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Research article

Sage and Rosemary Extract Gel: Anti-Aging Efficacy in D-galactose-Induced Skin Aging Model

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Abstract: This study aimed to develop a topical herbal gel incorporating Salvia officinalis Linn and Rosmarinus officinalis L. extracts to evaluate its anti-aging effects in a mouse model of skin aging. *Salvia officinalis* and *Rosmarinus officinalis* are rich in polyphenols and antioxidants, protecting against oxidative stress and inflammation, common factors in premature aging. The study involved formulating and characterizing twelve herbal gel variants containing methanol extracts from these plants. These gels exhibited desirable qualities, such as stability and viscosity, and HF4, containing carbopol 934, displayed superior release properties. HF4 demonstrated excellent extrudability, 103±1.82% w/w of net content, and 97.65 ± 1.63% cumulative release at 5 hours. In a d-galactose-induced skin aging mouse model, HF4 exhibited significant anti-aging effects, with increased dermal and epidermal layer thickness, elevated glutathione levels, and reduced malondialdehyde levels compared to the untreated group. In conclusion, this study successfully created a topical herbal gel with the potential to combat skin aging by enhancing skin structure and reducing oxidative stress. These findings suggest the promising anti-aging properties of *Salvia officinalis* and *Rosmarinus officinalis* herbal gel formulations.

Keywords: Anti-aging; Antioxidant; Rosemary; Salvia; Oxidative stress; Galactose

1. Introduction

With age and economic growth, people's life expectancies are rising, making them more concerned with their health and skincare because having healthy skin will boost their confidence. Much research has been done on creating skincare products, particularly for inhibiting and delaying skin aging. The natural and predictable aging process affects all living things, including humans. The part of the human body most obviously impacted is the skin. Skin function and aesthetic changes caused by intrinsic and extrinsic factors accelerate skin aging. Age-related intrinsic aging happens naturally, whereas extrinsic aging is brought on by outside factors, like solar ultraviolet B (UVB) radiation. Two types of skin aging can be distinguished: photoaging, which causes premature aging, and chronological aging, which is caused by aging [1]. The effects of photoaging, caused by environmental or external factors, include changes in pigmentation, a leathery appearance, and cavernous furrows [2, 3]. Several intrinsic and extrinsic factors, as well as UVB radiation, all play a role in the complex and multifactorial photoaging process. The most harmful radiation

is UVB, a high-frequency radiation associated with photocarcinogenesis [4]. Erythema, sunburn, edema, hyperplasia, melanoma, carcinogenesis, and hyperplasia can also result from prolonged skin exposure to UVB rays [4, 5].

The skin starts to wrinkle naturally as we get older. The dermis, epidermis, and subcutaneous tissue are the three separate layers of skin. Fibroblasts, collagen, elastin, and other proteins comprise the extracellular matrix (ECM), the top layer of skin. The ECM provides a structural foundation for the skin's elasticity and expansion and is crucial for maintaining the body's physiological processes. Skin wrinkles result from UVB radiation-induced premature aging, also known as photoaging, which reduces collagen biosynthesis and directly degrades collagen in the extracellular matrix (ECM). A few enzymes involved in the skin-aging process, such as collagenase, hyaluronidase, and elastase, have been linked to increased activity due to the degradation of the ECM, which is directly related to skin aging [3, 6].

UV radiation absorption by the skin can increase the production of ROS and oxidative stress. One type of oxidative damage that can happen is gene and protein modification, which can alter the structure and function of proteins [7, 8]. DNA and mitochondrial damage are other potential consequences. Increased ROS levels can activate the enzymes hyaluronidase, collagenase, and elastase, which can speed up the aging process of skin tissue [9]. In radiation-induced oxidative stress, UVB radiation can produce reactive nitrogen species (RNS) and reactive oxygen species (ROS), which can oxidize DNA proteins and peroxide lipids, indirectly damaging biomolecules.

According to studies, the Lamiaceae plant species *Salvia officinalis* Linn and *Rosmarinus officinalis* L. have high antioxidant concentrations and may be helpful in photoprotection and anti-aging. In our earlier studies, the anti-aging and antioxidant properties of sage and rosemary were extracted and evaluated. These plants were chosen based on their conventional, historical, and traditional use in cosmetics and previous reports of their antioxidant activity in research studies or the researchers' lab, even though these results have not yet been published. It has been demonstrated that these two plants have the power to scavenge free radicals and shield cellular components like proteins, lipids, and DNA macromolecules. They have also been shown to have the ability to change a variety of signaling pathways, including those involved in cell growth, apoptosis, and inflammation. Despite these well-known biological properties of *Salvia officinalis* Linn and *Rosmarinus officinalis* L., there are currently no studies or reports on its application in topical formulations to prevent UVB radiation-induced oxidative damage [10-16].

Therefore, in this present study, a topical herbal gel was developed using leaves & flowers methanol extract of *Salvia officinalis* Linn (MES) and *Rosmarinus officinalis* L. (MER), and anti-aging properties of both MES and MER in a topical gel are not reported in animal/preclinical studies, these plants were appraised for anti-aging action to discover the systematic evidence for their usage in the management, treatment, and deferring of aging and associated problems. MES and MER were fabricated as a gel because it is simple to administer, have a localized effect, don't hurt or irritate when applied, don't have a first-pass effect, don't initiate gastro-intestinal metabolic degradation, and can be applied right to the affected zone. The leaves & flowers of MES and MER were used to create a topical gel formulation because, although all parts of the plants are used as medicines, leaves and flowers are primarily rich in phenolic and flavonoid compounds. As evident from a plethora of literature, a higher dietary intake of carbohydrates, especially glucose and galactose, has been linked to skin aging. In fact, through nonenzymatic glycation, the covalent attachment of sugar to a protein, and subsequent production of advanced glycation end products (AGEs), carbohydrates can harm the essential components of the skin. Therefore, to evaluate the anti-aging potential of the topical gel fabricated to contain MES and MER, a d-galactose-induced skin-aging animal model was utilized.

2. Materials and Methods

2.1 Drugs and chemicals

Carbopol 934, carbopol 940, and propylene glycol were purchased from Sigma-Aldrich (St. Louis, MO, USA. DTNB solution (Ellman's reagent), thiobarbituric acid, TCA (trichloroacetic acid), disodium edetate, and triethanolamine were purchased from Himedia Laboratories in Mumbai, India. All other chemicals and reagents used in the study were of reagent grade and were commercially available (SRL Mumbai, E. Merck India).

2.2 Authentication and preparation of extracts

Salvia officinalis and *Rosmarinus officinalis* were collected in the late spring from the Dehradun region. A botanist authenticated the collected flowers and leaves, and voucher specimens (AKG/SO/2022/11 and AKG/RO/2022/12) were preserved for future reference. The flowers and leaves were dried in the shade and cut into small pieces. The powdered flowers and leaves (2000 gm) underwent maceration using petroleum ether at room temperature for three cycles of 48 hours each to remove fats. The resulting extracts were air-dried and further subjected to methanol extraction. The methanol extracts were collected and concentrated under reduced pressure at 40 to 50°C. This process yielded dark greenish-brown concentrated methanol extracts of Salvia officinalis (MES) and Rosmarinus officinalis (MER) leaves and flowers. The final extracts were stored at 4°C until they were used.

2.3 Fabrication of the gel base

To avoid agglomeration, carbopol 934 was gradually dissolved with stirring for an hour in 60 mL of demineralized water. Disodium edetate and triethanolamine were dissolved in 10 mL of demineralized water and stirred for 10 minutes. An aliquot of 4.83 mL of propylene glycol was added to 12 mL of demineralized water and stirred for 10 minutes. After adding the disodium edetate and triethanolamine solutions, the pH of the Carbopol solution was raised to 7.4 by stirring the mixture for ten minutes. A clear and uniform gel base was then achieved by adding the propylene glycol solution and stirring for 10 minutes [17].

Table 1. The composition for the herbal gel formulations.

	Gel Formulation code											
Ingredients	**HF1**	**HF2**	**HF3**	**HF4**	**HF5**	**HF6**	**HF7**	**HF8**	**HF9**	**HF10**	**HF11**	**HF12**
MES (g)	0.5	1	1.5	2	2.5	3	0.5	1	1.5	2	2.5	3
MER (g)	0.5	1	1.5	2	2.5	3	0.5	1	1.5	2	2.5	3
Carbopol 934 (g)	1.5	1.5	1.5	1.5	1.5	1.5	---	---	----	----	----	---
Carbopol 940(g)	---	----	----	----	---	----	1.5	1.5	1.5	1.5	1.5	1.5
Triethanol amine (g)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Disodium EDTA (g)	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Propylene Glycol (g)	5	5	5	5	5	5	5	5	5	5	5	5
D.M. water (100 g)	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

2.4 Preparation of gel formulation

Twelve topical gel formulations were prepared following the drug formulation manual [18]. For formulations HF1 to HF6, 1.5% of the carbopol 934 as gel base was used, while for formulations HF7 to HF12, 1.5% of carbopol 940 as gel base was utilized. MES and MER were separately weighed and slowly introduced into the Carbopol dispersion (the gel base), with continuous stirring to ensure thorough mixing and homogeneity. The specific composition details for each formulation can be found in Table I. Among the formulations, HF4 prepared using carbopol 934 exhibited superior quality characteristics and was selected for further evaluation of its anti-aging activity.

2.5 Characterization

2.5.1 Assessment of active constituents

The individual gel formulation was prepared by adding the required amount of methanol to 1 g of the formulation in a 50 mL volumetric flask. The flask was vigorously shaken to ensure the active ingredients in the methanol were dissolved. After that, Whatman filter paper was used to filter the resulting solution. The filtrate was carefully separated into an aliquot of 0.1 mL diluted with methanol to a final volume of 10 mL. The concentration of the active constituents in the solution was determined spectrophotometrically by utilizing a standard curve constructed at 280 nm, corresponding to the maximum absorbance (λmax) of the active constituents in the extracts [19].

2.5.2 Extrudability and measurement of the pH

The gel weighed approximately 20 g and was contained in a closed collapsible tube. A clamp was used to stop any rollback while applying firm pressure at the crimped end to extract the gel. After removing the tube's cap, the extruded gel was gathered and weighed. The weight of the extruded gel was then used to calculate the percentage of gel successfully extruded from the tube [20]. Using a digital pH meter, the gel's pH was determined. The pH meter's glass electrode was completely submerged in the gel system to ensure it was completely covered. The pH measurement was performed three times to obtain triplicate readings, and the average value of the three measurements was recorded [21].

2.5.3 Appearance, homogeneity and viscosity

The homogeneity and physical attributes of the prepared gels were evaluated visually. The viscosity of the gel was determined using a Brookfield viscometer. The measurement was conducted at 25°C, with the viscometer's spindle speed set at 12 rpm. This allowed the gel's viscosity to be determined [22].

2.5.4 Spreadability

Two glass slides of standard dimensions were utilized for this experiment. The herbal gel formulation was applied onto one of the slides, and the other slide was placed on top of the gel, creating a sandwich structure where the gel was enclosed between the two slides. The gel occupied a 7.3 cm area along the slides. A weight of 100 g was then placed on the upper slide to ensure uniform pressure, forming a thin layer of gel. The weight was subsequently removed, and any excess gel adhering to the slides was carefully removed. The two slides, now in position, were securely fixed to a stand without any disturbances. Only the upper slide was allowed to slide off freely due to the force exerted by the weight attached. A 20 g weight was carefully tied to the upper slide, and the time taken for the upper slide to travel the distance of 7.3 cm and separate from the lower slide under the influence of the weight was recorded. This experiment was repeated thrice, and the mean time was calculated for further analysis [23]. Spreadability was computed employing the succeeding formula: $S = m \times l/t$, and in the formula, S denoted spreadability, m denoted weight knotted to upper top slides (20 g), l denoted the length of the glass slide (7.3 cm), and t denotes the time taken in sec.

2.5.6 Diffusion study In vitro: permeation using rat skin

In vitro diffusion tests were carried out on all formulations using a Franz diffusion cell. The diffusion area was 3.9 cm2 in the diffusion cell apparatus, which had an open-ended cylindrical tube design and was made locally. Its area was 3.8983 cm^2, and its height was 100 mm. A phosphate buffer solution with a pH of 7.4 was used as the receptor media. As the dialysis membrane, rat abdominal skin (dorsal skin of the rat and 0.6 mm in thickness) was used. To ensure that the rat skin's stratum corneum side was in direct contact with the release surface of the formulation, it was carefully attached to the diffusion cell, specifically the donor cell. An isotonic phosphate buffer solution with a volume of 100 mL and a pH of 7.4 was added to the donor compartment before mounting the diffusion cell. The rat skin was applied with a weighed amount of the formulation that was 1 g of gel thick, slightly submerged in 100 mL of constantly stirring receptor medium. A constant temperature of 37±1 ^oC was maintained throughout the entire system. A 5 mL aliquot was taken at predetermined intervals up to 8 hours and spectrophotometrically estimated at 280 nm. The diffusion medium was replaced with an equal volume of fresh diffusion medium following each withdrawal. To evaluate the release of the active ingredients from the formulation, the cumulative percent release was calculated for each period (in hours) [23].

2.5.7 Release kinetics

The obtained data were fitted to various mathematical models to determine the release pattern of the active constituent from the herbal gel [24]. The concentration-dependent first-order kinetics model and the concentration-independent zero-order kinetics model were considered when analyzing the release behavior. Additionally, the possibility of drug release occurring through mechanisms such as swelling, erosion, or simple diffusion was taken into account [24]. Higuchi's model was employed to confirm the nature of the release reaction and validate the data.

2.5.8 Stability study

The main objective of stability testing or studies is to show how a drug product's quality changes over time, primarily due to temperature and humidity. A six-month stability study was carried out on a topical herbal gel formulation following the standards established by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The selected formulation, consisting of 2% MES and MER, was subjected to stability testing in a dedicated stability chamber. The stability chamber used for the study was a floor-standing model with three units, each equipped with an individual humidity and temperature controller. The chamber dimensions were 300 x 300 x 300 mm, and the temperature and humidity conditions were set as follows: 40°C ± 2°C/75% RH ± 5% RH, 32°C ± 2°C/60% RH ± 5% RH, and 25°C ± 2°C/60% RH ± 5% RH. During the stability study, samples were withdrawn at specific intervals, including the initial time point, and at the first, second, third, and sixth months. These samples were then evaluated for any changes in homogeneity, color, pH, odor, viscosity, microbial load, and net content and subjected to a sterility test. These evaluations aimed to assess the stability and quality of the herbal gel formulation over the designated period [17].

2.6 Anti-aging activity

2.6.1 Evaluation of the anti-aging activity of gel formulations

The anti-aging properties of the formulation were assessed using a galactose-induced aging model in albino mice, as described previously [25]. The study protocols were approved by the Institutional Animal Ethics Committee (2210/PO/Re/S/23/CCSEA). Male Swiss albino mice, aged 6-8 weeks, weighing between 20g and 30g, were used for the experiments. The mice were housed under standard conditions with a 12-hour light-dark cycle and provided with pellet food and water ad libitum. Except for the control group, all the mice were administered galactose (100 mg/kg body weight subcutaneously) once daily for 42 days, as reported earlier [25]. The normal control group received a daily subcutaneous saline injection (1 mL/kg body weight) for 42 days. In addition to galactose administration, the test group was also treated with topical applications of formulations (HF4) on a 2 mm × 2 mm square area located on the lower abdomen region of the mice. This treatment regimen was followed for 42 days. On the 43rd day, the mice were euthanized, and their skin was excised for further analysis of biochemical parameters, including glutathione (GSH) and malondialdehyde (MDA) levels, as well as histopathological examinations.

2.6.2 Histopathological studies

The collected skin samples were preserved and fixed in a 10% v/v formalin solution in neutral phosphate buffer to facilitate histopathological analysis, following the standard procedures outlined previously [26]. The formalin-fixed skin tissues were then embedded in paraffin using conventional techniques. Subsequently, a microtome cut sections of approximately 5 μm thickness from the embedded tissues. These sections were stained with haematoxylin and eosin (H&E) for histopathological examination. Microphotographs of the stained skin specimens were captured using a digital camera mounted on an Olympus BX51 microscope with a 2x objective lens. The obtained microphotographs were used to analyze the histopathological results. The Image J program, an image analysis system, was employed to calculate the thickness of the epidermal and dermal layers in micrometers (μm) based on the captured images.

2.6.3 Evaluation of antioxidant parameters

2.6.3.1 Estimation of the content of glutathione (GSH)

The skin samples obtained from the treated animals were processed following the procedure outlined elsewhere [27, 28]. Glutathione (GSH) content was measured in nanomoles (nM). To prepare the stock standard solution, 0.0307 g of GSH was dissolved in a final volume of 100 mL of 0.2 M EDTA solution, resulting in a concentration of 0.001 M. After the final treatment, all the animals in the different groups were euthanized 1 hour later. The freshly collected skin samples were weighed (Precisely weighed out around 0.5 to 1 gram of the excised skin tissue) and homogenized using a mixer with 1 mL of 0.2 M EDTA for 10 minutes. The homogenate was then centrifuged, and 0.5 mL of the supernatant was mixed with 2 mL of tris buffer. To each tube, 50 μL of DTNB solution (Ellman's reagent) was added and thoroughly mixed using a vortex mixer. The

absorbance of the samples was read at 405 nm within 2-3 minutes after the addition of DTNB. The results were expressed as nanomoles per milliliter (nmol/mL).

2.6.3.2 Estimation of the content of malondialdehyde (MDA)

For the determination of malondialdehyde (MDA), the lipid peroxidation method was employed [28, 29]. The experiment utilized an analytical kit from Himedia Laboratories in Mumbai, India. Briefly, fresh skin samples were weighed, homogenized, and centrifuged. The resulting supernatant (0.5 mL) was mixed with 2 mL of a reagent consisting of 0.5% TBA (thiobarbituric acid), 20% TCA (trichloroacetic acid), and 0.25 M HCl in a ratio of 1:1:1 (v/v). The mixture was then heated and cooled in a water bath for 15 minutes. The absorbance of the solution was measured at 532 nm using a spectrophotometer. The results were expressed as micromoles per milliliter (μmol/mL).

2.7 Statistical analysis

Data analysis was performed using the GraphPad Prism Software package. The results are presented as mean ± SD (standard deviation). A one-way analysis of variance (ANOVA) was conducted to compare multiple groups, followed by Dunnett's test as *post hoc* to determine the statistical significance. A *p*-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

Among various topical semisolid preparations, gel formulations are generally preferred for several reasons. They offer numerous advantages such as prolonged residence time on the skin, high viscosity, moisturizing effects for flaky skin due to their occlusive properties, enhanced bioadhesives, reduced irritation, independence from the water solubility of active ingredients, ease of application, and improved release characteristics [30, 31]. Numerous studies have highlighted the anti-inflammatory, antioxidant, protective, and anti-aging activities of flavonoids and phenolic compounds found in herbs. Moreover, these polyphenolic flavonoids, including rosmarinic acid and apigenin, have demonstrated the ability to penetrate the human skin [32-35]. Based on these findings, a topical herbal gel formulation was developed to incorporate these flavonoid and phenolic compound extracts to prevent and treat skin aging and associated conditions.

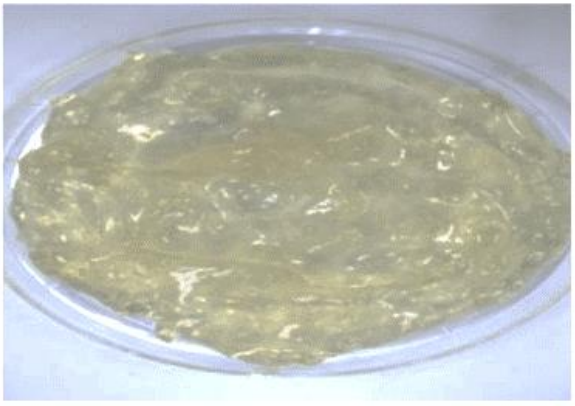

Figure 1. The gel formulation

3.1 Fabrication and evaluation of topical herbal gel

Diverse concentrations (0.5 to 3% w/w) of the extract (MES and MER) were used to fabricate a total of twelve gel formulations (HF1 to HF12). The gels were formulated with either 1.5% Carbopol 934 or 1.5% Carbopol 940 polymer. These polymers were chosen as gelling agents due to their desirable properties, such as biodegradability, bioadhesives, biocompatibility, lack of irritation, and inability to be absorbed into the body. Previous studies reported that Carbopol 934 exhibits superior gelling properties compared to Carbopol 940 (Blanco-Fuente et al., 1996), which aligns with our findings. In the gel formulation, the carbopol 934 polymer demonstrated promising potential as a carrier for the controlled release of active phytoconstituents.

