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Green Extraction of *Lawsonia Inermis L. Leaves* for Antimicrobial Active Packaging Film

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The demand for active antimicrobial packaging especially for perishable fresh food such as seafood is ever-increasing with the global health awareness. This work aimed to assess the feasibility of green extraction method for *Lawsonia inermis L.* leaves and the antimicrobial potential of extract as active packaging film. Green extraction of *Lawsonia inermis L.* leaves was done with distilled water using Soxhlet apparatus and the quantified lawsone was found comparable to *Lawsonia inermis L.* commercial powder extract. Different concentration of *Lawsonia inermis L.* leaves extract and commercial powder (0.30 to 0.90 g/mL) were incorporated into starch-based film for antimicrobial assay against *Pseudomonas sp*. Results revealed that *Lawsonia inermis L.* leaves extract exhibited better antimicrobial activity than commercial powder with 36 % enhancement at the highest concentration (0.90 g/mL). Antimicrobial film with the highest concentration of *Lawsonia inermis L.* leaves extract had the greatest antimicrobial activity against *Pseudomonas sp*. among all the samples. The antimicrobial assay of *Lawsonia inermis L.* leaves extract was also done on real food spoilage bacteria using fresh fish for 3 days storage. All active films exhibited comparable antimicrobial activity on day 2 (24.2 – 51.6 % reduction). Yet only the concentration with 0.90 g/mL *Lawsonia inermis L.* leaves extract still exhibited indistinguishable inhibition zone (49.8 % reduction) on day 3. This indicated that the highest concentration of green extracted *Lawsonia inermis L.* leaves extract could inhibit the real food spoilage bacteria more effectively to extend food shelf life. Taken together, the leaves extraction of *Lawsonia inermis L.* using greener method could be applicable as natural antimicrobial agent in food packaging to enhance food shelf life and food safety.

1. Introduction

Global foodborne diseases issue in which about 600 million people fall ill after eating contaminated food and cause 420,000 deaths annually (World Health Organization, 2022) has driven the development of food packaging industry. Food packaging plays a prominent role in ensuring food safety and quality especially for fresh products that involve minimal processing, easy preparation and ready-to-eat. Reduction of shopping trips during the Covid-19 pandemic indirectly encourages consumers to be more aware of the perishable fresh food with a longer shelf life and good product quality. One of the perishable fresh food that gained much interest is seafood. An estimated 33.7 Mt of fisheries and aquaculture production, accounted for 35 % of global harvest was lost or wasted yearly due to spoilage (FAO, 2020).

To enhance the shelf life and safety of fresh food, active packaging with antimicrobial functionality becomes one of the feasible and favourable options. Many antimicrobial agents were available to be utilized as single active ingredient (Vasile and Baican, 2021) or synergistic active ingredients (Ching, Keesan and Muhamad, 2022) for active antimicrobial packaging. With the emergence of antibiotic resistance and toxicity issue of commercial antibiotics, natural antimicrobial agents especially using green extraction (Foo, Salleh and Hana, 2017) gained much interest as alternative to synthetic antimicrobial agents. Plant extract from *Lawsonia inermis L (L. inermis)* is one of the promising natural antimicrobial agents. *L. inermis,* generally known as henna or inai, widely cultivated in warm and arid regions of oriental, Middle Eastern and northern African countries. They are predominantly applied as colouring dye for hair, skin, cosmetic, fingernails and fabrics. This is attributed to the

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principle dye molecule, lawsone (2-hydroxy-1,4-napthoquinone) derived from *L. inermis* leaves. Gradually, *L. inermis* becomes prominent medicinal plant as its phytochemically active compound, especially lawsone was found to exhibit great antimicrobial (Pasandi Pour and Farahbakhsh, 2020), antioxidant and anti-inflammatory properties (Sultana et al., 2021).

