavenumber. The results of the current study were in agreement with an FTIR analysis by Musa *et al*. (2019) on corn starch-glycerol and anthocyanin from *Hibiscus sabdariffa*. The strong bands were found at 3378.11 cm^{-1} (O-H stretching), 2937.11 cm^{-1} (stretching vibration of C-H), 1648.33 cm^{-1} (stretching vibration of C=O), and 1417.87 cm^{-1} (CH-CH_2 bending vibration).

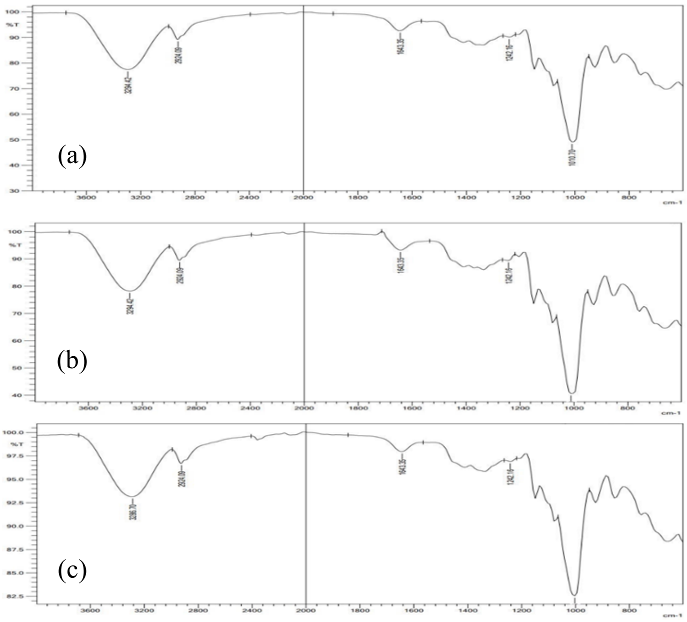

Figure 5. FTIR spectra of colourimetric indicator films with (a) 10% *A. leptopus* extract, (b) 30% *A, leptopus* extract and (c) 50% *A. leptopus* extract.

Figure 6 shows the maximum absorption for both colourimeter indicator films at 300 nm and 550 nm in the UV and Vis region. Those absorptions described the wavelength of maximum anthocyanidin detection. Qin *et al*. (2010) reported that 2 benzene rings will create a conjugated system in anthocyanin molecules in the UV-Vis region at maximum absorption around 280 nm and 500-550 nm respectively. In addition, Musa *et al*. (2019) also reported that the maximum absorbance at 520 nm indicated the presence of C=C conjugation in the anthocyanin structure. The presence of anthocyanin in colourimeter indicator film enabled the film to change colour in different pH values. However, this finding shows that the use of these films should be limited to avoid direct contact with hydrophilic chemicals, as the colour pigments may migrate to the product, which would be undesirable.

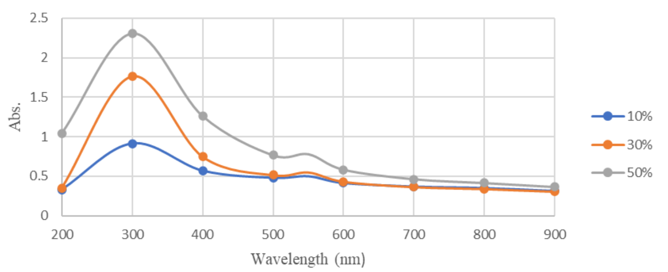

Figure 6. UV-Vis adsorption of colourimetric indicator films with 10%, 30% and 50% *A. leptopus* extract.

Furthermore, from this result, the absorbance increased as the concentration of *A. leptopus* extract was increased. This is because one of the factors that affect a sample's absorbance is its concentration. As the concentration is higher, more radiation is absorbed leading to the absorbance increase (Luong *et al*., 2011). As a result, the concentration is directly proportional to the absorbance.