The polymer's concentration was optimized by creating gels with various concentrations ranging from 0.5% to 2.5%. This optimization process determined that a gel formulation containing 1.5% of either Carbopol 934 or Carbopol 940 fulfilled the requirements for gel formulations and demonstrated compatibility with the desired characteristics.

According to the outcomes of the quality control tests, it was clear that Carbopol 934 (HF1 to HF6), the gelling agent used to create the gel formulations, exhibited superior qualities to Carbopol 940 (HF7 to HF12), except for the Spreadability parameters, where Carbopol 940 performed better. Therefore, the six herbal topical gel formulations (HF1 to HF6) made with Carbopol 934 were the only ones used in the in vitro diffusion studies. Furthermore, studies on in vitro release and stability were conducted specifically for the best herbal gel formulation, HF4. Studies have suggested that dimethylsulfoxide (DMSO) and propylene glycol are effective permeation enhancers in permeation enhancement [36]. However, due to reports of skin erosion caused by DMSO, propylene glycol was chosen as the permeation enhancer in preparing the gel formulation [37, 38]. Disodium edetate and triethanolamine were included in the formulation to adjust the pH. Overall, the selection of Carbopol 934 as the gelling agent, the use of propylene glycol as the permeation enhancer, and the inclusion of triethanolamine and disodium edetate to adjust pH contributed to the development of a superior herbal gel formulation with desirable characteristics for further in vitro study for release profile and stability.

3.2 Characterisation of the formulated topical gel

Using carbopol polymers, twelve gel formulations designated as HF1 to HF12 were fabricated, and their physical characteristics were evaluated. The evaluations included assessments of viscosity, pH, physical appearance, spreadability, extrudability, in vitro diffusion profile, and net content. The outcomes obtained from these evaluations were compared against the acceptable limits specified in the ICH guidelines, and the detailed findings are presented in Table 2. The prepared gel formulations exhibited homogeneity and a favorable appearance, demonstrating good consistency. Since the pH range for all of the formulations was between 7.33 and 7.69, they were unlikely to irritate the skin, a conclusion further supported by the skin irritation study conducted. The inclusion of polymers in the designed topical formulations was aimed to ensure the prompt release of the drug and maintain the drug concentration within the therapeutically effective range. No discernible difference in viscosity was seen by maintaining the polymer concentration at 1.5% in all gel formulations. Notably, a viscosity value between 0.38 and 0.39 poise has been reported as ideal for topical gel formulations developed using carbopol polymers [39]. The Spreadibility values obtained for the gel formulations demonstrated their ease of Spreadibility. More than 90% of the contents of the HF1 to HF6 gel formulations could be extruded, indicating excellent extrudability. However, in the case of HF1 and HF3, 80% of the contents were extrudable, which still falls within the excellent range (>80% extrudability: good). Overall, the gel formulations exhibited favorable extrudability characteristics, with the majority surpassing the 80% extrudability threshold.

3.3 In vitro diffusion profile and release kinetics

Table 3 presents the in vitro diffusion profiles of formulations HF1 to HF6. To carry out the gel formulations' in vitro release studies, a phosphate buffer saline solution with a pH of 7.4 was selected as the medium, considering that the pH range of the membrane used was 5 to 7.8. Notably, the drug release *in vitro* for all 6 formulations formulated with carbopol 934 demonstrated an impressive release performance, with nearly 100% of the formulation contents being released within 5 hours.

Table 2. Characterizations for the topical gel formulations fabricated with Carbopol 934 at 1.5% concentration.

Code	Conc (%)	pH*	Viscosity* (poise)	Spreadability* g cm/sec	Net content* % w/w	Extrudability*	Physical appearance
HF1	0.5	7.48 ± 0.98	0.3841 ± 0.0011	33.08 ± 1.30	99.82 ± 1.96	Good	Homogenous, translucent and dark-greenish
HF 2	1.0	7.48 ± 0.97	0.3853 ± 0.0022	46.14 ± 1.11	104.54 ± 2.79	Excellent	Homogenous, translucent and dark-greenish
HF 3	1.5	7.68 ± 0.94	0.3863 ± 0.0021	57.48 ± 1.33	104.23 ± 1.91	Good	Homogenous, translucent and dark-greenish
HF 4	2.0	7.51 ± 0.91	0.3874 ± 0.0022	65.12 ± 1.08	104 ± 1.992	Excellent	Homogenous, translucent and dark-greenish
HF 5	2.5	7.69 ± 0.98	0.3882 ± 0.0013	72.47 ± 1.42	103 ± 1.82	Excellent	Homogenous, translucent and dark-greenish
HF 6	3.0	7.33 ± 0.99	0.3902 ± 0.0020	76.83 ± 1.21	102 ± 1.92	Excellent	Homogenous, translucent and dark-greenish

Data are preseneted as Mean ± SD for n =3.

Table 3. *In vitro* diffusion profile of HF4

TIME (Hr)	Gel Formulations					
	% % Cumulative Release					
	HF1	**HF2**	**HF3**	**HF4**	**HF5**	**HF6**
1	7.45 ± 0.98	16.91 ± 0.99	9.63 ± 0.99	16.59 ± 1.15	10.46 ± 0.98	12.87 ± 1.08
2	15.52 ± 0.99	33.38 ± 1.08	23.84 ± 0.99	32.57 ± 1.27	25.63 ± 1.11	26.79 ± 1.32
3	31.61 ± 1.03	68.77 ± 1.29	46.82 ± 1.82	51.77 ± 1.42	49.68 ± 1.49	54.36 ± 1.77
4	40.12 ± 1.01	88.75 ± 1.14	62.54 ± 1.56	68.63 ± 1.57	66.94 ± 1.39	64.44 ± 1.59
5	64.91 ± 1.24	78.69 ± 1.52	77.79 ± 1.87	97.65 ± 1.63	82.91 ± 1.59	81.28 ± 1.85

Table 4. Kinetic modeling of *In vitro* release data of HF4

Code for Formulations	Zero-order	First Order	Higuchi diffusion model	Best fitted model
	Qt=Q0+k0t	lnQt=lnQ0+k1t	Qt=kHt	
	R^2	R^2	R^2	
HF1	0.918 ± 0.001	0.834 ± 0.001	0.639 ± 0.000	Zero-order
HF2	0.843 ± 0.001	0.834 ± 0.001	0.737 ± 0.001	Zero-order
HF3	0.973 ± 0.002	0.876 ± 0.001	0.721 ± 0.000	Zero-order
HF4	0.978 ± 0.002	0.827 ± 0.002	0.733 ± 0.001	Zero-order
HF5	0.975 ± 0.001	0.864 ± 0.002	0.723 ± 0.000	Zero-order
HF6	0.976 ± 0.001	0.889 ± 0.001	0.754 ± 0.001	Zero-order

Note: Qt = amount of drug released at time t, Q0 = initial amount of drug in the solution, k0 = zero-order release constant, k1 = first-order release constant and kH = Higuchi dissolution constant

The drug release *in vitro* of the fabricated topical gel exhibited promising characteristics comparable to the commercially available gel. Among the different formulations (HF1 to HF6), HF4 demonstrated superior release performance (97.65±1.63%) compared to HF1, HF2, HF3, HF5, and HF6 (as depicted table 3). According to our kinetic release study results, the HF4 formulation had zero order release kinetics, which is good for prolonged release. Therefore, the gel formulation with 2% of each MES and MER was chosen for additional in vivo research.

In contrast to the commercial gel formulation, which released roughly 90% of its content in 3 hours, the HF4 formulation with 2% of MES and MER showed a prolonged release of active ingredients for up to 5 hours (almost 100% release). Because of its prolonged release profile, the HF4 formulation can be used for sustained release, improving patient compliance. The gel formulation containing 2% of each MES and MER followed zero-order release kinetics, according to the release data obtained using various mathematical models (Table 4). For subsequent *in vivo* studies, the HF4 gel formulation was chosen.

3.4 Stability study

To ensure consistent quality, safety, and efficacy over its shelf life, a stability study was conducted per the guidelines established by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The HF4 formulation prepared using carbopol 934 demonstrated superior quality characteristics and was subjected to the stability study. Throughout the stability testing period of 0, 1, 2, 3, and 6 months, the topical herbal gel formulation exhibited no noticeable changes in color, odor, homogeneity, pH, viscosity, or net content. These parameters are crucial indicators of the formulation's

stability. Based on the results obtained from the study (refer to Table 5), it is evident that the formulated topical gel HF4 remains stable over time.

3.5 Anti-Aging activity

3.5.1 Appraisal of anti-aging activity: Histopathological assessment

The subcutaneous administration of galactose caused the aging of the skin for 42 days at a dose of 100 mg/kg body weight (bwsc). After 42 days, the disease control group saw a significant reduction ($p < 0.01$) in the thickness of the epidermal and dermal layers. The thickness of the epidermal layer decreased from 52.55 μm to 40.16 μm. The thickness of the dermal layer decreased from 656.9 μm to 587.0 μm when compared to the control group (Group 1) (Fig. 2). In Treatment Group 3, galactose was administered at a dosage of 100 mg/kg bwsc for 42 days, along with the topical application of the gel. The topical application of the gel formulations showed a significant increase ($p < 0.05$) in the thickness of the epidermal layer and a significant increase ($p < 0.05$) in the thickness of the dermal layer compared to the disease control group (Group 2) (Fig. 3). The epidermal thickness increased from 40.16 μm (in the disease control group) to 41.64 μm due to the topical application of the gel formulation. When the gel formulation was applied topically, the epidermal thickness increased from 40.16 μm (in the disease control group) to 41.64 μm. Due to the topical gel formulation, the dermal thickness increased from 587.0 μm (in the disease control group) to 862 μm.

Table 5. Stability study data of HF4

Storage condition 1						
S. N.	**Evaluation Parameters**	**Topical herbal gel formulation (HF4) (2% w/v of each MES and MER)**				
		25 °C ± 2 °C/60% RH ± 5% RH				
		Months				
		0	1	2	3	6
1	Color	No colour change				
2	Odor	No Odour change				
3	Homogeneity	Homogeneous				
4	pH	7.35 ± 0.98	7.33 ± 0.97	7.29 ± 0.98	7.28 ± 0.96	7.25 ± 0.921
5	Viscosity (poise)	0.384 ± 0.0020	0.384 ± 0.0021	0.382 ± 0.0022	0.374 ± 0.0011	0.372 ± 0.0020
6	Net content (%)	99.78 ± 1.91	99.84 ± 1.94	99.14 ± 1.69	98.82 ± 1.89	97.91 ± 1.98
7	Microbial load (Bacteria & Fungi)	Observed no microbial growth Time points: 24 h, 48 h and 72 h				
8	Sterility test	Observed no microbial growth Time points: 24 h, 48 h and 72 h				

Table 5. Stability study data of FH4

Storage condition 2						
				32 °C ± 2 °C/60% RH ± 5% RH		
				Months		
		0	1	2	3	6
1	Color			No colour change		
2	Odor			No Odour change		
3	Homogeneity			Homogeneous		
4	pH	7.35 ± 0.98	7.33 ± 0.97	7.34 ± 0.98	7.28 ± 0.96	7.25 ± 0.921
5	Viscosity (poise)	0.384 ± 0.0020	0.384 ± 0.0021	0.383 ± 0.0021	0.374 ± 0.0011	0.372 ± 0.0020
6	Net content (%)	99.78 ± 1.91	99.84 ± 1.94	99.36 ± 1.99	98.82 ± 1.89	97.91 ± 1.98
7	Microbial load (Bacteria & Fungi)			Observed no microbial growth Time points: 24 h, 48 h and 72 h		
8	Sterility test			Observed no microbial growth Time points: 24 h, 48 h and 72 h		
Storage condition 3						
				40 °C ± 2 °C/75% RH ± 5% RH		
				Months		
		0	1	2	3	6
1	Color			No colour change		
2	Odor			No Odour change		
3	Homogeneity			Homogeneous		
4	pH	7.33 ± 0.98	7.33 ± 0.98	7.33 ± 0.98	7.33 ± 0.98	7.33 ± 0.98
5	Viscosity (poise)	0.386 ± 0.0012	0.386 ± 0.0012	0.386 ± 0.0012	0.386 ± 0.0012	0.386 ± 0.0012
6	Net content (%)	99.89 ± 1.67	99.89 ± 1.67	99.89 ± 1.67	99.89 ± 1.67	99.89 ± 1.67
7	Microbial load (Bacteria & Fungi)			Observed no microbial growth Time points: 24 h, 48 h and 72 h		
8	Sterility test			Observed no microbial growth Time points: 24 h, 48 h and 72 h		

3.5.2 Evaluation of antioxidant parameters

In the disease control group, there was a significant reduction ($p < 0.05$) in the levels of glutathione (GSH) in the skin, measuring 88.72 µmol/mL, compared to the control group (Group 1) with levels of 114.67 µmol/mL. Nevertheless, topical application of the HF4 gel during treatment restored the skin's GSH levels. The GSH levels increased from 88.72 µmol/mL (in the disease control group) to 94.76 µmol/mL in Group 3 (Fig. 5). The induction of oxidative stress in the skin tissues as shown by an increase in malondialdehyde (MDA) levels occurred as a result of the administration of galactose at a dosage of 100 mg/kg bwsc. MDA levels rose to 0.088 mol/mL in the diseased group as opposed to 0.052 mol/mL in the control group. However, the levels of MDA were reduced after treatment with the gel formulations. The MDA levels decreased from 0.088 µmol/mL (in the disease control group) to 0.074 µmol/mL in Group 3 (Fig. 6).

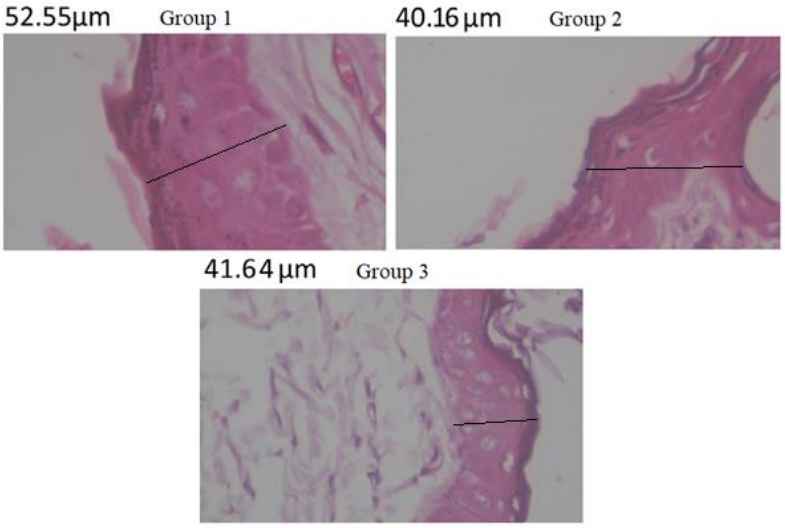

Figure 2. Effect of topical application of gel formulation containing MES and MER on galactose (100 mg/kg bwsc.), induced aging in mice skin epidermal layer thickness Group 1 served as normal control and received saline solution, Group 2 served as positive (disease) control received galactose, group 3 served as test group received formulation (HF4).

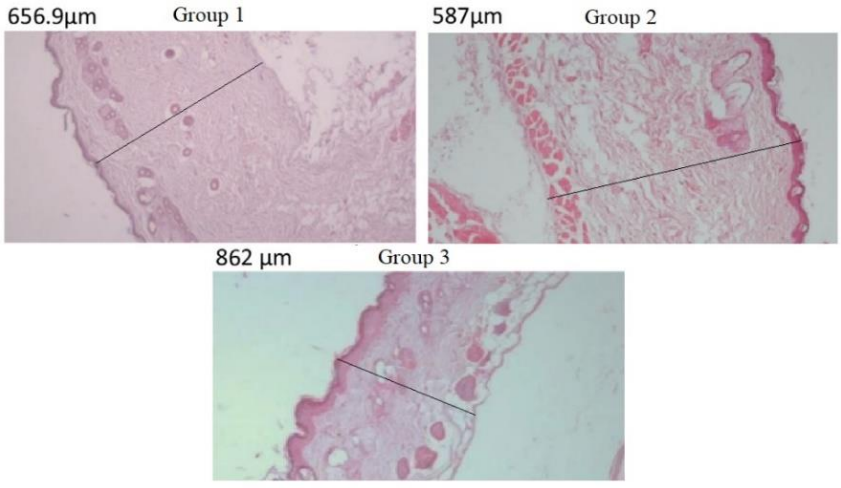

Figure 3. Effect of topical application of gel formulation containing MES and MER on galactose (100 mg/kg bwsc.), induced aging in mice skin dermal layer thickness Group 1 served as normal control and received saline solution, Group 2 served as positive (disease) control received galactose, group 3 served as test group received formulation (HF4).

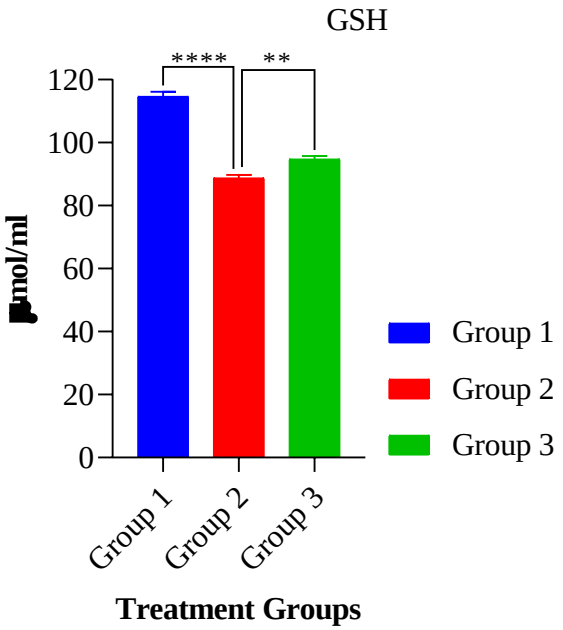

Figure 4. Effect of topical application of formulation (HF4) containing MES and MER on GSH levels in galactose-induced aging in mice skin: Group 1 served as normal control and received saline solution, Group 2 served as positive (disease) control received galactose, group 3 served as test group received formulation (HF4). Data are expressed as the mean ± SD for each group of mice (n = 6). **** $P < 0.05$ compared to normal control (Group 1) and ** $P < 0.05$, Comparison with Group 2 (Disease control).

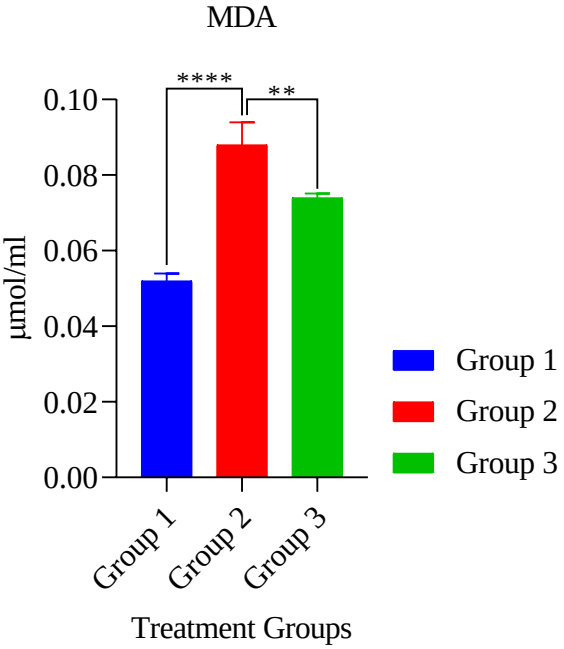

Figure 5. Effect of topical application of formulation (HF4) containing MES and MER on MDA levels in d-galactose induced aging in mice skin, Group 1 served as normal control and received saline solution, Group 2 served as positive (disease) control received galactose, group 3 served as test group received formulation (HF4). Data are expressed as the mean ± SD for each group of mice (n = 6). **** $P < 0.05$ compared to normal control (Group 1) and ** $P < 0.05$, Comparison with Group 2 (Disease control).

3.6 Summary

The findings of the study suggested that the herbal gel formulation code-named HF4, which is composed of 2% of methanol extracts from *Salvia officinalis* Linn. (MES) and *Rosmarinus officinalis* Linn. (MER) each, demonstrated a significant slowing down of skin aging by reducing oxidative stress in the skin. The anti-aging action of the fabricated topical gel might be attributed to the presence of several phytochemical compounds, including carnosic acid and rosmarinic acid, found in the methanol extracts of the leaves and flowers *of Salvia officinalis* and *Rosmarinus officinalis*. The developed formulation HF4 is demonstrated as a potential topical herbal gel for the treatment and prophylactic prevention of skin aging, capable of significantly reversing skin aging. The formulation contained 2% MES and MER, fabricated with 1.5% Carbopol polymers. Rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) are well known for their anti-aging properties due to their rich content of polyphenols and antioxidants. These compounds help reduce oxidative stress and enhance skin elasticity, thereby reducing signs of aging. Studies have shown that extracts from these herbs, viz. Rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) can increase collagen production and reduce wrinkle depth [40-42]. Additionally, their anti-inflammatory effects contribute to overall skin health and vitality. The application of rosemary and sage in skincare formulations is supported by their ability to protect against UV-induced damage and improve skin hydration, making them valuable in anti-aging treatments [40-42]. Additional research in clinical settings can support the use of this formulation in patients with skin aging and related problems, potentially validating its efficacy and safety for broader dermatological applications.