Since the study of *L. inermis* as antimicrobial agent in food packaging industry was scarce, this study aims to investigate the practicability of *L. inermis* leaves extract and starch matrix as active antimicrobial film for fish storage based on previous study (Khairuddin et al, 2017). Different strategies were adopted to extract the main ingredient, lawsone from *L. inermis* leaves include ultrasound-assisted extraction (Bennaceur et al., 2021), magnetic solid-phase extraction (Arkaban et al., 2021) and Soxhlet extraction (Nagarajan et al., 2013). This study implemented Soxhlet extraction method using distilled water in considering its cost-effectiveness, sustainability and feasibility. Lawsone amount was quantified and compared with *L. inermis* commercial powder. The antimicrobial efficacy of active film was examined on *Pseudomonas sp* and real food spoilage bacteria in fish storage. The pH changes of stored fish were measured as well to elucidate the deterioration process. These insights provided a strategy for fabricating active antimicrobial film for food packaging industry.

2. Methodology

2.1 Green extraction of *L. inermis* extracts

Fresh mature *L. inermis* leaves were collected in UTM, thoroughly washed with water and dried separately under shade for five days. Further drying was done at 60 °C in the oven for 5 h. The dried plant samples were ground into small particles using a mixer grinder and kept in an air-tight transparent plastic bag at room temperature before extraction. Approximate 10 g of dried samples were placed in the thimble filter and positioned into the central compartment of the Soxhlet column. 350 mL of aqueous solvent which is distilled water was placed into the borosilicate glass flask at the lower compartment. After 8 h of extraction, the solution from the Soxhlet was transferred to the rotary evaporator (Model RV 10 Digital, IKA, Malaysia) to remove the solvent through evaporation at 100 °C with maximum vacuum pressure of 24.0 mmHg for 2 h. The extract was placed in the Petri plate and stored at chiller at 4 °C. Aqueous extract of 0.1 g/mL was achieved by dissolving the extract in distilled water and centrifuging them to remove unwanted particles. As a comparison, 20 g of *L. inermis* commercial powder was dissolved in distilled water using homogenizer for 3.5 h to produce 0.2 g/mL solution. All aqueous extracts were stored in chiller prior to testing to avoid deterioration.

2.2 Quantitative analysis of lawsone

Approximate 0.1 g of *L. inermis* leaves extract was dissolved in 10 mL of distilled water and thereafter centrifuged at 5,000 rpm for 20 min to collect the clear supernatant. The absorbance of supernatant was read out at 452 nm wavelength. Similar procedure was done for the *L. inermis* commercial powder. The parts per million (PPM) value of lawsone was calculated by referring the calibration curve of concentration versus absorbance (linear regression line: y = 0.3037x – 0.0048) from Upadhyay et al. (2010). The lawsone content in the sample was then calculated using Eq(1) as follows (Korwar and Pratibha, 1999):

$$\textit{Lawsone in sample (mg/gm)} = \textit{PPM in test solution} \times \textit{Dilution factor} \quad (1)$$

2.3 Fabrication of starch-based antimicrobial film

The starch-based antimicrobial film was prepared by dissolving 5 g of starch (Merck, USA) in 100 mL of distilled water under mixing to get a homogenous mixture. When the mixture was completely dissolved, about 1.7 mL of glycerol (HmbG Chemicals, Germany) was added as a plasticizer and heated to mild boiling. *L. inermis* leaves extract and commercial *L. inermis* crude extract were varied parameters. They were added at different concentrations (0.30, 0.45, 0.60, 0.75, 0.90 g/mL extract) for a fixed 30 mL of casting mixture to perform homogenous dispersion and casting. The mixture was then casted on petri dish (10 cm diameter x 1.5 cm depth) and dried in oven at 37 °C for 48 h to produce antimicrobial active film. Pristine starch-based film without antimicrobial agents was used as control.

2.4 Antimicrobial assay

Antimicrobial assay of *L. inermis* was investigated on Gram-negative bacteria, *Pseudomonas sp* since it is primary cause of fish deterioration using agar diffusion method (Habbal et al., 2011). Spread plates were prepared by sampling 100 µL of bacteria culture. The antimicrobial active films were cut with 16 mm diameter and sterilized with UV light before placing onto the agar plate. A total of five concentrations and pristine film was studied for both *L. inermis* leaves extract and commercial powder up to 3 d to compare their effectiveness. All plates were incubated at 37 °C for 72 h and the inhibition zone measurement was done at 24 h interval.