3.4 Colour responses

Figure 7 illustrates corn starch-glycerol films containing 50% *A. leptopus* extract in solutions with pH values ranging from 3 to 10. When the pH was changed from 3 to 10, the colour change of the films was not obvious. This observation was proved by the finding of colour parameters, shown in Table 4. Table 4 displays the colourimetric parameters of corn starch-glycerol films containing 10%, 30% and 50% *A. leptopus* extract after soaking the films in several pH buffer solutions. There is no significant difference observed between the L*, a* and b* of colourimetric indicator films when analysed using one way ANOVA between the pH. However, previous studies reported by Luchese *et al*. (2017) had different results, who confirmed that the colourimetric indicator films are visually perceptible differences to the human eye between the samples (corn starch films containing blueberry powder). Besides that, Li *et al*. (2021) reported that the change in colour of the film with anthocyanin was from pink (pH = 3) to light

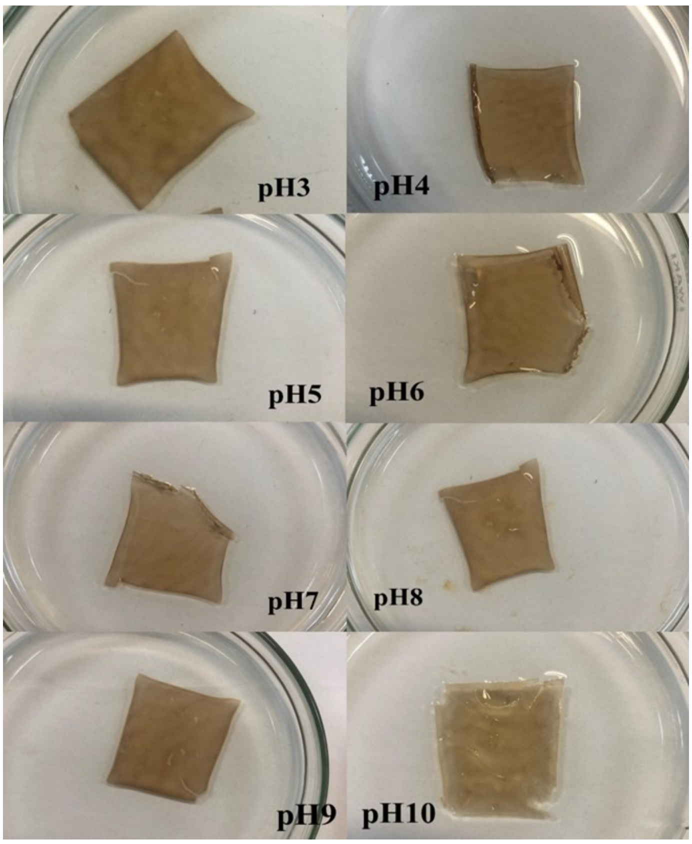

Figure 7. Colour responses of colourimetric indicator films containing 50% *A. leptopus* extract.

Table 4. Colourimetric parameters (CIELab*) of colourimetric indicator films containing 10%, 30% and 50% *A. leptopus* extract in different pH.

A. leptopus extract	pH value	Colour parameters		
		L*	a*	b*
10%	3	87.92±0.67[a]	2.23±0.08[a]	10.02±0.79[a]
	4	88.04±0.52[a]	2.35±0.10[ab]	10.28±0.30[a]
	5	87.70±0.30[a]	2.40±0.15[ab]	10.12±0.03[a]
	6	87.81±1.96[a]	2.48±0.13[ab]	10.62±0.83[a]
	7	87.37±0.82[a]	2.64±0.06[ab]	10.95±0.10[a]
	8	87.22±1.63[a]	2.71±0.21[ab]	11.02±1.19[a]
	9	86.96±1.06[a]	2.64±0.13[ab]	11.04±0.93[a]
	10	86.95±0.28[a]	2.82±0.23[b]	12.09±0.94[a]
30%	3	80.97±6.14[a]	4.49±1.87[a]	16.91±4.77[a]
	4	81.69±3.93[a]	4.23±1.50[a]	16.76±3.80[a]
	5	80.59±4.07[a]	4.43±1.39[a]	17.08±2.98[a]
	6	81.04±3.78[a]	4.43±1.35[a]	17.08±3.50[a]
	7	81.62±5.32[a]	4.06±1.54[a]	16.15±4.09[a]
	8	79.86±4.02[a]	4.65±1.37[a]	17.81±2.89[a]
	9	80.86±1.94[a]	4.32±0.82[a]	16.84±2.21[a]
	10	80.29±1.37[a]	4.46±0.65[a]	17.47±1.85[a]
50%	3	70.74±2.99[a]	7.53±2.35[a]	23.20±2.40[a]
	4	75.82±2.57[a]	6.78±2.32[a]	24.84±0.48[a]
	5	70.82±3.43[a]	8.33±2.30[a]	26.24±3.08[a]
	6	73.53±1.87[a]	7.66±0.85[a]	22.98±0.97[a]
	7	72.44±4.09[a]	7.67±1.62[a]	24.10±2.69[a]
	8	72.29±2.36[a]	9.54±2.66[a]	26.39±1.49[a]
	9	70.36±6.58[a]	9.19±2.87[a]	24.66±3.71[a]
	10	73.27±4.22[a]	9.41±2.69[a]	26.31±1.69[a]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different between sample by Fischer's Least Significant Difference (LSD) test, with $p < 0.05$ considered significant.

purple (pH = 5) to bright green (pH = 7) to light green (pH = 9) to light yellow (pH = 11).