4. Conclusions

The study's findings suggest that the herbal gel formulation code HF4, which contains 2% of MES and MER each, significantly slowed skin aging by reducing oxidative stress in the skin. Anti-aging action of the fabricated topical gel might be owing to the occurrence of several phytochemical compounds, including carnosic acid, rosmarinic acid, etc, in methanol extracts of the leaves and flowers of *Salvia officinalis* Linn. (MES) and *Rosmarinus officinalis* Linn. (MER). The developed formulation HF4 was demonstrated as a potential topical gel (herbal) for the treatment and prophylactic prevention of skin aging. It contained 2% MES and MER fabricated with 1.5% carbopol 934. Additional research in the clinical setting can support using this formulation in patients with skin aging and related problems.

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Research article

Performance of Hybrid and Inbred Rice Varieties under Vermicast Application

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Abstract: This study aimed to evaluate the performance of hybrid and inbred rice varieties using vermicast as fertilizer. Soil chemical properties, plant growth, and yield performance were observed and tested for significant differences. The experiment employed a Randomized, Completely Design-Factorial layout with three replications of 30 plots. Six check plots were also prepared as farmers fertilization practices for rice production. The total experimental area is 468 m², with a planting distance of 20m x 20m and a total plant population of 5,376. Factor A involved six rice varieties, and Factor B included varying vermicast levels. Growth parameters and yield components were assessed, and data were analyzed using two-factor ANOVA and LSD test. Results indicated significant differences among rice varieties in days to flowering, days to maturity, plant height, and grain yield per hectare. However, no significant differences were observed in productive tillers, filled grains, and thousand-grain weight. No interactions were observed between rice varieties and vermicast levels. Soil analysis revealed an increase in available phosphorus but a decrease in total nitrogen and potassium content in the soil after the experimental study. Based on the observed results, the study recommends utilizing the NSIC Rc314H variety in rice production with a vermicast application rate of 5 tons per hectare for better crop yield. Future research on vermicast application in lowland rice is highly recommended for more conclusive findings and valuable insights into optimizing rice cultivation with specific varieties and application for improved yield and sustainable agricultural practices.

Keywords: Rice; Vermicast; Performance

1. Introduction

Rice is the staple food for nearly half of Asia's world population, as it plays a more significant role in the world's present and future food security [1]. In the Philippines, rice is an important food staple for millions of Filipinos, with almost eighty percent spending one-fourth of their income on rice alone [2]. According to Tallada [3], the medium and high rice per capita consumption for the Philippine population in 2030 will be 128 million and 131 million, respectively, based on the 2020 census. Likewise, Filipinos consume an average of 110kg per capita of rice. On the production side, 48 million ha in Asia are used for rice production, equating to almost 30% of the world's rice harvest. The Philippines alone recorded 4.81 million hectares of area

harvested for rice and a total production of 19.96 million metric tons, with a value of Php 403.89 billion [5]. However, even with this level of rice production in the Philippines, rice is still the country's third most imported agricultural item [6].

Future food security would depend on an improved yield per unit area planted. In general, success in crop production depends on many factors, such as varietal improvement, which will effectively improve yield stability when other factors of production are sufficiently available [7]. Accordingly, more than 400 inbred and hybrid rice varieties were released from the mid-1960s to 2023 [8]. These varieties were developed to adapt to environmental factors with promising yields to boost farmers' rice production. However, despite the development of new and improved varieties, local rice production is not enough to meet growing demand due to challenges brought about by climate change, a growing population, declining soil fertility, the high cost of chemical fertilizers, poor drainage, and irrigation facilities that lead to rice self-sufficiency in the country, an elusive goal [9].

Essential nutrients are a significant limiting factor for rice crop production and tend to inhibit rice growth if their absorption is significantly reduced. Synthetic fertilizers are a costly input, such that their use limits the probability of rice farming for high- or low-input systems, and the use of fertilizers alone is sometimes exceptionally inefficient [10] as the losses of the primary nutrients are greater when not correctly applied. Using commercial fertilizers N, P, and K contributed to a massive crop yield increase that nourishes the world's population. However, the excessive use of these fertilizers has been cited as a source of pollution of surfaces, groundwater, and even soil acidity problems in rice fields [11]. Accordingly, modern rice production relies heavily on applying chemical fertilizers, and excess application increases production costs and causes severe environmental problems [12]. Ameliorating soil acidity in rice soils is now seriously considered to enhance soil fertility and productivity [13].

The increasing demand for healthy and sustainable food production practices leads farmers to use organic fertilizers [14]. Organic fertilization positively affects agroecosystems by stimulating plant growth, enhancing crop productivity and grain quality, and improving soil fertility [15]. Vermicast, or vermicompost, is an organic fertilizer and soil enhancer produced from biodegradable materials introduced to earthworms. Compared to chemical fertilizers and conventional compost used by farmers, vermicast restores and enhances natural soil fertility by increasing the physical, chemical, and biological properties without degrading the soil in the long run due to harmful chemical effects [16]. Vermicasts generally have a higher pH and moisture content than soil, along with more significant enrichment by carbon, nitrogen, phosphorus, ammonium, polysaccharides, exchangeable cations (Ca^{2+}, Mg^{2+}, and K^{+}), and humus. Higher nutrient content in vermicasts is associated with partial organic matter decomposition during gut passage, converting these nutrients into available forms [17].

In the study conducted by Ruan et al. [18], the application of vermicast exhibited better growth in terms of higher plant height, bigger stem diameter, and increased biomass of rice crops. Moreover, the multiple nutrients present in vermicompost enhance the uptake of nutrients such as nitrogen, phosphorus, potassium, iron, manganese, copper, and magnesium from vermicast, consequently enhancing the photosynthesis activity and increasing rice yield. Furthermore, Rahman and Barmon [19] also revealed that the application of vermicast to rice productivity is significantly higher than chemical fertilizer. Kheyri [20] also concluded that increasing the application of vermicast and vermicompost in three splits over the plant growth stages led to an increase in yield and yield components in rice. The application of vermicast is gaining interest as an alternative fertilizer input in rice production due to the increasing price of chemical fertilizer on the market.

Hence, this study on the performance of hybrid and inbred rice varieties under vermicast fertilizer application was conducted to evaluate which fertilizer application would give better growth and yield components. This study will also serve as an alternative fertilizer source for rice farmers, utilizing organic fertilizer in rice-based agricultural production to reduce the cost of fertilizer inputs.

2. Methodology

The study was performed using a two-factorial Randomized Complete Block Design (2 Factor-RCBD) with six promising high-yielding rice varieties, four of which were inbred rice varieties (NSIC Rc300, NSIC

Rc222, NSIC Rc238, and NSIC Rc216) and two hybrid rice varieties including NSIC Rc492H and NSIC Rc314H as Factor A and with five levels of vermicast fertilizer application (VL1 3 tons/ha, VL2 4 tons/ha, VL3 5 tons/ha, VL4 6 tons/ha, VL5 7 tons/ha) and inorganic fertilizer application as check plots locally known as farmers practiced (90- 60-60 kg/ha) as Factor B. Ninety-six experimental plots were prepared with a plot size of 1.5 m x 1.5 m. The rate of vermicast applied per plot and the time of application are indicated in Table 1. During the first basal application, vermicast was incorporated into the soil in each plot after the final land preparation. The second and third applications of vermicast were done by top dressing. Data were recorded on various parameters such as plant height at maturity, average days to flowering, days to maturity, average number of productive tillers per hill, weight of thousand grains, filled grains, and yield per hectare in tons. The recorded data was subjected to statistical analysis using the analysis of variance, and mean significant differences were subsequently analyzed using LSD [4].

Table 1. Fertilizer application guide for hybrid and inbred rice experiment

Level of Vermicast Fertilizer	Amount to be applied per plot	Time of Application		
		1st Basal	2nd PI	3rd Flowering
3 tons/ ha	0.70 kg	50%(0.35 kg)	25%(0.175 kg)	25%(0.175 kg)
4 tons/ ha	0.90 kg	50%(0.45 kg)	25%(0.225 kg)	25%(0.225 kg)
5 tons/ ha	1.13 kg	50%(0.57 kg)	25%(0.28 kg)	25%(0.28 kg)
6 tons/ ha	1.40 kg	50%(0.70 kg)	25%(0.35 kg)	25%(0.35 kg)
7 tons/ ha	1.60 kg	50%(0.80 kg)	25%(0.40 kg)	25%(0.40 kg)

3. Results and Discussion

3.1 Soil Characteristics of the Experimental Area

The analysis of soil chemical properties before and after the study is presented in Table 2. The soil chemical analysis before the conduct of the study indicated the following characteristics: pH value: 5.51; available nitrogen: 0.21 percent; total phosphorus: 5.51 ppm; exchangeable potassium: 0.22 meq/100 g; organic matter content of the soil: 11.44 percent; CEC: 16.40 Moreover, the texture is clay-loam soil.

The data shows that after the experimental study, the soil chemical analysis result shows that the nitrogen, phosphorus, and potassium experiments changed the numerical value. The amount of nitrogen after the experiment decreased from 0.21% to .12%, while there was an unprecedented increase in the total phosphorus content after applying fertilizer, from 5.51 ppm to 49.40 ppm. However, the exchangeable potassium decreased from 0.22 to 0.034 meq/100 g. Likewise, the organic content of the soil and Cations Exchange Capacity (CEC) also increases. Also, the data noted that the application of vermicast improved the pH value of the soil. The soil analysis result was supported by the study of Elissen et al. [21], which found that vermicomposting increases the breakdown of organic matter, increases plant-available phosphorus nutrient concentrations in the soils, and increases stable humic and fulvic acid content.

Table 2. Soil chemical Analysis before and after the study

Characteristics	Critical Level Range	Before Experiment	After Experiment
		Value	Value
Ph	5.5 – 8-5	5.51	5.6
Available N (%)	0.3-0.6	0.21	0.122
Available P (ppm)	20-30	5.52	49.40
Available K (meq/100g)	0.3-0.6	0.22	0.034
Organic Matter (%)	2-3	11.44	12.15
CEC	15-25	16.40	16.80
Soil Textural Class		Clay loam	Clay loam

** Source: Analytical Service Laboratory, Bureau of Soils and Management*

3.2 Average plant height at maturity

The average plant height at maturity of rice varieties applied with different levels of vermicast is presented in Table 3. The plant height of four inbred and two hybrid varieties was determined at maturity. The plant height of inbred varieties ranges from 77.27 cm to 81.87 cm. Among the four inbred varieties, NSIC Rc300 and NSIC Rc238 grew as high as 81.87 cm and 80.05 cm, respectively. At the same time, NSIC Rc222 and NSIC Rc216 registered the shortest plant height and grew only to a mean height of 79.00 cm and 77.27 cm, respectively. Across hybrid varieties, the plant height ranged from 85.80 cm to 88.27 cm. NSIC 492H obtained an average height of 88.27 cm compared to NSIC Rc314H, which registered a slightly shorter plant height with a mean of 85.80 cm, respectively. Regarding vermicast application, the plant heights of all treated inbred varieties were comparable, ranging from 80.78 cm to 83.45 cm. However, the plant height of hybrid varieties in the check plot was higher than that of the treated inbred varieties.

Statistical analysis showed that the plant height of inbred and hybrid rice varieties was statistically significant. However, the vermicast application rate did not significantly affect the plant height of all varieties tested. Furthermore, treatment means comparison revealed no significant differences between hybrid Rc492 and Rc314 and inbred Rc300, Rc222, Rc238, and Rc216, but significant differences were observed in hybrid variety Rc492 over inbred Rc216. In addition, the plant of height, both inbred and hybrid varieties, did not show significant interaction among the rates of vermicast applied to different rice varieties tested.

The result of the study emphasized that the height characteristics of both hybrid and inbred rice varieties were not affected by levels of vermicast application. It further explains that the significant difference was only observed in the different varieties tested. In addition, among the varieties tested, the hybrid rice varieties outgrew the inbred varieties in growth performance, and this is expected due to the inherent uniform growth characteristics of the hybrid rice, which are superior to those of the inbred rice varieties [22].

Table 3. Average plant height at maturity (cm) of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	87.00	81.33	80.00	85.00	82.33	80.67	**81.87^{ab}**
NSIC Rc222 (Inbred)	85.00	81.33	78.67	79.33	81.67	74.00	**79.00^{ab}**
NSIC Rc238 (Inbred)	92.00	83.00	81.67	79.67	79.00	77.00	**80.05^{ab}**
NSIC Rc216 (Inbred)	84.00	75.67	82.67	75.67	75.67	76.67	**77.27^{b}**
NSIC Rc492H	91.00	85.33	91.33	82.67	89.33	92.67	**88.27^{a}**
NSIC2 Rc314H	95.00	88.67	86.33	85.67	84.67	83.67	**85.80^{ab}**
Mean* (B)	**89.00**	**82.56**	**83.45**	**81.34**	**82.11**	**80.78**	

**-Means followed by the same letter were not significantly different from each other using the LSD test.*

3.3 Days to flowering

The study examined the effect of different levels of vermicast on the flowering times of four inbred and two hybrid rice varieties. The inbred varieties, particularly NSIC Rc216 and NSIC Rc238, displayed variations in flowering time, with NSIC Rc216 flowering earliest at 79.47 days, followed by NSIC Rc238 at 81.07 days. The other two inbred varieties took longer to flower, averaging 83.07 and 87.07 days. Among the hybrid varieties, NSIC Rc314H flowered earlier at 85.87 days compared to NSIC R492H at 87.67 days. The check plot consistently showed delayed flowering compared to the experimental plot.

However, the application of vermicast did not significantly affect the flowering time across inbred and hybrid varieties, as the average days to flowering ranged from 83.17 to 84.67 days, regardless of the vermicast application rate. Treatment comparison among rice varieties revealed that NSIC Rc314, NSIC Rc300, NSIC Rc492, and NSIC Rc222 did not differ significantly among themselves but were significantly different in terms of days to flowering compared to NSIC Rc238 and Rc216. Likewise, the study indicated that varietal differences significantly influenced flowering time, and the nutrients present and the slow release of nutrients from vermicast did not significantly affect both inbred and hybrid rice days to flowering. In general, there was

no significant interaction between the two factors (varieties and vermicast levels) in determining the flowering time of rice varieties.

The variation in days to flowering among varieties tested was related to the different physiological characteristics of every variety. Hybrid varieties with late physiological maturity tend to have late days to produce flowers compared to inbred varieties with short physiological maturity. Likewise, the result shows that differences in days to flowering were due to physiological characteristics and were not affected by the levels of vermicast application.

Table 4. Average days to flowering of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	89.00	89.00	88.67	84.33	85.33	88.00	87.07[a]
NSIC Rc222 (Inbred)	87.00	83.67	81.33	85.00	81.67	83.67	83.07[abc]
NSIC Rc238 (Inbred)	88.00	79.33	81.00	82.67	81.67	80.67	81.07[bc]
NSIC Rc216 (Inbred)	86.00	78.67	80.67	79.00	81.00	78.00	79.47[c]
NSIC Rc492H	93.00	87.00	89.33	89.67	87.33	85.00	87.67[a]
NSIC2 Rc314H	94.00	81.33	87.00	84.33	86.00	90.67	85.87[ab]
Mean* (B)	89.83	83.17	84.67	84.17	83.83	84.34	

**-Means followed by the same letter were not significantly different from each other using the LSD test.*

3.4 Number of days to maturity

The result of the study showed that the different varieties have different maturity periods, ranging from 104.20 to 111.93 days for the inbred varieties after planting. Among the inbred varieties, NSIC Rc216 matured early at 104.20 days, followed by NSIC Rc238 and NSIC Rc222 at 106.20 and 108.07 days, respectively. On the other hand, NSIC Rc300 matured later, with an average mean of 111.93 days after planting. For hybrid varieties, NSIC Rc314H matures within 110.87 days, while NSIC Rc492H has a delayed maturity period with an average of 112.67 days. The application of vermicast did not influence the maturity period of all inbred and hybrid varieties.

Statistical analysis showed that the maturity periods of inbred and hybrid varieties are significantly different, but the application of vermicast at different rates did not indicate a significant difference. Furthermore, no interaction was observed in the number of days to maturity of all inbred and hybrid varieties or in the application of different rates of vermicast. Differences in the maturity of different varieties could be attributed to the inherent physiological maturity dictated by the genotype of each variety and its interaction with the environment.

Table 5. Average days to maturity of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	119.00	114.00	113.67	108.67	110.33	113.00	111.93[a]
NSIC Rc222 (Inbred)	117.00	108.67	106.33	110.00	106.67	108.67	108.07[abc]
NSIC Rc238 (Inbred)	118.00	104.33	106.67	107.67	106.67	105.67	106.20[bc]
NSIC Rc216 (Inbred)	116.00	104.00	105.67	103.00	106.00	102.33	104.20[c]
NSIC Rc492H	123.00	112.00	114.33	114.67	112.33	110.00	112.67[a]
NSIC2 Rc314H	124.00	106.33	112.00	109.33	111.00	115.67	110.87[ab]
Mean* (B)	119.50	108.22	109.78	108.89	108.33	109.22	

**-Means followed with the same letter/superscript were not significantly different from each other using the LSD test.*

3.5 Number of productive tillers

Results showed that the number of productive tillers was comparable among the inbred and hybrid varieties tested. On average, NSIC Rc216 had a mean of 12.13 productive tillers per hill, followed by NSIC Rc300 with 12.47 productive tillers per hill. The two other inbred varieties (NSIC Rc222 and NSIC Rc238)

obtained an average number of 13.13 tillers and 13.47 productive tillers per hill, respectively. The two hybrid varieties also produce productive tillers comparable to those of inbred varieties. Likewise, the application of vermicast, at any rate, did not influence the tillering ability of different inbred and hybrid varieties except in the check plot. In comparison, the check plot attained the highest number of productive tillers, with an average mean of 15.66 per hill. Furthermore, no interaction effect was noted between the different varieties and the rate of vermicast applied to the different rice varieties. The result clearly shows that hybrid and inbred varieties had comparable productive tillers despite the advantage of hybrid varieties in terms of growth and yield characteristics. Furthermore, the tillering ability of each variety was not affected by the different rates of vermicast and check plots or the application of inorganic fertilizer.

Table 6. The average number of productive tillers of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	11.00	12.67	11.67	12.67	13.00	12.33	12.47
NSIC Rc222 (Inbred)	17.00	15.33	11.33	13.33	14.00	11.67	13.13
NSIC Rc238 (Inbred)	15.00	13.67	13.33	14.00	13.33	13.00	13.47
NSIC Rc216 (Inbred)	13.00	11.67	12.33	12.00	12.00	12.67	12.13
NSIC Rc492H	15.00	13.00	14.67	12.67	13.33	16.67	14.07
NSIC2 Rc314H	23.00	14.00	14.00	14.33	14.33	12.33	13.80
Mean* (B)	15.66	13.39	12.89	13.17	13.33	13.11	

**-Means followed by the same letter were not significantly different from each other using the LSD test.*

3.6 Average Weight of Thousand Grains

Table 7 presents the average weight of a thousand grains of rice varieties applied with different levels of vermicast. Statistical analysis showed that the weight of a thousand grains in grams of inbred and hybrid varieties was not significantly different. On average, NSIC Rc300 had a mean of 27.60 grams, followed by NSIC Rc238 with a mean of 27.93 grams, and the other two inbred, NSIC Rc222 and NSIC Rc216, obtained an average mean of 30.93 and 31.13 grams, respectively. The two hybrid varieties weigh a thousand grains more than the inbred varieties. NSIC Rc314H had an average mean of 34.20 g, and NSIC Rc492H had an average mean of 35.60 g, respectively. Meanwhile, applying vermicast at different rates did not significantly affect the grain weight in grams of the different hybrid and inbred varieties in both the treated and the check plots. Likewise, statistical analysis has shown no interaction effect between inbred and hybrid varieties and the rate of vermicast applied as fertilizer in the different rice varieties.

The result on the weight of a thousand grains shows that all the tested varieties, the hybrid and inbred varieties, had comparable weights. Inbred rice varieties had similar performances despite the inherent advantage of hybrid rice varieties.