2.5 Fish freshness, fish storage and shelf-life study

Fish storage and shelf-life study was conducted according to Khairuddin et al (2017). Fresh fish was bought from the nearest market. About 10 g of fish was blended and dissolved in 100 mL distilled water under mixing. The mixture was stored at 25 °C and the pH changes of mixture was observed every 6 h for 3 d to investigate the fish freshness. For fish storage and shelf-life study, fish was placed in room and was left for three days purposely for deterioration process. Subsequently, about 3 g of fish was blended and dissolved in 30 mL sterile distilled water with mixing. The fish solution was centrifuged to remove particles and collect the supernatant. Approximate 1 mL of fish solution was pipetted to 9 mL nutrient broth in universal bottle. The nutrient broth suspension was vortexed for 10 s to ensure homogenization. After two times of dilution, 150 µL of bacteria suspension was spread on the agar plate. Sterilized antimicrobial active film with 16 mm diameter was placed onto the agar plate and incubated at 37 °C for 72 h. The inhibition zone for *L. inermis* leaves extract was measured at five different antimicrobial concentrations and pristine film as control. Liquid culture test was done by immersing antimicrobial active films (1.5 cm x 1.5 cm) into 20 mL nutrient broth containing 150 µL bacteria suspension. The universal bottles were placed in orbital shaker with rotation at 37 °C and 200 rpm. The OD readings at 600 nm were taken using spectrophotometer (Model UV-160, Shimadzu, Japan) for every 2, 4, 8, 12, 24, 48, and 72 h during the incubation to obtain microbial growth profiles. The testing was repeated for other concentrations and pristine film as control.

3. Results and discussion

3.1 Quantification of lawsone

Lawsone content in leaves extract and commercial powder was quantified at 22.37±0.27 and 24.58±0.11 mg/gm. These were comparable to the lawsone extracted from leaves by Sarang et al., (2017) in their HPLC profile. *L. inermis* leaves extract had lesser lawsone content compared to the commercial powder which was in a good agreement with the study done by Bakkali et al. (1997). *L. inermis* commercial powder was normally added with other chemical compounds to give a better colour and it may affect the effectiveness of antimicrobial agent activity. There were several antimicrobial substances in *L. inermis* which were phenolic compound such as tannic acid, mucilage acid, and gallic acid. Sudharameshwari and Radhika (2007) stated that the antimicrobial substances in *L. inermis* was not only having phenolic compound but also containing traces of alkaloids and xanthoprotein. It could be deduced that lawsone alone was not responsible for the antimicrobial activity but with the presence of other compounds along such as phenolic and alkaloids compound.

3.2 Result of antimicrobial assay

A good antibacterial activity could demonstrate at least eight mm or greater inhibition zone (for the case inhibition zone diameter minus sample diameter) (Ali et al., 2001). Based on this calculation, results in Figure 1 showed that active film with more than 0.45 g/mL leaves extract concentration exhibiting good antimicrobial activity on day 2 and 0.9 g/mL achieved the greatest antimicrobial activity. These findings corroborated previous findings by Habbal et al., (2011) for *L. inermis* extracts from different Oman regions in inhibiting *Pseudomonas aeruginosa*.

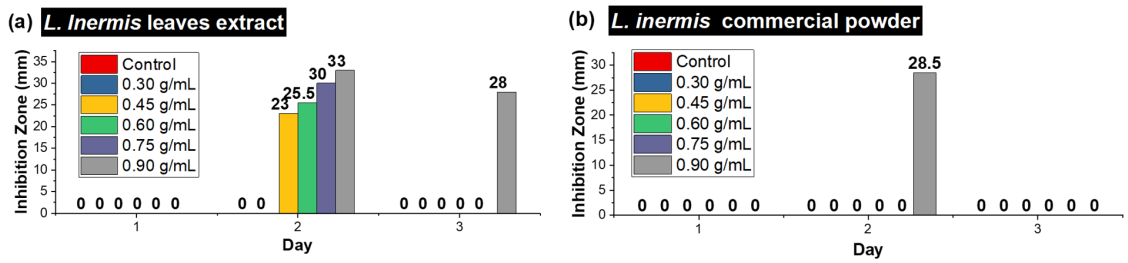

Figure 1: Antimicrobial activity of active films with (a) L. inermis leaves extract and (b) commercial powder against Pseudomonas sp

It was also noted that the antimicrobial activity improved with the increasing concentration of leaves extract. Low concentration (0.3 g/mL) of leaves extract did not perform antimicrobial activity, deducing that *L. inermis* leaves extract had low concentration of active ingredient, lawsone. Lawsone (2-hydroxy- 1,4-naphthoquinone) is the main active ingredient as antimicrobial agent to inhibit microbial growth. The free hydroxyls in lawsone are susceptible to interact with the bacterial cell wall proteins and polysaccharides (Kathem K *et al.*, 2008). Their

interaction or attachment to the enzymatic site of bacteria would then render them inactive to grow or proliferate, leading to bacterial death. It can be inferred that the higher the concentration of leave extract, the higher the lawsone concentration to exhibit better antimicrobial property.