The reason why the colour change of films was not obvious may be due to the degradation of anthocyanin that is present in the *A. leptopus* extract. The stability of anthocyanin is influenced by a number of factors such as pH, light, temperature, co-pigmentation, sulfites, ascorbic acid, oxygen and enzymes. This degradation of anthocyanin that is present in the *A. leptopus* extract may be caused by improper storage temperature of *A. leptopus* extract. Kırca *et al*. (2006) reported that the degradation of anthocyanins was clearly affected by storage temperature. The breakdown of anthocyanins was substantially faster at 37°C than it was at 4°C when stored refrigerated. Moreover, light also accelerates the degradation of anthocyanins present in *A. leptopus* extract. Anthocyanins are affected by light in 2 ways. Anthocyanin production is aided by light, but it also accelerates their destruction. Enaru *et al*. (2021), report that the greatest substantial loss of anthocyanins occurs when pigments are exposed to fluorescent light. To prevent the harmful effects of light on anthocyanins, *A. leptopus* extract should be stored in a container composed of materials that can block light from the visible spectrum, particularly the ultraviolet field of the spectrum, producing a protective barrier.

3.5 Application of the colourimetric indicator films in monitoring fish spoilage

To validate the colourimetric indicator films, this study was done using cornstarch-glycerol films with three different concentrations (10%, 30% and 50%) of *A. leptopus* extract in detecting fish deterioration. Figure 8 shows that the 50% *A. leptopus* extract level of colourimeter indicator film exhibits the highest colour change rate, followed by 30% and then 10% of *A. leptopus* extract. Among the colourimeter indicator films, the ΔE* value of the 50% *A. leptopus* extract of colourimeter indicator film expresses the highest difference between before and after storage. The ΔE* value of colourimeter indicator films with 50% *A. leptopus* extract concentration increased from 74.63 to 77.19 (Table 5). Previous studies have reported different results; in some, the colourimeter indicator film with 30% anthocyanin had the highest rate of colour change, followed by 50% anthocyanin and no variation in 10% of anthocyanin (Wu *et al*., 2019). Zhai *et al*. (2017) reported that a higher colour variation rate occurred in

Table 5. Colourimetric parameters (CIELab*) of colourimetric indicator films containing 10%, 30% and 50% *A. leptopus* extract after 14 days.

A. leptopus extract (%)	L*	a*	b*	ΔE*
10	87.73±0.73[c]	1.88±0.32[a]	12.72±1.11[a]	88.67±0.71[c]
30	77.28±3.81[b]	5.69±1.79[b]	24.00±2.03[b]	80.93±2.53[b]
50	70.52±5.35[a]	9.61±2.77[c]	29.71±2.23[c]	77.19±3.47[a]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different between sample by Fischer's Least Significant Difference (LSD) test, with $p < 0.05$ considered significant.

the colourimetric films with fewer anthocyanin and this was due to the fact that the discoloured anthocyanin took higher proportions of the total anthocyanins within the same reaction time. Colourimeter indicator films' colour fluctuation mechanism responds quickly to TVB-N in the container this can indicate fish freshness in the early phases of storage. TVB-N hydrolysed to produce hydroxyl ions after they diffused into the films, causing the environment of the film to become alkaline, and the latter induced the colour change of anthocyanins (Zhai *et al*., 2017). TVB-N value, pH value, firmness and microbial content of Indian mackerel samples were also determined.

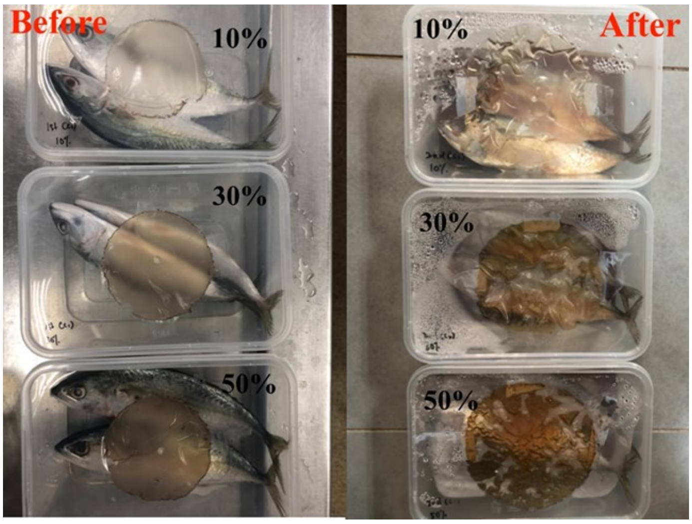

Figure 8. Colour change of colourimetric indicator films after 14 days.