Table 7. The average weight of a thousand grains of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	28.10	26.67	29.00	28.33	27.33	26.67	27.60
NSIC Rc222 (Inbred)	31.00	25.33	29.00	33.33	30.00	37.00	30.93
NSIC Rc238 (Inbred)	28.95	35.00	24.33	26.00	24.33	30.00	27.93
NSIC Rc216 (Inbred)	31.20	35.00	32.00	32.33	32.00	24.33	31.13
NSIC Rc492H	38.00	36.00	41.33	35.00	31.33	34.33	35.60
NSIC2 Rc314H	37.00	28.33	33.33	36.33	33.00	40.00	34.20
Mean* (B)	32.38	31.06	31.50	31.89	29.67	32.06	

**-Means followed by the same letter were not significantly different.*

3.7 Percentage of filled grains

Results of the study of different inbred and hybrid rice varieties showed that the percentage of filled grains ranged from 93.20 to 95.73 percent for the inbred varieties. However, non-significant results were obtained between varieties and levels of vermicast application. Among the inbred varieties, NSIC Rc300 and NSIC Rc222 obtained the highest mean percentage of filled grains. Likewise, hybrid varieties, NSIC Rc429H, obtained the highest percentage of filled grains than NSIC Rc314H, with a mean percentage of 95.94%. However, varieties in the check plot applied with inorganic fertilizer have similar percentages of filled grains, with an average mean of 94.74 % filled grains. Statistical analysis showed that the percentage of filled grains in both inbred and hybrid varieties is not significantly different. The application of vermicast did not show significant differences in filled grains. Furthermore, no interaction was observed in the number of days for all inbred and hybrid varieties applied with different levels of vermicast. The study results imply that the hybrid and inbred rice varieties, at any rate of vermicast application, will produce a comparable percentage of filled grain. In addition, the filled grains performance of each rice variety is higher among varieties, and it was a good indicator that the varieties responded uniformly to factors outside their genetic control.

Table 8. Percentage of filled grains of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	98.36	94.88	96.27	97.01	95.63	94.86	**95.73**
NSIC Rc222 (Inbred)	87.50	96.63	95.13	97.09	95.23	94.27	**95.67**
NSIC Rc238 (Inbred)	99.00	94.76	95.24	95.91	93.54	94.93	**94.88**
NSIC Rc216 (Inbred)	90.91	91.57	95.23	91.78	95.09	92.34	**93.20**
NSIC Rc492H	99.00	95.62	96.57	96.03	96.57	96.92	**96.34**
NSIC2 Rc314H	93.67	94.76	95.44	95.70	97.11	96.68	**95.94**
Mean* (B)	**94.74**	**94.70**	**95.65**	**95.59**	**95.53**	**95.00**	

**-Means followed by the same letter were not significantly different from each other using the LSD test.*

3.8 Yield per hectare in tons

Table 9 presents the computed yield per hectare in tons of rice varieties applied with different levels of vermicast. As shown in the table, the data on the grain yield of the four inbred and two hybrid varieties ranged from 3.63 to 4.26 tons per hectare for the inbred varieties. Grain yield ranges from 4.75 to 4.83 tons per hectare for hybrid varieties. Among the inbred varieties, NSIC Rc216 had an average yield of 3.63 tons/ha, significantly different among inbred varieties. On the other hand, the hybrid varieties obtained slightly higher grain yields than the inbred varieties. NSIC Rc314H had an average grain yield of 4.83 tons per hectare. Statistical analysis showed that grain yield in tons of inbred and hybrid varieties differed significantly. However, the vermicast application rate did not significantly affect the grain yields of all varieties tested. Moreover, the treatment mean comparison result disclosed that hybrid varieties NSIC Rc492H, NSIC Rc314, and Inbred NSIC Rc300, NSIC Rc222, and NSIC Rc238 did not show significant differences. Still, significant results were observed between hybrid NSIC Rc314 and inbred NSIC Rc216. Likewise, both inbred and hybrid grain yields did not show a significant interaction between the rate of vermicast applied. The result of the study shows that the yield performance of the different varieties of inbred and hybrid rice had a lower yield performance based on its physiological yield potential. However, the varieties surpassed the country's average yield of 3 tons per hectare. Likewise, differences in yield performance were attributed to their genetic differences that affect their performance when interacting with the environment.

Table 9. Yield per hectare in tons of rice varieties applied with different levels of vermicast

Rice Varieties	Check Plot	Vermicast levels (tons/ha)					Mean* (A)
		3 tons	4 tons	5 tons	6 tons	7 tons	
NSIC Rc300 (Inbred)	4.44	4.18	4.19	3.96	4.04	3.91	4.06^{ab}
NSIC Rc222 (Inbred)	4.44	4.96	3.54	4.37	4.54	3.91	4.26^{ab}
NSIC Rc238 (Inbred)	4.46	4.74	3.90	4.56	3.67	3.78	4.13^{ab}
NSIC Rc216 (Inbred)	4.0	3.42	4.22	3.38	3.61	3.50	3.63^{b}
NSIC Rc492H	6.22	4.46	4.83	4.28	4.71	5.48	4.75^{ab}
NSIC2 Rc314H	8.0	4.44	4.65	5.64	5.07	4.33	4.83^{a}
Mean* (B)	5.26	3.67	4.22	4.37	4.27	4.15	

**-Means followed by the same letter were not significantly different from each other using the LSD test.*

4. Conclusions

The study evaluates the agronomic parameters of inbred and hybrid varieties, including significant variations in plant height at maturity, average number of days to flowering, average days to maturity, and grain yield per hectare in tons. Notably, inbred and hybrid varieties were the same concerning the average number of productive tillers, the average percentage of filled grains, and the computed weight of a thousand grains in grams. Additionally, applying different levels of vermicast did not influence the parameters of both inbred and hybrid varieties. Likewise, no interaction was observed between the inbred and hybrid varieties and the levels of vermicast across all observations. Interestingly, findings included the higher grain yield in tons per hectare of the hybrid varieties (NSIC Rc314 and NSIC Rc492H) and inbred varieties (NSIC Rc238, NSIC Rc216, and NSIC Rc300) compared to NSIC Rc216. Furthermore, the study observed changes in soil chemical components, with a significant increase in exchangeable phosphorus and a decline in nitrogen and potassium. The plants consumed these nutrients in large amounts. These findings contribute valuable insights to understanding crop performance and soil dynamics, offering implications for future agricultural practices and research endeavors.

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Research article

Antioxidant Activity of Banana Peel Waste, the Development and Stability Evaluation of Facial Toner Containing Banana Peel Extract

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Abstract: The banana processing industry produces many by-products, mainly banana peel. Banana peel waste, after banana processing, can be a good source of bioactive compounds. Extraction of phenolic compounds using optimum extraction conditions enhances the yield and quality of the products. This study evaluated optimizing the extraction time for the maximum extraction yield from banana peels, consequently lowering the overall process cost. In addition, the application of increasing the value of banana waste was also evaluated. The optimum conditions were achieved using 95% ethanol with a solid-to-liquid ratio of 1:5, 25 ± 5 °C under various extraction times (0-96 h). The responses, such as total phenol content (TPC), total flavonoid content (TFC), and DPPH inhibition activity, were measured by spectrophotometric analysis. The optimal incubation time at 72 h was found to be more effective compared to the others. The values of TPC, TFC, and DPPH inhibitory activity at optimized conditions were 8.23 ± 0.42 mg gallic acid equivalent (mg GAE)/g, 8.18 ± 0.08 mg quercetin equivalent (mg QE)/ g, and 93.96%, respectively. The ethanolic extract of banana peel had an inhibition concentration (IC_{50}) of 48.35 ± 0.88 μg/mL. After that, banana peel extract was subjected to study the potential for cosmetic application. The extract was applied as an ingredient in facial toner. The toners were preliminarily characterized. Banana peel waste possesses reasonable antioxidant activity and shows high stability over time. The results showed high potential for cosmetic applications using banana peel extract. In addition, using banana peel waste reduces agricultural waste in the environment.

Keywords: Antioxidant activity; Banana peel; Banana processing; Cosmetic product; Facial toner

1. Introduction

The fruit processing industry produces a large volume of fruit waste, such as seeds, peels, pomaces, and press cakes. More than 25-40% of fruit residues were left after fruit process [1]. Most fruit residues are often thrown into the landfill without proper treatment. Therefore, it produces air pollution and greenhouse effect [1]. Banana chips, a famous product from Phatthalung

province (Thailand), also produce a lot of peel. It has been reported that 40% of the overall weight of fresh bananas belongs to banana peel [2]. Recently, research and development focused on transforming agricultural waste into valuable products. Namwa (Musa ABB cv'. Kluai Namwa) is a significant fruit rich in antioxidant compounds and natural bioactive such as phenolic, flavonoids, minerals, vitamins, and anthocyanin [3]. These compounds exhibit potential application in various fields, such as the cosmetic, pharmaceutical, and food industries [4]. Therefore, the main focus is the extraction of bioactive materials such as phenolic and flavonoids in banana peels. Therefore, the optimization condition which produces a high yield is still required. Islam et al. [1] reported that products' extraction yield and quality increased when banana peel was extracted under suitable conditions.

Furthermore, the potential application of banana extract is also an exciting topic. The application of plant extract in the cosmetic field is increasing due to the high growth of the cosmetic and beauty market [5]. Banana peels (especially yellow-colored peels) are rich in flavonoids and phenolics. In addition, banana peel components are hydrophilic (producing an attractive force on water molecules), which is suitable for application in the cosmetic fields, such as lotion and moisturizer [6]. It has been reported that banana extract-containing lotion can be controlled and cure the damage and inflammation of free radicals in cells. Lotion supplemented with banana extract is soft, smooth, non-sticky, and works at the cellular level. The pH of the lotion was between 5.0 and 5.5, within the pH range of the human skin acid mantle (4.5-6.5). Therefore, the lotion will not harm the skin acid mantle layer [7]. The moisturizer supplemented with banana peel had no side effects on human skin was reported. It was an oil-in-water emulsion type within 4-5 months of shelf life [8]. The moisturizer prepared from banana peels (Kepok) effectively prevented xerosis (skin dryness). The addition of banana peel in herbal face packs and masks was also reported. The herbal face pack has enhanced the smoothness and softness of the face. The face pack was affordable, had no side effects, and was eco-friendly [9]. A gel face mask of banana peel (Ambon variety) was found easy to peel off. It is a rich source of vitamin B6, sugars, proteins, phosphorus, vitamin C, saponins, and tannins. This mask helped to reduce fine lines and wrinkles and glow the skin [10]. Banana peel-off gel mask and shampoo were made in Indonesia with the Kepok variety, a rich source of antioxidants [11]. The extraction of bioactive compounds was done using the conventional maceration method. Antioxidants shield the scale from UV radiation and free radicals so scalp health can be maintained. Banana peels have the potential to be used as a raw material for anti-hair-loss shampoos since flavonoids stimulate and repair damaged hair structure [12]. Facial toner, liquid, is often used daily to clean and refresh the skin. In addition, it helps to balance the facial pH after cleaning with soap. However, there are some reports that commercial facial toners irritate the skin. Therefore, the addition of plant extract with antioxidant activity shows several advantages. Banana peel extracts contain various bioactive compounds that have nutritional and medicinal properties. The key components in banana peel extract include phenolic compounds, vitamins, carotenoids, minerals, phytosterols, tryptophan, and antioxidants. Therefore, there are numerous benefits to using banana toners, such as hydrating skin, balancing pH levels, reducing aces and pores, neutralizing free radicals and protecting cells from oxidative damage, anti-aging, anti-inflammatory, and leaving skin feeling refreshed [13]. Therefore, this current study aimed to increase the extraction yield of banana peel waste by varying the incubation time. After that, evaluation of antioxidative activities. In addition, banana extract was used as an ingredient in facial toner. The stability and antioxidant activity of the toner were determined.

2. Materials and Methods

2.1 Raw materials processing

Fresh, ripe, and disease-free bananas (Musa ABB cv'. Kluai Namwa) were kindly provided by Pansa Interfood, Co. Ltd. (Phatthalung, Thailand). The banana peel waste, the residue after banana processing, was collected and cleaned to eliminate all contaminants using tap water. Furthermore, the cabinet drier (50 ± 5 ^{o}C) removed superficial water. The peel was dried until the 13% of moisture content was obtained. Afterward, the dried sample was ground, sieved, and packed in a high-density polyethylene bag. The ground peel was kept at -18 ^{o}C until use [1].

2.2 Effect of incubation time

The effect of time was studied. Ten grams of peel powder was added to an Erlenmeyer flask and shaken in the water bath. Then, the effect of incubation time was varied from 0-96 h while the other parameters were fixed using 95% ethanol with a solid-to-liquid ratio of 1:5, 25 ± 5 °C [1]. The optimal time, which yields the highest content of extraction yield, was selected and used throughout this study. The percentage of extraction yield was evaluated and compared.

$$\text{The percentage of extraction yield} = \frac{\text{Weight of solvent-free extract (g)}}{\text{Weight of dried extract (g)}} \text{ x } 100$$

Afterward, the sample was extracted under optimal conditions, filtrated, and evaporated using a rotary evaporator (49 °C). After that, propylene glycol was added to adjust the sample volume to 10 mL. The banana was extracted at 4 °C for an analysis of phenolic, flavonoid, and antioxidant activity.

2.3 Total phenolic content (TPC) determination

Folin-Ciocalteu assay was used to determine the TPC content [14]. Sample (0.5 mL), Folin-Ciocaltue reagent (0.5 mL), and 7.5% Na_2HCO_3 solution (1 mL) were added. Distilled water was added to adjust the mixture volume to 10 mL. Afterward, the vortex was used to mix the sample (3 s). The mixture was left in the dark (35 min) before centrifugation at 4,000 xg for 10 min. The TPC was determined at 750 nm using a UV-Vis spectrophotometer. The standard curve (R^2 = 0.9986) of various concentrations of gallic acid from 0 to 200 µM was used to calculate TPC. The unit of TPC was expressed in mg GAE/g.

2.4 Total flavonoid content (TFC) determination

The TFC was determined in a falcon tube by mixing banana extract (1 mL), distilled water (4 mL), and 5% $NaNO_2$ (0.3 mL). The mixture was left at room temperature for 5 minutes before adding 10% AlCl3 (0.3 mL). Then, the sample was kept at room temperature (1 min) before 1 M NaOH (2 mL) and distilled water (2.4 mL) was added [15]. The sample was mixed using a vortex and then centrifugated at 4,000 *x*g for 10 min. The mixture was left in the dark (15 min) before being determined at 510 nm using a UV-Vis spectrophotometer. The standard curve (R^2 = 0.9930) of quercetin at various concentrations from 0 to 100 µM was used to determine TFC. The unit of TFC was expressed in mg QE/g.

2.5 Antioxidant activity determination

DPPH scavenging assay was used to determine antioxidant activity in this current study [16]. Firstly, 0.1 mL of various concentrations of banana peel extract from 62.5 to 500 mg/mL was added and mixed with 0.1 mM DPPH solution (0.2 mL) in methanol [16]. The sample was left at room temperature for 1 h in a dark place (25 ± 5 °C). The antioxidant activity was measured at 517 nm using a UV-Vis spectrophotometer and calculated.

$$\text{The percentage of scavenging of DPPH} = \frac{\text{The initial absorbance at 0 h} - \text{The final absorbance at 1 h}}{\text{The initial absorbance at 0 h}} \text{ x } 100$$

In addition, the IC_{50} value of peel extract was calculated. All samples were analyzed in triplicate and on average. The concentration of peel extract, which inhibits a percentage of DPPH scavenging by 50%, was compared with standard ascorbic acid.

2.6 Functional group determination

The Fourier determined the functional group of peel extract transform infrared spectrometry (Perkin Elmer, USA) at a wavenumber between 4,000 to 400 cm^{-1} at room temperature (25 ± 5 °C) [17].

2.7 Facial toner containing banana peel extract preparation

The various formulas for facial toners were designed following Sadsyam et al. [5] (Table 1). Then, each formula was characterized, and the suitable formula was chosen due to the suitable value of polydispersity

index (PDI) and zeta potential. In this current study, particle size (PS), PDI, and zeta potential were determined using photon correlation spectroscopy (PCS) [18].

2.8 Physical and stability of facial toner determination

The toner was prepared and kept for 24 hours before determining physical characteristics, including color, texture, pH, odor, and phase separation [19]. In addition, the stability of the facial toner was tested using a three-cycle acceleration test. For the 1st cycle, the sample was placed at 4 °C for 48 h and at 45 °C for 48 h. The similar steps were repeated for 2nd and 3rd cycles. The physical characteristics and antioxidant activity were determined after the end of each cycle [19].

2.9 Statistical analysis

Extraction yield, TPC, TFC, and antioxidant activity were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to determine whether there were significant differences in mean values between different samples, followed by Tukey's honestly significant differences (HSD) multiple rank test at 95% ($p < 0.05$).

Table 1. The formula design of facial toner containing banana peel extract.

Materials	Unit	Concentration (%w/v)						Utility
		S1	S2	S3	S4	B1	B2	
Banana peel extract (0.1 mg/mL)	mL	0.001	0.001	0.002	0.002	0.000	0.000	Active ingredient
Olive oil		2	2	2	2	2	2	Solvent
Propylene glycol		2	4	2	4	2	4	Co-solvent, Preservative
Glycerine		4	4	4	4	4	4	Humectants, Preservative
70% Sorbitol		3	3	3	3	3	3	Humectant, Thickening agent
Phenoxyethanol		0.3	0.3	0.3	0.3	0.3	0.3	Preservative
Vitamin E		0.3	0.3	0.3	0.3	0.3	0.3	Antioxidant
Purified water was added to		100						
Adjust pH to		4.7-5.7						

While S1 and S2 contained similar volumes of banana extract (0.001 mL) with different volumes of propylene glycol at 2 and 4 mL, respectively.
S3 and S4 contained similar volumes of banana extract (0.002 mL) with different volumes of propylene glycol at 2 and 4 mL, respectively.
B1 and B2 contained no banana extract with different volumes of propylene glycol at 2 and 4 mL, respectively.

3. Results and Discussion

3.1 Effect of incubation time

The effect of incubation time was demonstrated. The extraction yield increased with the increased time from 0 to 72 h. The highest yield (5.36%) was observed at 72 h incubation time (Table 2). However, the extraction yields slightly decreased after 96 h of incubation time. The prolonged incubation time may result in a decrease in yield. This is caused by oxidation and degradation of the desired compound [20].

Table 2. The effect of incubation time on banana peel extraction yield.

Time (h)	Extraction yield (%)
0	2.08 ± 0.98[a]
12	2.32 ± 1.00[b]
24	3.02 ± 0.58[c]
48	3.96 ± 0.88[d]
72	5.36 ± 0.94[f]
96	4.55 ± 0.79[e]

Data are represented as mean ± standard derivation (SD); letters in the same column with different superscript(s) are significant ($p < 0.05$).

The extraction yield was not different from a previous study in which 2.54-5.76% of the extraction yield was obtained using ethanol as a solvent [19]. As a result of this study, extraction time highly affected the yield. It provided extracts with greater yield and antioxidant activity for extended periods. Some bioactive compounds might require longer to dissolve fully in the solvent, leading to a high concentration of extraction yield and antioxidants. A similar result was observed in lemongrass (*Cymbopogon citratus*) leaves, which gave the highest yield and TPC at 93.8 °C, 3.7 min [21]. However, a longer extraction time at 96 h proportionally decreased the yield in the extract. Certain compounds, such as polyphenols, are sensitive to heat, light, and oxygen. Extended extraction times can cause oxidative degradation, reducing the overall yield and antioxidant activity. However, a lower yield was obtained in this study compared to others. The extraction of banana peel using the vacuum microwave method gave the highest yield at 13.03% with conditions at 60 °C and 20 min [22]. In addition, the yields of the extractions from three varieties of banana peels, including *Musa acuminata, Musa sapientum* L, and *Musa balbisiana*, ranged from 6.67-9.80% using 95% ethanol for 3 weeks. Therefore, the extraction yield is mainly affected by various parameters, including banana species, extraction solvent, and method [23]. Therefore, the incubation time at 72 h was selected and used for banana peel extract in this study.

3.2 Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity

The TPC of banana peel extract using 95% ethanol as a solvent at 72 h incubation time was determined. The TPC was 8.32 ± 0.42 mg GAE/g. Zhang et al. [24] reported that the TPC in banana peel was 4.95-47 mg GAE/g dry matter. Therefore, the TPC in this current study was in the range. However, the TPC in banana peel was 1.5-3 fold higher than pulp [25]. High TPC in banana peel waste showed an excellent ability to apply peel waste in the cosmetic sector. Adeel et al. [26] reported that 100% ethanol is suitable for extracting TPC from banana peel. The highest TPC was obtained when 100% ethanol was used as extraction solvent, followed by 100% acetone. At the same time, 80% methanol yielded the lowest TPC [27]. In addition, TFC was also determined in banana peel extract. Flavonoids, important phytochemical compounds, have been reported for their ability to be utilized in the cosmetic industry [1]. The TFC extracted from banana peel waste was 8.18 ± 0.08 mg QE/g. However, the TPC and TFC of banana peel waste were lower than those obtained from the enzymatic-assisted method [1]. The high TPC (25.37 mg GAE/g) and TFC (13.99 mg QE/ g) were obtained. The TPC, TFC, and antioxidant activity from banana peel were summarized (Table 3), and the values were compared with other reports. This result is due to the effect of extraction conditions, which play a vital role in the extraction of TPC and TFC [28].