3.3 Fish shelf-life evaluation

Two phases of pH changes in stored fish were illustrated in Figure 2. During the initial stage, the initial pH value was 6.78, which was very close to the neutral pH. Yet the pH decreased slightly every 6 h from pH 6.66 to pH 6.37 at 30th h. The pH increased from 6.37 to 6.61 at 48th h in second phase, followed by a non-remarkably pH change (pH 6.61-6.63) afterwards. The flesh's pH becomes slightly acidic in the first phase because the enzymes break down the adenosine triphosphate (ATP) to the compound that was associated to off flavoured. Prior to the bacterial activity, ATP-related molecules were degraded via autolysis to produce adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx). The autolysis then induced the bacterial spoilage in second phase of pH. Bacterial spoilage involved the decarboxylation of amino-acids and production of biogenic amines such as histamine, putrescine, cadaverine and tyramine which reduced the nutritive value of the fish significantly. The increment of pH in second phase was attributed to microbial metabolites that deteriorate the flesh. Similar positive trend was depicted for common carp and rainbow trout (Moradi et al., 2019), the pH of flesh in this study was predicted to increase continuously.

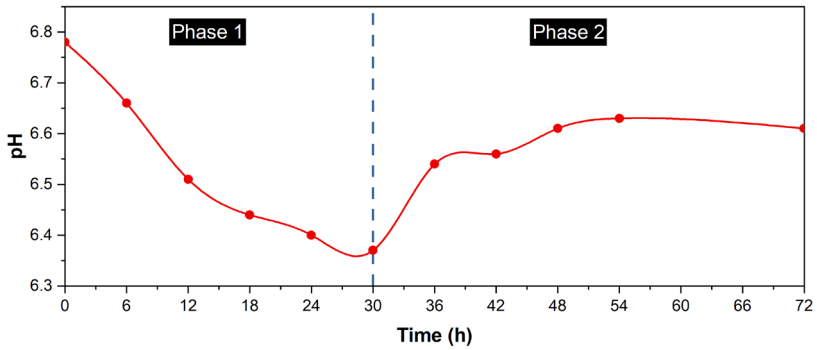

Figure 2: pH changes of stored fish

The antimicrobial activity of active films against real food spoilage bacteria was depicted in Table 1. All samples had good antimicrobial activity on day 2 but no clear inhibition zone observed on day 1. In contrast to the positive trend in previous antimicrobial study against *Pseudomonas sp*, the antimicrobial trend against real food spoilage bacteria on day 2 was fluctuated across all the concentrations. The highest inhibition zone was observed at 0.75 g/mL (3.9 % higher than 0.90 g/mL). No clear inhibition zone was also measured for all samples on day 3 except the concentration of 0.90 g/mL. Although the inhibition zone of 0.90 g/mL slightly decreased by 9.09 % on day 3, it was still effective in inhibiting microbial growth for a longer period.

Table 1: Antimicrobial activity of active films with L. inermis leaves extract against real fish spoilage bacteria

Leaves extract (g/mL)	Inhibition Zone (mm)		
	Day 1	Day 2	Day 3
0	n.d	n.d	n.d
0.30	n.d	32.5	n.d
0.45	n.d	27.5	n.d
0.60	n.d	34.8	n.d
0.75	n.d	40.0	n.d
0.90	n.d	38.5	35

*n.d= not defined

The liquid culture test further evidenced this by converting the OD to CFU/mL in accordance to the previous study (Dong-ju Kim, 2012). Since *Pseudomonas sp* is the dominating spoilage bacteria in fish products (Lone and Hans Henrik, 1996), the linear relationship between OD and CFU/mL with slope of 2.04×10^8 CFU/mL/OD (linear regression line: $Y = 2.04 \times 10^8 X + 4 \times 10^{-6}$) was referred. The changes of spoilage bacteria in all stored fish was presented in Figure 3.