Table 6 shows the changes in fish freshness after 14 days. The initial TVB-N value of the Indian mackerel sample was 7.60 mg/100 g then it increased with storage time and reached 179.2 mg/100 g after 14 days. The Chinese standard for fish (GB 2733-2015) specified that the TVB-N content of fresh fish should be less than 15 mg/100 g (National Standards of People's Republic of China, 2015). European Union Commission Regulation (EC) no. 2074/2005 (Annex II) reported that the consumption limit of TVB-N value ranges from 25 to 35 mg/100 g, which indicates that the quality of Indian mackerel has declined and is not suitable for consumption (European Union, 2005). TVB-N value is one of the most widely used methods to determine the freshness and spoilage of fish. TVB-N is an off-flavour substance that is produced by enzymes or microbial degradation of protein-rich foods which include ammonia, dimethylamine, trimethylamine and so on. The pH of the sealed package may change due to the formation of TVB-N (Zhai *et al*., 2017). Kyrana *et al*. (1997) reported that the high value of TVB-N is attributed to the high degree of protein degradation. Low molecular weight nitrogenous compounds were broken down and volatile base nitrogen was produced. The formation of volatile nitrogenous compounds causes the formation of an alkaline environment on the films (Ma *et al*., 2018). Moreover, microorganisms also cause biogenic amines and result in a rise in pH value. The pH of Indian mackerel samples was 5.67 at the start of storage and increased to 7.53 at the end. This is related to the development of alkaline substances such as ammonia compounds and TMA, which are mostly formed from microbial action at refrigeration (Chudasama *et al*., 2018). These alkaline compounds have a high pH, which causes anthocyanins to generate carbinol bases, resulting in film colour shifts.

Table 6. The change in fish freshness after storage.

	Day 0	Day 14
TVB-N content (mg/100 g)	7.60±0.24[b]	179.2±2.85[a]
pH	5.67±0.17[b]	7.53±0.28[a]
Microbial content (CFU/g)	-	1.835×10^6
Firmness (N)	1872.38±218.21[b]	1490.21±509.04[a]

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different between sample by Fischer's Least Significant Difference (LSD) test, with $p < 0.05$ considered significant.

The total aerobic mesophilic count on the spoiled fish sample was 1.835×10^6 CFU/g. The standard count method relies on bacteria growing a colony on a nutrient medium (Kuley *et al*., 2017). The nutrient agar that is used in this research for standard plate count is Plate Count Agar (PCA). PCA is not a selective medium. Bacterial growth nutrients are provided by peptone, yeast extract and glucose. Yeast extracts are the main source of B-complex vitamins. Glucose is a carbohydrate that provides energy. These nutrients, together with the nutrient factors present in the products to be evaluated will support the growth of most organisms found in the product. In a solid sample, the number of bacteria is measured in colony forming units per gramme (CFU/g).

In addition, some parasites can cause tissue breakdown, leading to undesirable texture defects in fish (Batt and Patel, 2014). Changes in texture have a direct impact on seafood freshness. The firmness of Indian mackerel changes from 1872.38 N to 1490.21 N. Prabhakar *et al*. (2020) also reported that one of the factors that affect the textural quality changes of fish is due to autolysis that produces hypoxanthine and formaldehyde.

4. Conclusion

Colourimetric indicator films containing 10%, 30%, and 50% *A. leptopus* extract were found to be compatible with corn starch and glycerol in this study. These colourimetric indicator films containing *A. leptopus* extract can be used to monitor the freshness of fish. The 50% *A. leptopus* extract film showed the most significant colour change after fish preservation. These findings imply that *A. leptopus* is a good source of anthocyanins and that colourimetric indicator films containing 50% *A. leptopus* extract can be used to determine the freshness of marine commodities like fish. However, due to improper storage of *A. leptopus* extract, the colourimetric indicator films were not responsive to colour change, and this was not noted throughout the investigation.

Conflict of interest

The authors declare no conflict of interest.

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