In this current study, 93.96% of DPPH scavenging activity was detected from peel extract. High antioxidant activity is related to the amount of TPC and TFC in extracts [14]. It can be concluded that banana peel extract showed great bioactive properties directly related to the ability to eliminate free radical reactions [14]. A linear regression method calculates the IC50 value of banana extract and standard ascorbic acid. The concentration of banana peel was varied from 5-100 µg/mL. The IC_{50} values of the tested extract and standard ascorbic acid were 48.35 ± 0.08 and 0.97 ± 0.08 µg/mL, respectively (Table 4). The TPC, TFC, and antioxidant activity indicated that banana peel extract contained bioactive compounds and could be applied in cosmetics.

Table 3. The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of banana peel extract.

Banana species/Extraction condition	TPC (mg GAE/g)	TFC (mg QE/g)	Antioxidant activity (%)	Ref
Musa acuminata cv. Sagor/1.0% Viscozyme® concentration, 9 h, 55 °C, the solute-liquid ratio 1:25	25.37	13.99	81.59	[7]
Ripe *Musa acuminata* L, cv cavendshii /80% methanol, room temperature, 3 h	5.85	2.26	45.08	[29]
Ripe *Musa acuminata* colla. AAA, cv 'Berangan'/80% methanol, room temperature, 3 h	0.92	0.72	40.01	
Musa sp./Ethyl acetate	4.63	5.86	-[a]	[30]
Musa omini (paranta)/70% ethanol, soxhlet 10 to 15 cycles	3.83	2.53	25.44	[31]
Dwarf cavandish/70% ethanol, soxhlet 10 to 15 cycles	3.36	2.43	30.27	
Musa ABB cv'. Kluai Namwa/95% ethanol, 72 h, solid-to-liquid ratio of 1:5, 25 ± 5 oC	8.32 ± 0.42	8.18 ± 0.08	93.96	**This study**

-[a]: not report

Table 4. The percentage of inhibition concentration of banana peel extract.

Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)	
		Banana peel extract	Standard (Ascorbic acid)
5	13.62	48.35 ± 0.08	0.97 ± 0.08
10	18.06		
30	36.77		
40	44.42		
80	75.42		
100	91.34		

3.3 Functional group characterization of banana peel extract

The FT-IR spectrum of banana peel extract is shown in Fig 1. The presence of strong hydroxyl groups, C-H stretching, and the carbonyl group stretching was detected at 3355, 2922, and 1708 cm^{-1}, respectively. The band of absorbed water (1644 cm^{-1}) and C-H bending (1375 cm^{-1}) were also observed. Moreover, the stretching of C-O and C-OR at 1245 and 1035 cm^{-1} were also presented, respectively. A similar spectrum was also reported by El-Din et al. [28]. The most intense and broadest peak at 3355 cm^{-1} indicates coinciding vibrations of O-H (hydroxyl) stretching of alcohols and phenol and N-H (amine) of amino acid. Therefore, the extract had antioxidant activity.

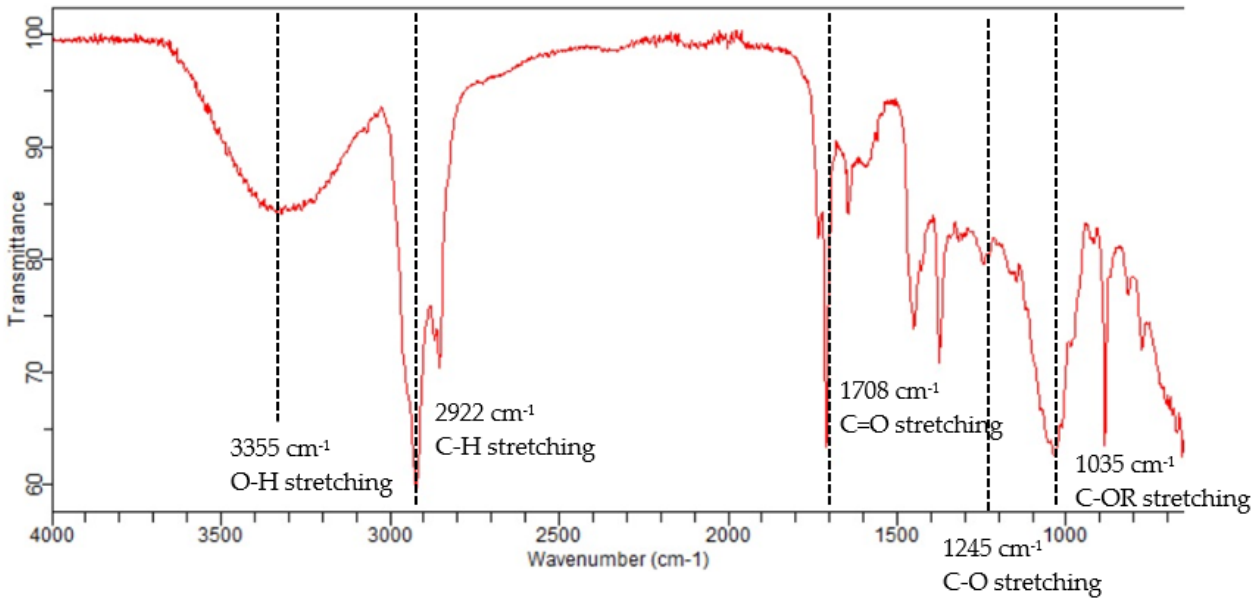

Figure 2. FT-IR spectrum of banana peel extract using 95% ethanol, solid-to-liquid ratio 1:5, 72 h incubation time.

3.4 Facial toner preparation

A facial toner containing banana peel extract was prepared using a different formula (Table 1). The physical characteristics of all formulas are yellow and mildly fragrant. In addition, all products are smooth, non-sticky, and easy to absorb (Fig 3). The pH for all products was in the range of 5.0-6.5. pH value is crucial because it guarantees the toner is in the proper pH range for the best possible skin health and efficacy. Therefore, all formulas are suitable for use. Therefore, the particle size and PDI were characterized.

All toner products showed an average particle size, PDI, and a zeta potential within the 1229-1740 nm range, 0.74-1.00 and -35.2 to -23.6 mV, respectively (Table 5). The value of PDI was limited from 0-1 for homo and hetero size distributions of emulsion. The PDI is related to particle size, while the large one is strongly scattered light more than the small one [32]. A PDI lower than 0.2 means a homogeneous size distribution of the product. At the same time, the heterogeneous size of particles in the product gives a higher PDI. Therefore, it is crucial to select a minimum PDI [33]. Zeta potential corresponds to the stability of facial toner [34]. Therefore, zeta potential is also another important selected criterion. The zeta potential at +/- 30 mV indicates no particle surface change, stable colloidal dispersion, and good facial toner performance. Therefore, low PDI (0.87) and the most potent negative zeta potential (- 26 mV) were found in the S2 formula. Therefore, the S2 formula was selected and used for the stability study. Several factors influence particle sizes, PDI, and zeta potential, such as pH, chemical composition and concentration, agitation, and temperature. Each of these parameters can interplay with the others [35].

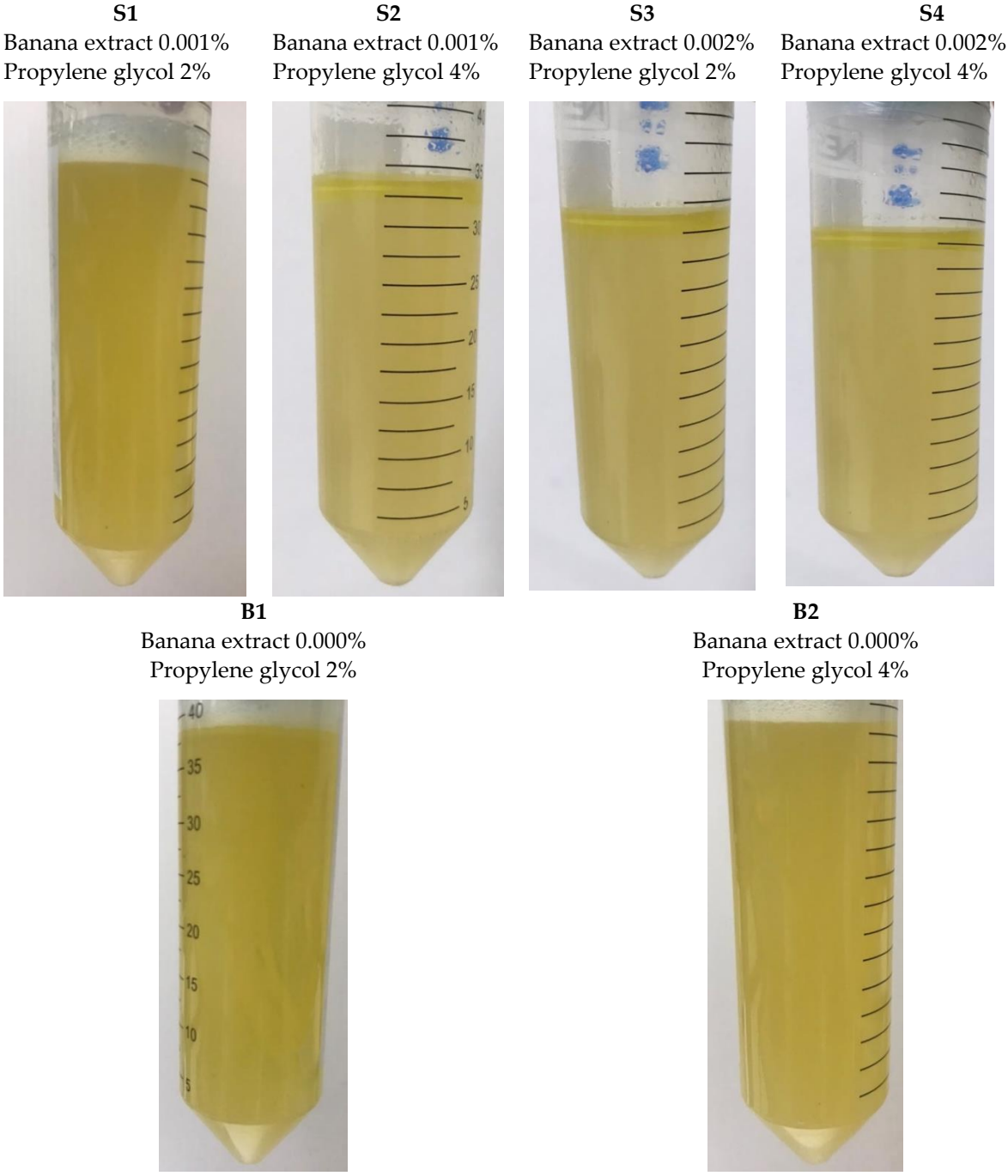

Figure 3. The facial toner contains banana peel extract, different extracts, and propylene glycol.

Table 5. Average droplet size (z-average), polydispersity index (PDI), zeta potential, and conductivity of facial toner in different formulas.

Formula	Z-Average size diameter (nm)	Polydispersity Index (PDI)	Zeta potential (mV)	Conductivity (mS/cm)
S1	1229 ± 99.46	0.89 ± 0.17	-24.5 ± 0.27	0.07 ± 0.000
S2	1740 ± 73.59	0.87 ± 0.16	-26.3 ± 0.42	0.05 ± 0.001
S3	1433 ± 69.76	0.74 ± 0.12	-23.6 ± 0.48	0.10 ± 0.002
S4	1513 ± 74.96	1.00 ± 0.00	-35.2 ± 0.46	0.09 ± 0.002
B1	482.9 ± 24.45	0.66 ± 0.10	-57.5 ± 0.47	0.07 ±0.002
B2	3477 ± 96.02	0.58 ± 0.14	-29.3 ± 0.65	0.12 ± 0.001

Data are represented as mean ± standard derivation (SD)

3.5 Stability test

The facial toner (S2 formula) was subjected to a stability test using the accelerated stability test. The test was repeated by heating/cooling at 4 °C and 45 °C for three cycles. For the 1st cycle, the sample was placed at 4 °C for 48 h and at 45 °C for 48 h. The similar steps were repeated for 2nd and 3rd cycles. The product that retains the desired properties after three cycles may have a reasonable degree of confidence in its stability. After that, the stability of facial toner, including physical characteristics and antioxidant activity, was measured. The pH values of the S2 formulation remained 5.20-5.50 (Table 6). However, the pH decreased from 5.50 to 5.20 with an increase in the tested cycle. The pH was similar to the range of facial skin pH (5.0-6.0). The remaining pH of the facial toner at 5.0-6.0 indicated the product's potential to maintain the physiological processes and provide an effective barrier. Moreover, the pH stability of the toner product showed the forming of a stabilized double-lamella structure in these mildly acidic conditions, and micellization occurs for pH > 6.0. In contrast, a disordered structure occurs for pH < 4.5 [36]. The stability of banana toner was also compared with commercial toner (BP pineapple toner). A similar result was obtained.

Table 6. Stability test results of the developed facial toner containing banana peel extract.

Cycle	**0**	**1st**	**2nd**	**3rd**
pH (Average ± SD)	5.50 ± 0.02	5.31 ± 0.01	5.20 ± 0.02	5.20 ± 0.02
Color	Yellow	Yellow	Yellow	Yellow
Odor	Mildly fragrant	Mildly fragrant	Mildly fragrant	Mildly fragrant
Turbidity	No	No	No	No
Phase separation	No	No	No	No
Precipitation	No	No	No	No
TPC (mg GAE/g)	8.23 ± 0.42	3.62 ± 0.18	3.59 ± 0.10	3.60 ± 0.12
Antioxidant activity (%)	93.96 ± 0.12	90.33 ± 0.10	90.15 ± 0.21	88.99 ± 0.13

Data are represented as mean ± standard derivation (SD)

After the third cycle of the stability test, no changes were observed in color and odor. In addition, the facial toner had no turbidity, phase separation, or precipitation. The TPC and antioxidant activity of the facial product were also measured. The result found that the TPC and antioxidant activity decreased with an increase in the tested cycle. The TPC increased from 8.23 ± 0.42 mg GAE/g to 3.62 ± 0.18, 3.59 ± 0.10 and 3.60 ± 0.12 mg GAE/g after 1st, 2nd and 3rd cycle, respectively. However, a slight decrease in antioxidants from 93.96 ± 0.12% to 90.33 ± 0.10, 90.15 ± 0.21, and 88.99 ± 0.13% was detected after 1st, 2nd and 3rd cycles, respectively. The high stability of facial toner was detected under three cycles of accelerated heating/cooling analysis.

The use of banana peel can have positive implications. Firstly, the banana peel may benefit the environment by using secondary processing materials. Secondly, it may provide a new perspective for consumers and producers concerning developing value-added products. In addition, banana peel showed the potential economic implications while it can be a suitable, less costly alternative to chemical-based substances. Recycling banana peels can effectively reduce waste and help implement efficient waste management practices. These by-products have been reported to be abundant and available for industrial scale-up [6].

4. Conclusions

This study showed the benefits of banana peel extract, which can be used as an ingredient in facial toners. The optimal incubation time obtained for maximum yield was observed at 72 h. High values of TPC (8.23 ± 0.42 mg GAE/g), TFC (8.18 ± 0.08 mg QE/g), and antioxidant (93.96%) were observed. In addition, the IC_{50} of banana extract was similar to ascorbic acid. Therefore, banana peels could be a good source of bioactive compounds that can be extracted and utilized in various fields, especially cosmetic applications. The facial toner preparation also showed significantly high stability, with a strong antioxidant effect. The banana (Musa ABB cv'. Kluai Namwa) peel, a massive by-product, is an excellent source of high-value raw materials for cosmetic industries. This is the first report to reveal the use of banana peel extract in facial toner by recycling agricultural waste. The results enhanced the reliability of banana peel extract and helped them become premium commercial products in the future. However, the properties of peel extract, such as antimicrobial and anti-aging activity, should be studied in more detail. This will provide a more comprehensive understanding of the potential benefits of using banana peel extract in skincare products. In addition, using

banana extract in the cosmetic field requires careful consideration to ensure safety, efficacy, and stability. Therefore, using plant extracts in cosmetic formulations needs to be ensured, maximizing their beneficial properties while minimizing risks to consumers.

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Research article

Metabolite Profiling and Morphological Screening of *C. militaris* Fruiting Bodies Extracts using UHPLC-QTOF-IMS and GC-MS Analysis

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Abstract: The medicinal mushroom *C. militaris* has several health advantages and has been utilized for many years throughout Asia as a component of traditional medicine systems. It can be used as a functional food and in nutraceutical products. This study investigated the morphological characteristics of *C. militaris* during the large-scale cultivation and metabolic profiling of the ethanolic and aqueous extracts of their fruiting bodies. The cultural and morphological characteristics of *C. militaris* were studied during the growth of this mushroom in terms of production of mycelial growth and fruiting bodies by conventional microbiological techniques. Cordycepin content in the aqueous and ethanol extracts of fruiting bodies was evaluated using UHPLC-QTOF-IMS analysis. The detection of metabolites in the ethanol extract was done by GC-MS analysis. The cordycepin content in the ethanol and aqueous extracts of the fruiting bodies was found to be 16.92 mg/g and 10.88 mg/g, respectively. GC-MS spectra analysis of the *C. militaris* fruiting bodies ethanolic extracts indicated the existence of eighteen metabolites such as 3,4-Dihydroxymandelic acid-terms, n-Hexadecanoic acid, Ethyl pentadecanoate, 1, E-11, Z-13-Octadecatriene, 9,12-Octadecadienoic acid (Z, Z)-, I-9-Octadecenoic acid ethyl ester, 9,12-Octadecadienoic acid (Z, Z)-, Trimethyls, 9(11)-Dehydroergosterol tosylate, Ergosterol, Silane, (phenyloxiranylidene) bis[trimethy, Neophytadiene, 1-Octadecyne, n-Hexadecanoic acid, Ethyl 9-hexadecenoate, 2,5-Diiodo-9-oxabicyclo [4.2.1] nonane, i-Propyl 9,12,15-octadecatrienoate, Ergosta-4,7,22-trien-3.beta.-ol, and TMS Palmitic acid. Evaluating cordycepin content and other bio components of C. militaris will help exploit this mushroom for potential medicinal benefits and develop reasonable quality pharmaceutical, nutraceutical, and functional food products.

Keywords: *Cordyceps militaris*; Caterpillar Fungus; Cordycepin; Metabolite; GC-MS study

1. Introduction

The medicinal mushroom *C. militaris* is an entomopathogenic fungus and has been a part of traditional medicine systems in Asian countries for many years. This crucial medicinal fungus offers the body several vital health advantages [1]. This fungus produces various bioactive compounds as secondary metabolites known for their potential health benefits, including

immunomodulatory, hepatoprotective, anti-inflammatory, anti-diabetic, antimicrobial effects [2] and anticancer effects [3]. Recently, there has been a global increase in the interest of individuals towards traditional and alternative medicine systems [4]. *C. militaris* contains many bioactive components, including cordycepin, polysaccharide, and ergosterol, and is widely used in various medical applications [5]. Due to its many bioactive components, this mushroom has potential health benefits, making it a suitable functional food ingredient. Cordycepin is a nucleoside derivative found in *C. militaris* and is one of the main bioactive metabolites found in this mushroom. This is a low molecular weight compound with several medicinal effects [5, 6]. Cordycepin is also known to suppress the growth of cancer cells by targeting cancer stem cells, inducing cell cycle arrest and upregulation apoptosis of cancer cells [4]. The studies revealed that *C. militaris* might be a promising mushroom for the neuroprotection of the hippocampus and recovery of neuroinflammation [7]. Several studies demonstrate that the bioactive compound cordycepin helps improve antioxidant enzyme levels and has anti-neuroinflammatory effects. Due to the significant anti-neuroinflammatory effect of cordycepin, this compound could help develop promising novel treatment strategies against neurological disorders like Alzheimer disease [8]. *C. militaris* harbors several beneficial biometabolites that can be used to develop herbal medicines [9]. These compounds have several beneficial biological activities [10]. UHPLC-QToF-IMS analysis is a rapid, reliable, and reproducible method developed and validated for simultaneously determining various nucleosides, including cordycepin [11]. The metabolite profiling using various techniques such as NMR, GC-MS and LC-MS analysis may be very useful for the identification of bio components [12]. In the present study, the morphological characteristics of *C. militaris* cultivated on rice medium were studied along with the evaluation of the metabolites detected in the ethanol and aqueous fruiting body extracts.