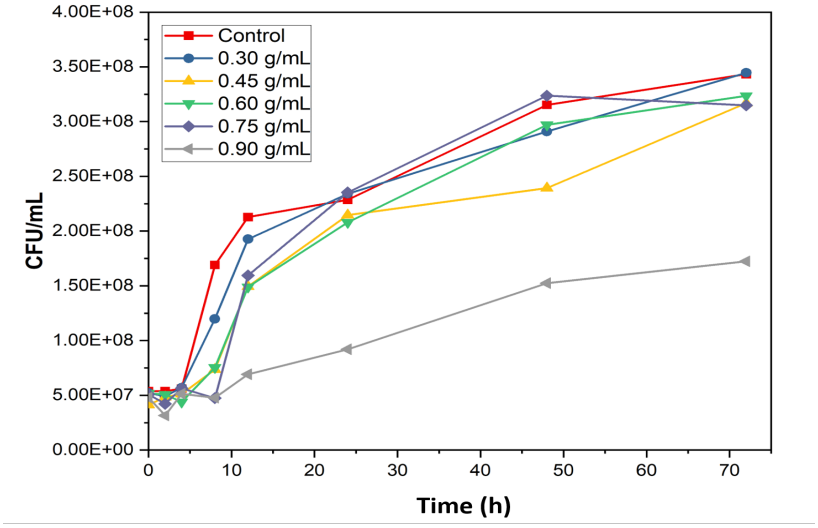

Figure 3: Antimicrobial activity of active films against real food spoilage bacteria in stored fish

The initial concentration of spoilage bacteria was approximately 4.96×10^7 CFU/mL and increased to 3.03×10^8 CFU/mL at the end of experiment. No significant difference was observed in their microbial growth profile of all active films except the film with 0.90 g/mL. It had the greatest reduction on bacteria, achieving 49.85 % of antibacterial rate. Antimicrobial activity was similar to the *L. inermis* leaves extraction reported by Pasandi Pour and Farahbakhsh (2020) and Shivsharan and Kothari (2020). Great reduction was mainly attributed to more available hydroxyls in higher concentration of lawsone that inhibit the bacterial growth. Although active film with 0.90 g/mL leave extract performed a significant antibacterial rate (59.65 %) on day 1, it was not reflected in the agar diffusion method as no clear inhibition zone was observed. This might be owing to the hydrophilic nature of starch-based active film where antimicrobial agents could be released more rapidly from film to environment in non-static liquid culture. The released antimicrobial agents could act by release-killing mechanism to inhibit microbial growth more effectively. The antimicrobial agents might also act by surface contact-killing mechanism in agar diffusion method where obvious inhibition zone was seen upon contact with bacteria. Both agar diffusion method and liquid culture test had confirmed the ability of *L. inermis* leaves extract against real food spoilage bacteria. This study also inferred that high concentration of *L. inermis* leaves extract effectively prevented fish spoilage and prolonged the fish shelf life by utilizing antimicrobial film.

4. Conclusion and recommendation

Leaves extract from *L. inermis* using greener and cost-effective distilled water with Soxhlet apparatus was shown to have indistinguishable lawsone content (8.9 % lower) with *L. inermis* commercial powder. Green extracted *L. inermis* leaves extract exhibited 36 % greater antimicrobial activity than *L. inermis* commercial powder as commercial powder might contain other chemical compounds that affect its effectiveness. A novel starch-based antimicrobial film was fabricated successfully by utilizing *L. inermis* leave extract as antimicrobial agent. Results revealed that high concentration of *L. inermis* (0.90 g/mL) had the greatest antimicrobial activity (50.7 % averagely) against *Pseudomonas sp*. The application of antimicrobial film on stored fish also confirmed the effectiveness of *L. inermis* leaves extract to inhibit real time food spoilage bacterial activity for 3 d. Antimicrobial film containing *L. inermis* leaves extract could be used as eco-friendly active packaging to enhance food shelf life, particularly, seafood. More discovery should be done on the optimization study of phytochemicals of *L. inermis* leaves extract and their effect on antimicrobial property to open up for wider applications.

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