2. Materials and Methods

2.1 Characterization of the *C. militaris*

C. militaris (KBRC-1163) was cultured in rice medium from KBRC Laboratory, Bilaspur (H.P.). Large-scale cultivation was carried out using brown rice cultivation medium in glass containers. In addition to cultural and morphological studies, *C. militaris* was investigated to learn more about how this fungus produced mycelia and fruiting bodies as it grows.

2.2 Preparation of extracts

The aqueous and ethanolic extracts of fruiting bodies were prepared per the methodology described elsewhere [13] with certain modifications. The fruiting bodies of *C. militaris* were cleaned and shade-dried, grounded using a mechanical grinder into a coarse powder. Coarse powder (50 g) was filled in two different soxhlet apparatuses containing 150 ml of solvents, water, and ethanol. The extraction was performed for 8 hrs at 80 ^{0} C for ethanol and 100 ^{0} C for water [13].

2.3 Evaluation of cordycepin content by UHPLC-QToF-IMS

A precise and reliable estimation of bioactive components such as cordycepin content in the *C. militaris* extracts was carried out using UHPLC-QToF-IMS analysis as per the methodology [12]. At IHBT, Palampur, Himachal Pradesh, samples were analyzed to assess cordycepin content. Hydrophilic interaction chromatography was used to achieve LC separations using an Eclipse plus C18 RRHD column (2.1 x 50 mm). Elution was monitored at 280 nm using a PDA detector at 30 °C. Mass spectrometry was performed on a high-resolution 6560 ion mobility Q-TOF LC/MS equipped with an Agilent 1290 Infinity II UHPLC system. The mass spectrometer was scanned from 100 to 1700 m/z in full scan mode with a scan rate of 1.50 spectra/s. The 121.050873 m/z ion was chosen for mass calibration to eliminate systematic errors in the reference solution. Internal standard (NMAE = N6-methyladenosine) concentrations were used to normalize metabolite concentrations. Unknown metabolites other than the target were identified from the collected data and matched against the METLIN database [14].

2.4 GC-MS study

Plant material was dissolved in ethanol to prepare the extract for GC-MS analysis, which was carried out according to standard technique [12-14]. Filtration and potential concentration were then carried out. The

extract is separated by raising the gas chromatograph temperature after introducing a little aliquot. The mass-to-charge ratios of the chemicals are then measured when they are ionized in the mass spectrometer. The resultant mass spectra are compared to standard libraries like NIST for identification. This technique successfully identified the extract's volatile constituents.

3. Results and Discussion

Research has shown that the well-known traditional Chinese medicine *C. militaris* possesses anticancer, immunostimulatory, and neuroprotective properties [15]. The application of herbal biotechnology in drug development, employing the medicinal fungus *C. militaris*, is steadily increasing. Technological developments have expanded the potential use of this fungus in goods made with herbs [16]. Highly pleomorphic morphologies are displayed by the medicinal mushroom Cordyceps militaris when cultivated in different culture conditions. As seen in Fig. 1(a) and (b), when Cordyceps was cultivated on nutrient-rich Sabouraud dextrose agar (SDA) for three days, the culture turned turbid as spiny blastospores emerged from the tips of the branching mycelia. Fungal cultures grew flaky mycelia, colored yellow-orange, and generated round to ovoid conidia when cultivated on rice medium for up to three weeks before stroma development (Fig. 1b and Fig. 2a). In rice medium, stromal development happens up to 4 weeks following growth (Fig. 2b). After 5–6 weeks, *C. militaris* complete fruit body formation happens on rice medium with perithecia (Fig. 3a and b). Using light and scanning electron microscopy, the morphological traits of *C. militaris* were studied based on the production of conidial from ascospores that germinated from vegetative hyphae. It has been noted that the physical characteristics of *C. militaris* sexual stage can be used to differentiate it from other species [17]. Numerous bioactive substances are the cause of these possible health advantages. One of the primary ingredients in this mushroom that supports the biological activity and the therapeutic effect is cordycepin. This could be the reason for the potential use of *C. militaris* mushrooms as functional foods and medicinal use [17]. Recent studies have also demonstrated that this mushroom has tremendous health benefits, including COVID-19 [15, 18]. In the present study, the cultivation of *C. militaris* mycelium using artificial media has lately been developed in this mushroom. Numerous investigations have shown that *C. militaris* is the species that produces the most cordycepin and can be grown in artificial environments. This is the primary cause of the mushroom's widespread usage in functional meals and herbal remedies [19].

The cordycepin content of the *C. militaris* fruiting body extracts was another objective of our study. The cordycepin content in the ethanolic and aqueous extracts of the fruiting bodies was evaluated using UPLC-QTOF-MS DAD chromatography, and maximum cordycepin content was found in the ethanolic extract (16.92mg/g) while minimum in aqueous extract (10.88mg/g) **(Fig 4 a and b, Table 1**). The UPLC-QTOF-MS DAD chromatograms of cordycepin standard and aqueous and ethanolic extracts of fruiting bodies of *C. militaris* samples are represented in **Fig 4.** The cordycepin content evaluated using UHPLC-QToF-IMS analysis was a fast, reliable, and reproducible method, which was developed and validated for simultaneous determination of various nucleosides such as adenine, adenosine, cordycepin, and guanosine, inosine, thymidine, thymine and uracil in medicinal fungi [12] and evaluated the bioactive components of the *C. militaris* after extraction into digestive juices in the artificial gastrointestinal tract model. The research aided in determining how bioavailable, bioactive ingredients are to the human body. The highest amount of cordycepin was found in fruiting bodies grown on-site (25.8 mg/100 g d.w.) and those purchased commercially (25.9 mg/100 g d.w.). The research aided in determining how bioavailable, bioactive ingredients are to the human body. These researchers showed that C. militaris fruiting bodies and mycelium were suitable for dietary supplements and positively impacted human health [20].

Table 1 Cordycepin content found in extracts of *C. militaris* fruiting bodies.

Sr. No.	*C. militaris* extract	Cordycepin content (mg/g)
1.	Aqueous extract (AE)	10.88
2.	Ethanolic extract (EE)	16.92

In a similar study [21], the cordycepin content of *C. militaris* was determined using reversed-phase HPLC. These workers demonstrated that the HPLC method is fast, simple, accurate, and reproducible for determining cordycepin content. In another study, [22] an improved HPLC method detected the major bioactive ingredients of mycelial and fruiting bodies of C. sinensis and C. militaris. These workers observed that cordycepin content was higher in *C. sinensis* than in *C. militaris*, whereas adenosine content was higher in *C. militaris* [23]. Nonetheless, this investigation assessed *C. militaris* fruiting body extracts. Also, it used reversed-phase HPLC to quantitatively assess the *C. miliatris* extracts and discovered that the fungal mycelium's 60% ethanol extract and 100% ethanolic extract had the highest cordycepin levels. According to their research, this is a valuable analytical technique for determining the best extraction conditions [24]. Several other methods such as Near-infrared (NIRS) spectroscopy in combination with partial least squares regression were also employed to determine cordycepin level in fruiting bodies of *C. militaris* [25]. Another study used the HPLC-based method combined with hierarchical cluster analysis and quantitative analysis of multi-components by single marker (QAMS) to evaluate bioactive components in the fermented *Cordyceps* products. There was no significant difference in the results; therefore, they were found valid for systematic quality control assessment [25]. The development of novel *C. militaris* strains with increased cordycepin content through genetic recombination or increasing cordycepin content by application of growth supplements during submerged cultivation may help to exploit the pharmaceutical and nutraceutical potential of this mushroom [26]. The sample was sent to Jawaharlal Nehru University, New Delhi for analysis. The chemical composition of the ethanolic extract of *C. militaris* was studied by gas chromatography- mass spectroscopy (GC/MS). Eighteen phytoconstituents were identified in the sample representing 27.48% of Octadecatriene, 9,12-Octadecadienoic acid (18.60%), Linoleic acid (18.60%), and isopropyl linolenate (17.59%) were the major constituents as shown in **Table 2.** The identification of various compounds was confirmed by comparing their mass spectra to those in the NIST libraries.

Table 2. IUPAC names, common names, RT, Area%, and chemical structures of compounds identified in ethanolic extracts of *C. militaris* fruiting bodies by GC-MS analysis.

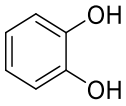
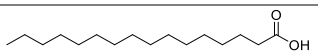
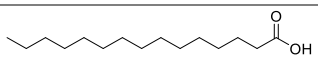
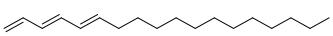
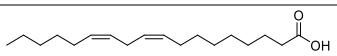
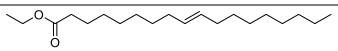
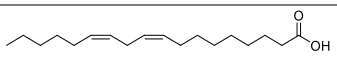
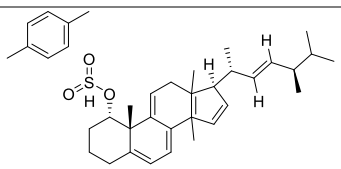
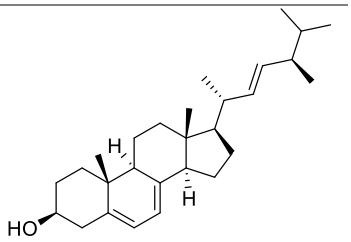
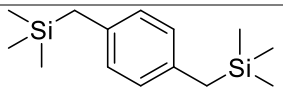
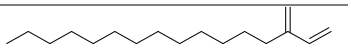
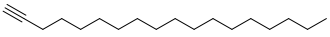
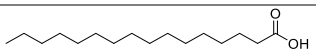
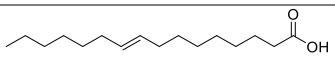
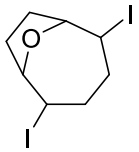
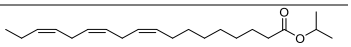
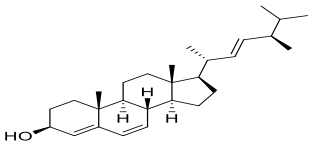
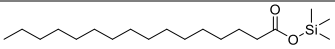
IUPAC Names	Common Names	RT	Area%	Chemical Structures
3,4-Dihydroxymandelic acid-tetratms	**Catechol, mandelic acid**	**12.330**	**1.24**	
n-Hexadecanoic acid	Palmitic Acid	16.136	15.04	
Ethyl pentadecanoate	Pentadecanoic acid	16.341	12.76	
1,E-11,Z-13-Octadecatriene	Octadecatriene	17.768	27.48	
9,12-Octadecadienoic acid (Z,Z)-	9,12-Octadecadienoic acid	17.899	18.60	
I-9-Octadecenoic acid ethyl ester	9-Octadecenoic acid, ethyl ester is a fatty acid ester.	17.950	2.62	
9,12-Octadecadienoic acid (Z,Z)-, Trimethyls	Linoleic acid	18.317	18.60	
9(11)-Dehydroergosterol tosylate	Dehydroergosterol tosylate	25.009	1.64	

Table 2. IUPAC names, common names, RT, Area%, and chemical structures of compounds identified in ethanolic extracts of *C. militaris* fruiting bodies by GC-MS analysis. (Contiune)

IUPAC Names	Common Names	RT	Area%	Chemical Structures
Ergosterol	**Ergosterol**	**28.709**	**11.60**	
Silane, (phenyloxiranylidene)bis[trimethy	1,4-Bis[(trimethylsilyl)methyl]benzene	11.375	1.14	
Neophytadiene	3-methylidenehexadec-1-ene	15.775	4.61	
1-Octadecyne	Octadecyne	16.222	1.34	
n-Hexadecanoic acid	Palmitic acid	17.142	12.49	
Ethyl 9-hexadecenoate	Palmitelaidic acid	18.959	2.42	
2,5-Diiodo-9-oxabicyclo[4.2.1]nonane	9-Oxabicyclo	26.022	1.51	
i-Propyl 9,12,15-octadecatrienoate	isopropyl linolenate	27.415	17.59	
Ergosta-4,7,22-trien-3.beta.-ol	Ergosta-4,6,22-triene-3beta-ol	29.706	12.81	
TMS Palmitic acid	Hexadecnoic acid trimethylsilyl ester	16.791	2.36	

GC-MS and Spectra analysis of the *C. militaris* fruiting bodies ethanolic extracts indicated the existence of various biocomponents **(Fig. 5 a and b)**. By comparing their mass spectra to those in the NIST libraries, given in **Table 2**, the 18 compounds were identified and characterized. **Table 3** lists the various biocomponents and their biological activities [27]. A total of eighteen compounds, for instance, 3,4- dihydroxymandelic acid-tetratms, n-hexadecanoic acid,ethyl pentadecanoate, 1,E-11,Z-13-octadecatriene, 9,12-octadecadienoic acid (Z,Z)-, 1-9-octadecenoic acid ethyl ester, 9,12-octadecadienoic acid (Z,Z)-, trimethyls, 9(11)-Dehydroergosterol tosylate, ergosterol, Silane, (phenyloxiranylidene)bis[trimethy, Neophytadiene, 1-Octadecyne, n-Hexadecanoic acid, ethyl 9-hexadecenoate, 2,5-Diiodo-9-oxabicyclo[4.2.1]nonane, i-Propyl 9,12,15-octadecatrienoate, Ergosta-4,7,22-trien-3.beta.-ol, and TMS Palmitic acid were detected. Oh and co-workers 2019, evaluated the metabolites of *C. militaris* fruiting bodies by gas chromatography-mass spectrometry (GC-MS) analysis

method based on their developmental phases. In this study, 39 components were identified associated with carbohydrates and amino acid metabolism. It has been observed that the bioactive compounds such as cordycepin, mannitol, and xylitol accumulated in stage four of the cultivation of this mushroom. However, our study evaluated the ethanolic extracts of dried fruiting bodies and identified eighteen compounds along with cordycepin level. In a similar study, NMR and GC-MS with multivariate statistical analysis were applied to detect biometabolites in *Cordyceps pruinosa* mycelia. This study identified seventy-one metabolites, such as alcohols, amino acids, purines, pyrimidines, fatty acids, and organic acids. These bio-components may vary at different growth conditions [28]. Studies are still required to predict the optimum amount of these compounds in the mushroom and affect different supplementation substrates on the concentration of these compounds during the cultivation. In a similar study conducted by [8], the metabolic profiling of ethanol extracts of C. militaris fruiting bodies was done using ^{1}H-NMR analysis. A total of 44 metabolites were identified. It has been observed that in the aging period of development of *C. militaris* fruiting bodies, higher levels of biometabolites were present. In another study, a simultaneous distillation-extraction (SDE) and gas chromatography-mass spectrometry (GC-MS) method was developed for the profiling of volatile components in the products prepared from *C. sinensis.* In this work, 64, 39, 56, 52, and 44 components were identified in the five different products. 5,6-dihydro-6-pentyl-2H-pyran-2-one, and fatty acids were mostly present [29]. In our study, noradrenalin, fatty acids, and sterols were the most dominating compounds among the identified bio metabolites in *C. militaris* fruiting bodies of ethanolic extract.

The medicinal mushroom *C. militaris* is a rich source of beneficial biometabolites for developing various herbal drug formulations. Due to the potential health benefits associated with this mushroom, it has gained significant importance in biotechnological and clinical applications [10]. *Cordyceps* species are also of great interest because of the broad spectrum of biological effects associated with its bio metabolites, such as cordycepin [30]. Many studies have demonstrated the therapeutic potential of this compound, and therefore, advancements in the development of techniques to produce products with higher cordycepin content have been observed [11]. Detecting metabolites in various products using NMR, GC-MS, and LC-MS analysis is beneficial for investigating bioactive components and their potential application in drug discovery and food products [11]. The metabolic components and biochemical activity indicate the potential application of *C. militaris* in the pharmaceutical and food industry [31].

Table 3. GC-MS analysis shows the nature and biological activity of ethanolic extracts of C. militaris fruiting bodies.

IUPAC Name	Common Names	Retention Time	Molecular weight	Nature of compound	Biological activity	Reference
3,4-Dihydroxymandelic acid-tetratms	Catechol, mandelic acid	12.330	472	Nor adrenalin metabolite	Higher free radical scavenging activity	[32]
n-Hexadecanoic acid	Palmitic Acid	16.136	256	Fatty acid	Anti-inflammatory compound	[33]
Ethyl pentadecanoate	Pentadecanoic acid	16.341	270	Fatty acid	Antibacterial and antifungal activity	[34]
1,E-11,Z-13-Octadecatriene	Octadecatriene	17.768	248	Fatty acid	Antibacterial property	[35]
9,12-Octadecadienoic acid (Z,Z)- is to be revised to 9,12-Octadecadienoic acid (Z,Z)-Trimethyls.	9,12-Octadecadienoic acid	17.899	280	Lineolic acids and derivatives.	Antimicrobial property	[36]
I-9-Octadecenoic acid ethyl ester	ethyl ester, 9-Octadecenoic acid,	17.950	310	Fatty acid ester	Antibacterial property	[36, 37]
9,12-Octadecadienoic acid (Z,Z)- Trimethyls,	Linoleic acid	18.317	352	Lineolic acids and derivatives.	Antioxidant activities	[37]
9(11)-Dehydroergosterol tosylate	Dehydroergosterol tosylate	25.009	548	Sterol	Immuno modulatory, Anti-inflammatory, activity	**[38]**

Table 3. GC-MS analysis shows the nature and biological activity of ethanolic extracts of C. militaris fruiting bodies. (Contiune)

IUPAC Name	Common Names	Retention Time	Molecular weight	Nature of compound	Biological activity	Reference
Ergosterol	Ergosterol	28.709	396	Sterol	Membrane fluidity, regulation, activity and distribution of integral membrane proteins	[22]
Silane, (phenyloxiranylidene)bis[trimethy	1,4-Bis[(trimethylsilyl)methyl]benzene	11.375	264	Benzene derivatives	Antimicrobial effects	[39]
Neophytadiene	3-methylidenehexadec-1-ene	15.775	278	Diterpenes	Antibacterial activity, treatment of headache, rheumatism and some skin disease	[30]
1-Octadecyne	Octadecyne	16.222	250	Long-chain hydrocarbon and an alkene	Anticancer, antioxidant and antimicrobial activities	[40, 41]
n-Hexadecanoic acid	Palmitic acid	17.142	256	Fatty acid	Balance membrane physical properties	[27]
Ethyl 9-hexadecenoate	Palmitelaidic acid	18.959	282	Fatty acid esters	Antioxidant and Antiandrogenic activity	[34]
2,5-Diiodo-9-oxabicyclo[4.2.1] nonane	9-Oxabicyclo	26.022	378	Lactones	Antibacterial activity	[22]
i-Propyl 9,12,15-octadecatrienoate	isopropyl linolenate	27.415	320	Fatty acid	Antioxidant effects	[34]

Table 3. GC-MS analysis shows the nature and biological activity of ethanolic extracts of C. militaris fruiting bodies. (Contiune)

IUPAC Name	Common Names	Retention Time	Molecular weight	Nature of compound	Biological activity	Reference
Ergosta-4,7,22-trien-3 beta-ol	Ergosta-4,6,22-triene-3beta-ol	29.706	396	Sterol	Anti-fatigue activity, and protection of hepatic and muscle activity	[15]
TMS Palmitic acid	Hexadecanoic acid trimethylsilyl ester	16.791	328	Fatty acid	Anticancer, beneficial for cardiovascular diseases and obesity	Mancini et al. (2015

Figure 1. *C. militaris* (a) yellowish-orange mycelial mat produced two weeks post-inoculation on rice medium (b) stromata formation from the yellowish-orange mycelial mat produced on rice medium.

(a) (b)

Figure 2. *C. militaris* (a) premature fruiting body with perithecia on rice medium (b) mature fruit bodies.

Figure 3. Dried fruiting bodies of *C. militaris*

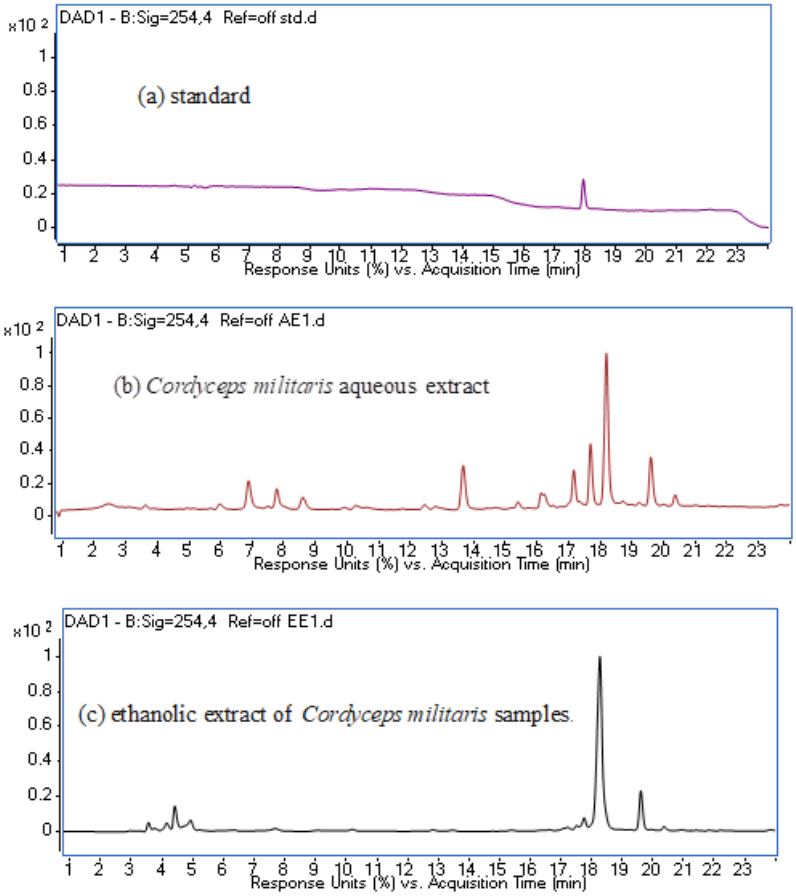


Figure 4. UPLC-QTOF-MS DAD chromatograms of cordycepin (a) standard, (b) *C. militaris* aqueous extract, and (c) *Cordyceps militaris* ethanolic extract.

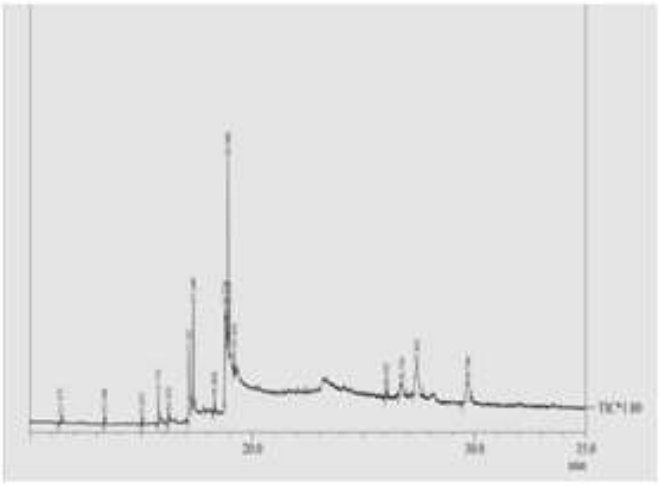


Figure 5. GC-MS chromatogram of ethanol extract of *C. militaris* fruiting bodies.

4. Conclusions

C. militaris have many therapeutic benefits and are commonly used in traditional medicinal systems. Due to various active phytoconstituents, *C. militaris* can be used to produce pharmaceutical and functional food products. The morphological traits change depending on the stage of cultivation. Cordycepin, a main bioactive component found in this mushroom, evaluated using the extracts from fruiting bodies, revealed that the higher cordycepin content was in the ethanolic extract in comparison to the aqueous extract, by UHPLC-QTOF-IMS analysis. Also, the ethanol extract showed the presence of eighteen compounds, and fatty acids and sterols were the most predominant among these compounds by GC-MS analysis. The characterization of *C. militaris* will help exploit this mushroom to develop herbal-based formulations and nutraceuticals. Detecting metabolites using UHPLC-QTOF-IMS and GC-MS analysis is beneficial for detecting bioactive components. These studies may help assess the potential application of these fungi in drug discovery and the food industry.

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Author Contributions: S. T. contributed to the study design, was the major contributor to the present study, performed experiments, analyzed the data, and prepared the manuscript. M. P. was the major contributor to the present study, analyzing the data and preparing the manuscript. P. G. was the major contributor to the present study, performing experiments, analyzing the data, preparing the manuscript, and checking the manuscript for plagiarism. P. B. helped in the analysis of the data and also reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Research article

Toxicity Evaluation of Copper, Nickel, and Mixture on *Daphnia magna*

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Abstract: This study conducted acute (48 hours) and chronic (21 days) toxicity tests of nickel (Ni^{2+}), copper (Cu^{2+}) ions, and a mixture of the Ni^{2+} - Cu^{2+} ions on *Daphnia magna* (*D. magna*) under M4 medium. The acute test results showed that the toxicity of Cu^{2+} was about 15 times higher than Ni^{2+}, demonstrated by the 50% effect concentration (EC_{50}) value of 185.2 $\mu g.L^{-1}$ and 2706.97 $\mu g.L^{-1}$. In addition, the results also illustrated that the mixture of the Cu^{2+} – Ni^{2+} ions was more toxic to *D. magna* than a single metal with an EC_{50} value of 175.22 $\mu g.L^{-1}$. Acute toxicity tests showed that the metal affected the viability of *D. Magna*, while the organism maturation and reproduction were affected under chronic exposure. Chronic test results showed that the toxicity of Cu^{2+} was higher than that of Ni^{2+} with EC_{50} values of 9.06 $\mu g.L^{-1}$ and 162.12 $\mu g.L^{-1}$; noticeably, the toxicity of the mixture of the two metals is higher than that of the single metal. In particular, Ni^{2+} at a concentration of 100 $\mu g.L^{-1}$ stimulated the maturation, survival, and reproduction of *D. magna*. but at higher concentrations (> 100 $\mu g Ni.L^{-1}$), Ni^{2+} would bind to Cu^{2+}, which exerted a more substantial effect on the test organism. This study initially evaluated the toxicity of Cu^{2+} and Ni^{2+} on microcrustaceans *D. magna*, which is the premise for further studies on the genotoxicity of heavy metals on microcrustaceans in general and *D. magna* in particular to ensure the quality of the ecosystem.

Keywords: *Daphnia magna*, acute, chronic, mixed metal toxicity, 50% effect concentration (EC_{50})

1. Introduction

Industrial, municipal, and municipal stormwater typically contains a mixture of metals such as copper (Cu^{2+}), nickel (Ni^{2+}), lead (Pb^{2+}), and zinc (Zn^{2+}) along with organic matters, all of which can be discharged directly or indirectly into aquatic systems [1, 2]. Consequently, aquatic ecosystems are contaminated by a mixture of chemicals, increasing worldwide concern [3]. Heavy metals such as Cu^{2+} and Ni^{2+} are often present in low concentrations in aquatic ecosystems, so they do not seriously affect human health. However, due to human impact, high concentrations of heavy metals have been detected in surface waters (lakes, lagoons, rivers and streams) [4-6] making artificial pathways the primart form of intrusion. Despite the known effects of heavy metals on humans and mammals, little is known regarding the modes of action of toxins in aquatic ecosystems.

In aquatic environments, metals rarely exist in isolation but rather as mixtures. Therefore, a broader perspective on water quality regulations is needed

better to understand the toxicity of metal mixtures to aquatic organisms. Numerous studies have demonstrated the relationship between water quality and metal toxicity [7, 8]. Water chemical parameters generally help organisms resist metal toxicity by forming complexes with metals or competing with metals for binding at toxic action sites in organisms [9]. However, the interactions between metal mixtures, water physicochemical parameters, and aquatic organisms are poorly understood. Previous reports have assessed the aquatic toxicity of metals using a variety of aquatic organisms like fish, protozoa, nematodes, and crustaceans [10]. Due to its sensitivity, water flea, *Daphnia magna* (*D. magna*), is a commonly used species for studying metal pollution and metal mixtures [11]. Moreover, *D. magna* is more sensitive than fish and other plankton [12–14]. As a crucial trophic level in the aquatic food chain, exposure of *D. magna* to heavy metals can result in harmful effects such as impaired growth, reduced reproductive capacity, and mortality [15]. Interestingly, most studies have concentrated on physiological characteristics such as death and reproduction, neglecting developmental endpoints [16, 17]. Winner and Farrell [18] tested the acute and chronic toxicity of Cu on four species of *Daphnia*, including *D. magna*, *D. pulex*, *D. parvula*, and *D. ambigua*. All four species had lower survival rates at Cu concentrations > 40 $\mu g.L^{-1}$. Additionally, Xiao and Peijnenburg [19] evaluated the toxicity risk of Cu metal nanoparticles on *D. magna*, revealing that the concentration causing a 50% effect on test organisms increased 12-fold when DOC levels rose from 0 to 10 $mg.L^{-1}$ in the exposure environment.

In parallel with acute exposure, there is a vital need for additional knowledge on the toxic effects of metal mixtures on aquatic organisms under chronic exposure conditions. However, the toxicity of some metals is known to be variable due to physicochemical factors in the environment, including pH, hardness, and DOC [20]. Therefore, a metal toxicity assessment must consider pH, hardness, and DOC. Understanding the mechanism of toxicity of metals in acute and chronic exposure, along with the effects on biological populations, allows scientists to make a more accurate risk assessment of metals. At the same time, this stimulates the development of more stringent water quality criteria for metal exposure. Therefore, this study was conducted to characterize and differentiate acute and chronic effects of Cu^{2+}, Ni^{2+}, and bimetallic mixtures on *D. magna* under laboratory conditions.

2. Materials and Methods

2.1 Test chemicals

The study used chemicals $NiCl_2$ (Ni^{2+}) và $CuCl_2.2H_2O$ (Cu^{2+}) (Merck, Germany), stored at 5°C to conduct the toxicity test of single metals and a mixture of Cu^{2+} – Ni^{2+}.

2.2 Expermental organism

D. magna was isolated and cultured at the laboratory of the Institute of Environmental Science, Engineering and Management - Industrial University of Ho Chi Minh City. Briefly, *D. magna* was grown in M4 medium according to ISO 6341:2012 at about 21 ± 1°C in a 16-8-hour light-dark cycle, light intensity 500 - 800 lux. *Chlorella vulgaris* was used as a food source, and 3 mL (concentration of 10^7 $cells.mL^{-1}$) of *Chlorella vulgaris* was added to each culture cup of adult *D. magna* three times a week. *D. magna* < 24 hours of birth were separated from the adult ones daily using plastic pipettes for toxicity tests.

2.3 Acute toxicity test

Set up the test in an M4 environment according to ISO 6341:2012, including a control sample (without chemicals added), the concentration range of Ni^{2+} (300, 600, 1200, 2400, and 3600 $\mu g.L^{-1}$) and Cu^{2+} (50, 100, 150, 200, and 250 $\mu g.L^{-1}$) were used to determine the 50% influence (EC_{50}) test individual range of each metal. Corresponding to each metal concentration, 9 *D. magna* were put into a beaker containing 40 mL of the medium prepared above. Each concentration is repeated three times. The number of immobilized/dead individuals after 48 hours of exposure was monitored and recorded.

The Ni^{2+} concentration range that caused mortality below and above 50% of *D. magna* individuals was 2400 $\mu g.L^{-1}$ and 3600 $\mu g.L^{-1}$, respectively. From the values just determined, the EC_{50} interval was subdivided into exposure concentrations of 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3400, and 3600 $\mu g.L^{-1}$. The range of Cu^{2+} concentrations causing mortality under and over 50% of *D. magna* was 150 $\mu g.L^{-1}$ and 250 $\mu g.L^{-1}$, respectively. From the values just determined, the EC_{50} interval was subdivided into an exposure concentration range of 40, 90, 130, 180, 220, 270, 310, and 360 $\mu g.L^{-1}$. The acute concentration mixture of Cu^{2+}–

Ni^{2+} was determined by using the concentration range of Ni^{2+} and choosing the EC_{50} value of Cu^{2+} as the fixed concentration. The experiment temperature was maintained at 21±1 °C. The medium pH, DO, hardness, and alkalinity were measured at the start and end of the test [21].

2.4 Chronic toxicity test

The experiment was set up and conducted similarly to that reported by Son, Chi [22], combined with the acute toxicity results. The experiment was conducted at different concentrations of Cu^{2+} (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 $\mu g.L^{-1}$), Ni^{2+} (0, 10, 20, 40, 80, 100, 120, 160, and 200 $\mu g.L^{-1}$); and the mixture of Cu^{2+} – Ni^{2+} were determined similarly to the acute toxicity test. Each trial batch was replicated with 9 D. magna three times and fed with *Chlorella vulgaris* every day [22] for 21 days of testing [23]. The endpoint of the chronic metal toxicity test for *D. magna* included survival (mortality rate), maturity (date of onset of egg-bearing), and fertility (number of offspring produced). The experiment temperature was maintained at 21±1 °C.

2.5 Data analysis

Experimental results were presented as the mean ± standard error. Sigma Plot 14.0 statistical software (Systat Software Inc., CA, USA) was used to create graphs. EC_{50} values were estimated through JMP Pro 16.0 software. At the same time, one-way ANOVA was used to evaluate the influence of Cu^{2+} and Ni^{2+} on the maturation of *D. magna* compared with the control. All statistical analyses were based on a significance level < 0.0001.

3. Results and Discussion

3.1 Physicochemical properties of the test medium

The M4 medium quality parameters were agreed upon during acute and chronic testings: temperature was 21 ± 1 °C; DO ranges from 7-8 $mg.L^{-1}$; pH was 8.2 ± 0.2; alkalinity was 90 $mgCaCO_3.L^{-1}$; hardness and DOC was 240 $mgCaCO_3.L^{-1}$ and 8.9 $mg.L^{-1}$, respectively. Similar to the present study, a pH range of 7.9 to 8.3 is considered favorable for the growth of *D. magna* [14]. Rodriguez and Arbildua [24] and Renzi and Blašković [25] also showed that high pH values, hardness, and dissolved substances can decrease the toxicity of metals to *D. magna*. Therefore, water physicochemical parameters such as hardness, pH, salinity, alkalinity, and DOC can modulate the toxicity of metals to aquatic organisms [26, 27].

3.2 Acute toxicity of metals to D. magna

The mortality rate in *D. magna* after 48 hours of exposure increased with the test metal concentration (Figure 1).

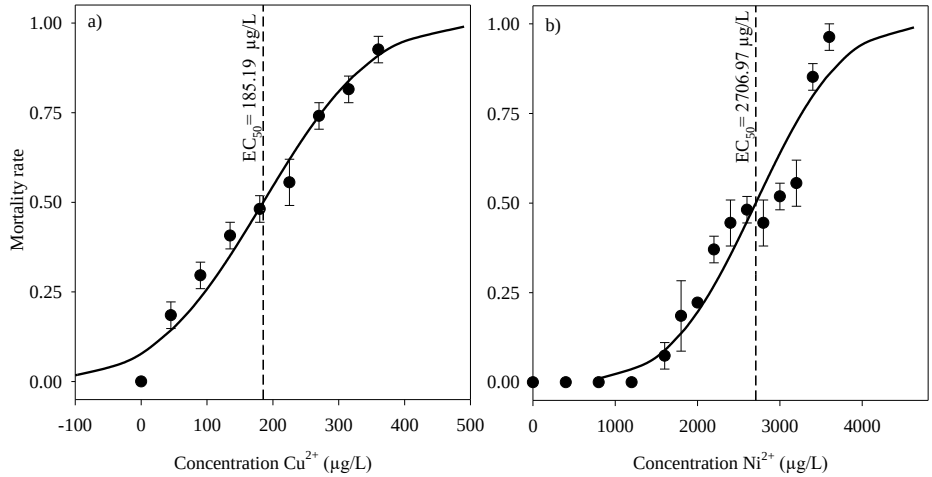

Figure 1. The mortality rate of *D. magna* upon exposure to a) Cu^{2+} and b) Ni^{2+} for 48 hours; n = 3, error bar: ± SE.

The mortality rate of *D. magna* due to Cu^{2+} reached 50% (EC_{50}) when the Cu^{2+} concentration increased to 185.19 $\mu g.L^{-1}$ and peaked at the estimated concentration of 490.29 $\mu g.L^{-1}$ (100%), which demonstrated that

the viability of the test organism was inversely proportional to the experiment with Cu^{2+} concentration (Figure 1a). Similar to the toxicity of Cu^{2+}, the mortality rate of *D. magna* also increased gradually with the concentration of Ni^{2+} metal exposure. At the concentration of 2706.97 µgNi.L^{-1}, it reached 50% and the highest at 4627.95 µgNi.L^{-1} (96%) after 48 hours (Figure 1b). The evaluation results showed that Cu^{2+} was more toxic to *D. magna* than Ni^{2+} (the lower the EC_{50}, the higher the toxicity) ($p < 0.0001$) (Figure 1).

The EC_{50} value of Cu^{2+} and Ni^{2+} in this present study was 13 and 650 µg.L^{-1}, respectively (hardness was 240 mg$CaCO_3$.L^{-1}) was higher (lower toxicity) reported by Meyer and Ranville [28] for Cu^{2+} (103 µg.L^{-1}) (hardness < 100 mg$CaCO_3$.L^{-1}), and for both Cu^{2+} and Ni^{2+} metals of Negin and Pedram [29] were 0.667 and 50.06 µg.L^{-1} (hardness of 88 mg$CaCO_3$.L^{-1}), Okamoto, Yamamuro [30] were 13 and 650 µg/L (hardness of 45 – 240 mg$CaCO_3$.L^{-1}), Lari, Gauthier [31] were 34.5 and 1502.5 µg.L^{-1}, respectively (hardness < 100 mg$CaCO_3$.L^{-1}). This demonstrates that metal toxicity increases as water hardness decreases, leading to variations in EC_{50} results between studies [32]. At the same time, from the tests and acute toxicity studies of the two metals, the toxicity of Cu^{2+} was higher than that of Ni^{2+} after 48 hours of exposure to *D. magna*. The results of the acute toxicity test of the mixture of Cu^{2+} – Ni^{2+} with a fixed concentration of 185.19 µgCu.L^{-1} are shown in Figure 2. EC_{50} of the mixture (175.22 µg.L^{-1}) was about 15 times lower when compared with Ni^{2+} (2706.97 µg.L^{-1}) and about 1 µg.L^{-1} compared with Cu^{2+} (185.19 µg.L^{-1}), indicating that the toxicity of mixture of Cu^{2+} - Ni^{2+} mixture (with 185.19 µgCu.L^{-1}) was higher than single Ni^{2+} and Cu^{2+} (Figures 1, 2).

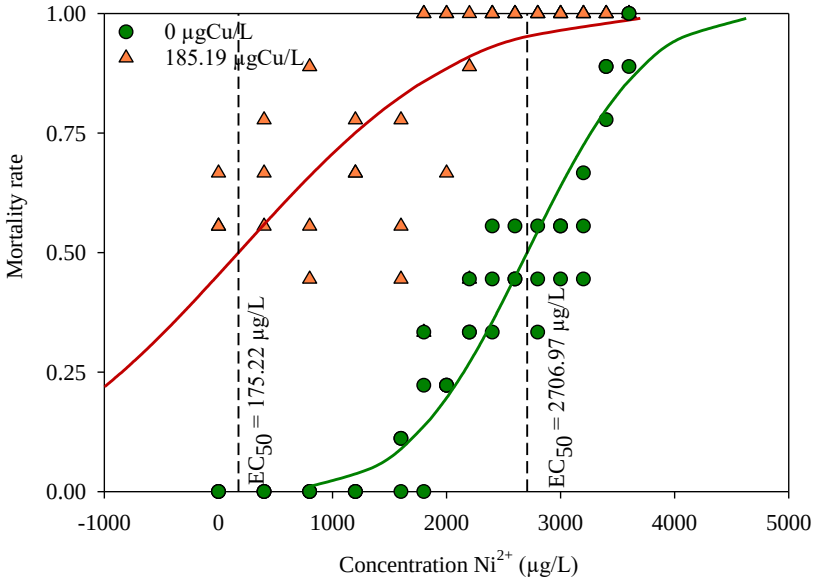

Figure 2. The mortality rate of *D. magna* in 48 hours of exposure to mixtures of Cu^{2+} – Ni^{2+}; n = 3, error bar: ± SE.

3.3 Chronic toxicity of metals to D. magna

3.3.1 Effect on the survivability of D. magna

After chronic exposure (21 days) to Cu^{2+}, the EC_{50} was 9.06 µg/L (Figure 3a). For Ni^{2+}, the EC_{50} obtained was 162.12 µg/L (Figure 3b). Evaluation results showed that the toxicity of *D. magna* with Cu^{2+} was higher than with Ni^{2+} under the same time and test conditions.

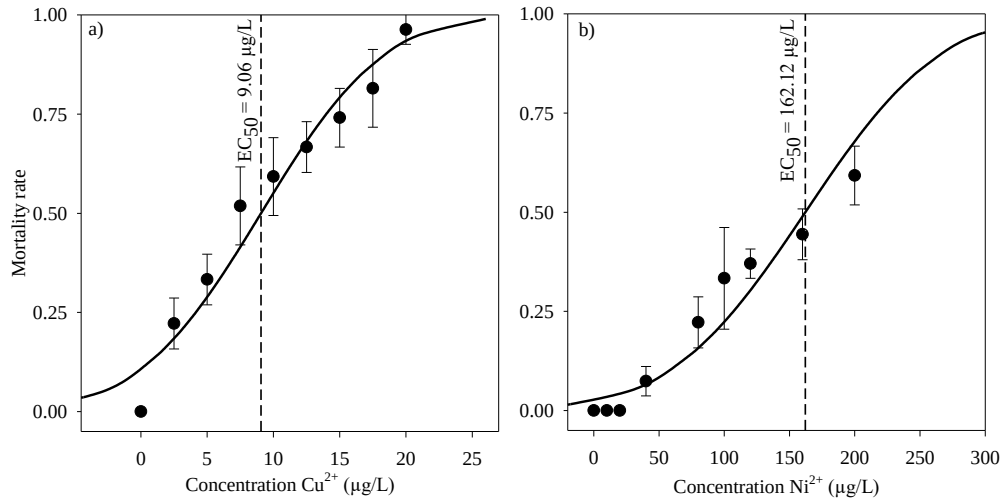

Figure 3. The mortality rate of *D. magna* upon exposure to a) Cu^{2+} and b) Ni^{2+} for 21 days; n = 3, error bar: ± SE.

In the chronic toxicity test of the mixture of Cu^{2+} – Ni^{2+}, the mortality rate of *D. magna* by metal concentration was shown in Figure 4.

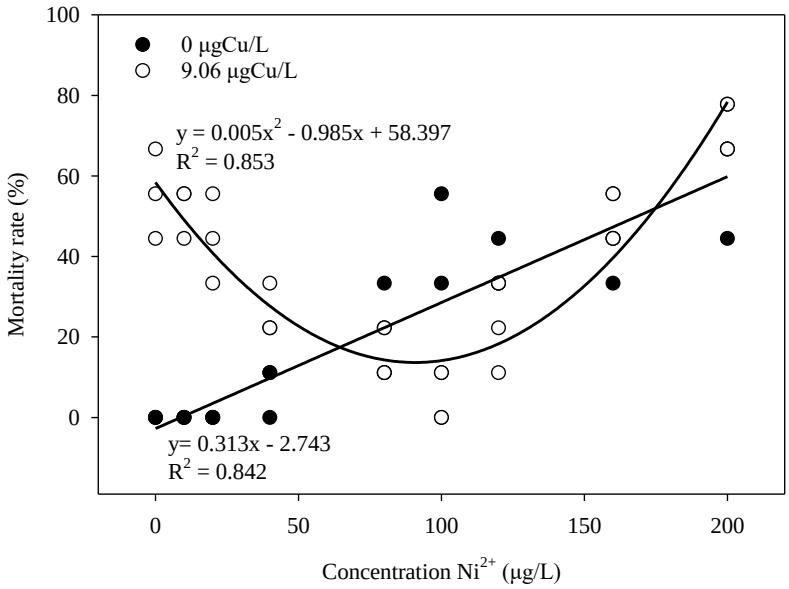

Figure 4. The mortality rate of *D. magna* in 21 days when exposed to Ni^{2+} and the mixture of Cu^{2+} – Ni^{2+}; n = 3, error bar: ± SE.

The mortality rate of *D. magna* after 21 days of exposure to Cu^{2+} - Ni^{2+} mixture increased high at concentrations of 10 and 20 µgNi.L^{-1}, but when the concentration of Ni^{2+} in the mixture increased (80 - 120 µgNi.L^{-1}), the mortality rate of *D. magna* tended to decrease significantly and was comparable to the control concentration (Figure 4). However, when the Ni^{2+} concentration increased rapidly from 160-200 µgNi.L^{-1}, *D. magna* reacted strongly with the metal, causing higher mortality than the single Ni^{2+} concentrations.

The results of the current study align with the observation that the chemical interactions of organisms in water exposed to high levels of toxicants result in more significant toxicity in Cu^{2+} - Ni^{2+} mixtures [12]. However, once the protective concentration threshold for D. magna is exceeded, Ni^{2+} combines with Cu^{2+} to produce a more pronounced toxic effect on the test organisms than individual metals. Deleebeeck and De Schamphelaere [33] used *D. magna* to evaluate Ni^{2+} toxicity and showed that the EC_{50} value after 21 days of exposure was 23 µg.L^{-1} at low hardness and EC_{50} from 59 – 365 µgNi.L^{-1} at medium hardness from moderate to high. This was consistent with the current research results with high hardness (240 mg$CaCO_3$.L^{-1}), with EC_{50}

value reaching 162.1 μg.L^{-1}. Nam and Son [34] evaluated the chronic toxicity of Ni^{2+} at a concentration of 5, 65, and 254 μg.L^{-1} to *D. lumholtzi* had a mortality rate of 20, 0, and 27.3%, indicating that *D. magna* was more sensitive than *D. lumholtzi* to exposure.

3.3.2 Effect on the maturation of D. magna

The chronic toxicity assessment of Cu^{2+}, Ni^{2+}, and bimetallic mixtures on *D. magna* maturation was shown in detail in Figure 5.

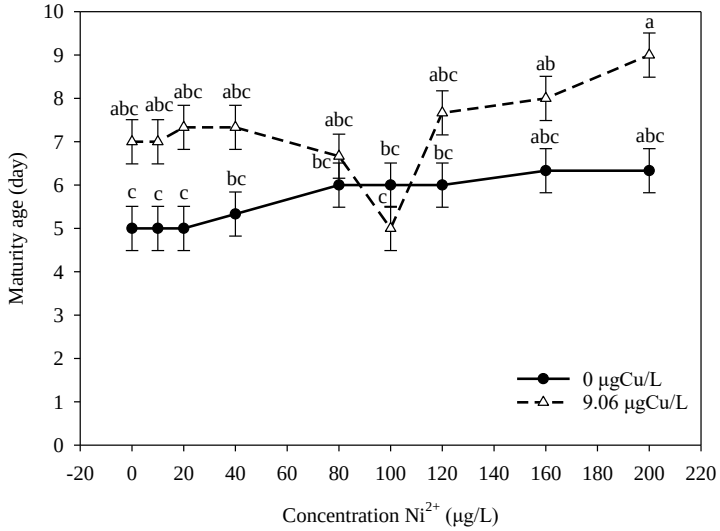

Figure 5. Maturity age of *D. magna* after 21 days when exposed to Ni^{2+} and the mixture of Cu^{2+} - Ni^{2+}; n = 3, error bar: ± SE.

The first *D. magna* matured on day 5 at control concentrations (0 μgCu.L^{-1}) and concentrations of 10 – 40 μgNi.L^{-1}, with no difference in statistical significance ($p < 0.05$) (Figure 5). When the concentration of Ni^{2+} increased from 80-200 μgNi/L, the adult age of *D. magna* showed signs of increasing by 1 day. In particular, in the Cu^{2+} – Ni^{2+} mixture (9.06 μgCu.L^{-1}) at the first three concentrations, maturation occurred on day 7 (Figure 5). As the concentration in the mixture increased (80-100 μgNi.L^{-1}), the maturation age of *D. magna* was on day 5. Nevertheless, at concentrations of 120, 160, and 200 μg.L^{-1}, the maturity date of *D. magna* tended to last up to 8 and 9 days (Figure 5). Up to this limit, the combination of Ni^{2+} and Cu^{2+} strongly affected the maturation.

The results of the present study showed that the first individual *D. magna* matured at day 5 of age (similar to the circadian cycle of *Daphnia*) in control and four concentrations: 10, 20, 40, and 80 μgNi.L^{-1} (Figure 5); which was higher than that of Nam and Son [34], reporting a concentration of 0, 5, 65, and 254 μgNi.L^{-1} of mature *D. lumholtzi* on day 4.

3.3.3 Effect on the fertility of D. magna

In a single Ni^{2+} chronic toxicity test (0 μgCu.L^{-1}), after 21 days of exposure, the number of *D. magna* juveniles decreased with the increase in Ni^{2+} concentration ($p < 0.0001$) (Figure 6). For the mixture of Cu^{2+} - Ni^{2+} (9.06 μgCu.L^{-1} and 0 - 100 μgNi.L^{-1}), the amount of *D. magna* produced gradually increased but tended to decrease sharply from the concentration of 120 - 200 μgNi.L^{-1} ($p < 0.0001$) (Figure 6).

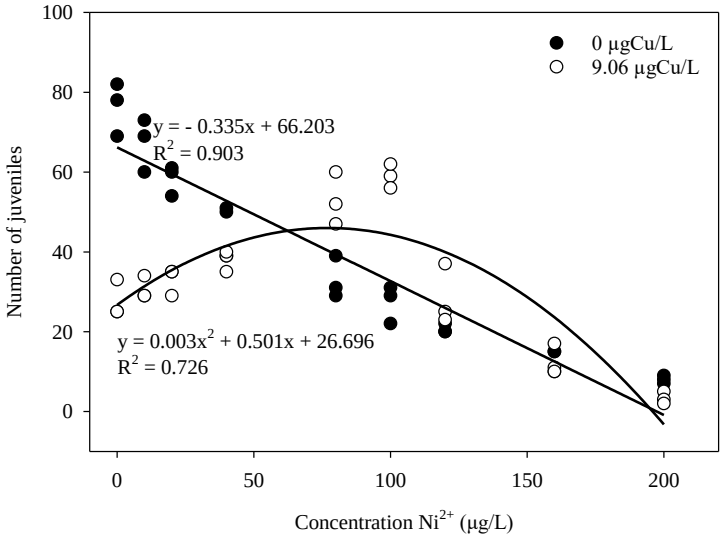

Figure 6. The number of juveniles *D. magna* after 21 days of exposure to Ni^{2+} and the mixture of Cu^{2+} - Ni^{2+}; n = 3, error bar: ± SE.

In the trial with single Ni^{2+} (0 µgCu.L^{-1}), the number of *D. magna* juveniles gradually decreased as the Ni^{2+} concentration increased. No statistically significant difference was observed between the number of juveniles in control at the concentration of 10, 20 µgNi.L^{-1} (Figure 6). Besides, the number of juveniles at Ni^{2+} concentrations of 10 and 20 µg.L^{-1} was higher than in the mixture. However, at 40 – 200 µgNi.L-1 concentration, the number gradually decreased with the lowest value of 8 juveniles (200 µgNi.L^{-1}) (Figure 6). For the chronic toxicity test of the Cu^{2+} – Ni^{2+} mixture (containing 80 and 100 µgNi.L^{-1}), the amount of *D. magna* produced was significantly higher than the control concentration($p < 0.0001$) (Figure 6). At the same time, the research results showed that the amount of *D. magna* generated in the mixture of Cu^{2+} - Ni^{2+} was higher than that of the single Ni^{2+}. However, when the Ni^{2+} concentration was at 120-200 µg.L^{-1}, the number of *D. magna* juveniles decreased gradually to the lowest level of 3 juveniles. Taylor reported the fertility of Daphnia under the chronic effects of Cu2+ and Ni2+, which had a higher number of juveniles > 40 individuals than in the current study [26]. The results demonstrated that different environments and crustaceans directly affected the toxicity of metals to test organisms. Notably, *D. magna* can switch from female to male under stress conditions and continue normal development without reproducing [36]. This phenomenon helps explain the differences in reproductive outcomes observed in *D. magna* following metal exposure. In addition, Pane and Smith [37] provided evidence that Mg^{2+} antagonism was a mechanism of acute and chronic toxicity of Ni^{2+} in water to *D. magna*. For Cu^{2+}, the effects observed on *D. magna* showed the simultaneous involvement of several toxic mechanisms, such as increased metabolic time. This process decreased energy intake (through inhibiting enzyme activity and digestion), reproductive inhibition (via inhibition of cell formation), and molting [38].

4. Conclusions

The results of toxicity assessment on microcrustaceans *D. magna* after 48 hours of exposure to metals in the M4 medium showed that Cu^{2+} was more toxic than Ni^{2+}. When combining two metals, the toxicity of the mixture increased as the concentration of Ni^{2+} increased, meaning that Ni^{2+} had no protective effects on *D. magna* from Cu^{2+} toxicity but combined to create more toxicity compared to Cu^{2+} with a single metal. Chronic toxicity of Cu^{2+} and Ni^{2+} affected the survival, maturation, and reproduction of *D. magna*. In particular, the presence of Cu^{2+} in water at a concentration of 20 µg.L^{-1} caused more deaths than 95% of tested *D. magna* individuals. When combined, the chronic toxicity of the two metals Cu^{2+} – Ni^{2+} was higher than that of the corresponding single metal. However, the protective effect of Ni^{2+} on *D. magna* could be observed at sufficient concentrations (80, 100, and 120 µgNi.L^{-1}); Cu^{2+} in the mixture increased toxicity to the organism expressed through the degree of influence on survival, maturation, and reproduction. Therefore, further studies are needed to fully understand the effects related to gene expression and the accumulated metal content in *D. magna* after acute and chronic exposure.

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Conflicts of Interest: The authors declare no conflict of interest.

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Research article

Antiproliferative Activity and GC–MS Analysis from the Leaves Extract of Different Cultivars *Carica Papaya*

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Abstract: Papaya is the fruit of the *Carica papaya* plant. Several secondary metabolites from the *Carica* genus have been reported to exhibit interesting biological activities. Its leaves are normally considered discarded. The research aimed to examine the antiproliferative and antioxidant effects of *Carica papaya* leaves from four different cultivars- Cocoa, Holland, Khaek Dam, and Red Lady and evaluate the chemical composition of the extracts through GC–MS analysis. The MTT assay evaluated the antiproliferative activity of all extracts. Red Lady exhibited higher effectiveness against MCF-7, SW620, and Vero cell lines compared to Khaek Dam, with IC_{50} values of 90.88 ± 0.39, 258.45 ± 2.16, and 301.73 ± 0.73 μg/mL, respectively. Cocoa and Holland extracts showed no cytotoxic effects on the mentioned cell lines. Antioxidant activity, measured through DPPH radical scavenging assays, revealed that Red Lady had the highest antioxidant capacity (IC_{50} 163.87 μg/mL), followed by Khaek Dam, Cocoa, and Holland. As a result, the GC–MS analysis concentrated on the extracts of Lady and Khaek Dam. The chromatograms revealed that the extracts from Red Lady displayed 23 components, while those from Khaek Dam contained 22. The primary metabolite produced in Khaek Dam were n-hexadecanoic acid (17.53%), 1-heptadecanecarboxylic acid (6.86%), and loliolide (5.58%), while in Red Lady 9-octadecenamide (20.82%), n-hexadecanoic acid (8.26%), palmitoleamide (5.43%) were produced. This indicates a difference in the chemical composition between the two cultivars. It is clear from this study that the chosen species included a range of potent phytochemicals with antiproliferative characteristics.

Keywords: *Carica papaya*; phytochemical constituents; antiproliferative activity; antioxidant activity

1. Introduction

lants are excellent resources for the development of medicinal chemicals and drugs. Natural products could eventually serve as human or livestock medications. These goods and their equivalents may serve as bridges in producing

beneficial medications. Plant-derived biochemical compounds, including phenols, flavonoids, anthocyanins, and terpenoids, offer a wide range of potential applications, including anti-inflammatory, anti-tyrosinase, antioxidant, and anti-cancer properties.

Carica papaya, commonly known as papaya, is a member of the *Caricaceae* family with a rich history of medicinal use. Different parts of the plant, including the leaves, fruit, seeds, bark, and roots, have been traditionally used to treat illnesses such as dengue fever, jaundice, sinusitis, eczema, malaria, and digestive disorders [1–4]. In traditional medicine, papaya leaves have been used for generations in folk remedies to treat various health problems, including inflammation, digestive disorders, infectious diseases, malaria, constipation, irregular menstruation, eczema, diabetes, hypertension, and dengue fever [5-6]. Papaya leaf extract has shown significant antiplasmodial, antibacterial, antiviral, antitumor, hypoglycemic, anticancer, antidiabetic, and anti-inflammatory activities [6-8]. Extracts from various parts of the papaya obtained using different extraction methods are continuously being investigated for their biological activity and phytochemistry. Phytochemical analysis from papaya has revealed flavonoids, phenols, alkaloids, steroids, glycosides, and phenols [9–10]. These substances are recognized for their antioxidant, antimicrobial, antiviral, antibacterial, anti-inflammatory, and anticancer activities [11–14].

In Thailand, several papaya varieties are cultivated, each with distinct characteristics such as flesh color, skin color, and size. The fruit is edible, while the leaves are typically considered as waste and usually thrown away. A recent study investigated the total phenolic and flavonoid contents of extracts from the leaves of three papaya varieties, Khaek Dam, Red Lady, and Holland, to identify beneficial bioactive metabolites. The study found that the ethanol extracts from papaya leaves exhibited robust antioxidant activity [15]. Surprisingly, there are minimal findings on papaya varieties' phytochemical analysis and antiproliferative activity: Cocoa, Holland, Khaek Dam, and Red Lady. This work aimed to evaluate the potential antiproliferative and antioxidant activities of the extracts derived from four different cultivars of *C. papaya*: Cocoa, Holland, Khaek Dam, and Red Lady. Specifically, we aimed to identify the extracts that showed significant activity against cancer cells for further analysis of their chemical composition by GC–MS.

2. Materials and Methods

2.1 Plant material

The leaves of four cultivars of *C. papaya* (Cocoa, Holland, Khaek Dam, and Red Lady) were collected from Hua Sai District and Pakphanang District, Nakhon Si Thammarat province.

2.2 Plant extraction

The samples of all four varieties of papaya leaves were cut into small pieces and ground thoroughly, then dried in a hot air oven at 60°C for 17 hours. Four cultivars of papaya (Cocoa, Holland, Khaek Dam, and Red Lady) leaves weighing 10 grams each were extracted with a soxhlet extractor using 200 mL of ethanol for 4 hours. Then, the solvent was evaporated using a rotary evaporator.

2.3 Cell culture conditions and in vitro cytotoxicity testing

The American Type Culture Collection (ATCC, Manassas, VA, USA) produced the breast cancer cell lines (MCF-7). Professor Dr. Surasak Songkhathat (Faculty of Medicine, PSU, Thailand) provided colorectal adenocarcinoma (SW620) cell lines for research. Kidney epithelial cells (Vero line) were provided by Associate Professor Dr. Potchanapond Graidist (Faculty of Medicine, PSU, Thailand). Cells were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% fetal bovine serum (Invitrogen), 50 μg/mL penicillin (Invitrogen), and 50 μg/mL streptomycin (Invitrogen) at 37 °C, containing 5% CO_2 and 95% of the air humidity. The cytotoxicity of the extracts and fractions was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as first described. Half maximal inhibitory concentration (IC_{50}) values below 20 μg/mL were considered to have in vitro cytotoxic activity [16]. Cell viability was checked after treatment with the positive control drug doxorubicin against MCF-7, SW620, and normal monkey kidneys. The selectivity index (SI) was utilized to evaluate the selectivity of the extracts, as first described [17]. The SI of the most active fraction was calculated by dividing the IC_{50} value of the extract on a normal cell line by the IC_{50} value of the extract on each cancer cell. An SI value greater than 3 was considered indicative of an anticancer drug.

2.4 DPPH radical scavenging assay

To evaluate the antioxidant activity, the percentage of inhibition and the IC_{50} value of papaya leaf extract are calculated about a Trolox standard solution. Free radical inhibition was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. A slightly modified approach was applied, following the methodologies described by Wasman, Mahmood, Chua, Alshawsh, & Hamdan (2011) [18] and Palafox-Carlos et al. (2012) [19]. A 0.2 mM DPPH methanolic solution was combined with varying extract concentrations (ranging from 0.1 to 0.5 mg/mL). After allowing the mixture to sit in a dark environment at room temperature for 30 minutes, the optical density was recorded at 517 nm using a UV-Vis spectrophotometer. Each experiment was performed in triplicate. The results were expressed as the IC_{50} value, representing the sample concentration that leads to 50% inhibition. IC_{50} values were calculated by extrapolating the linear regression equation from a graph plotting percent inhibition against sample concentrations.

$$\% \text{ inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100.$$

2.5 Gas chromatography-mass spectrometry (GC–MS) analysis

GC-MS was used to analyze the composition of the extracts using an Agilent 7890B GC with an Agilent 5977A single quadrupole MS (Agilent Technologies, Santa Clara, CA, USA). A VF-WAXms column (30 m length, 0.25 μm, ID 0.25 mm) was used as the analytical column. The GC was run in splitless injection mode, with the front inlet temperature set at 250°C and an injection volume of 1.0 μL. The oven was set to 60°C for two minutes, then raised to 250°C in twenty minutes at a rate of 5°C per minute. At 70 eV, electron ionization (EI) was employed for MS detection. The interface, MS quad, and MS ion source temperature were set to 250°C, 230°C, and 150°C, respectively. The scan mass range was 30 to 500 m/z, and the solvent delay was 6 minutes. The components were identified by analyzing the mass spectra using NIST14 and Wiley 10 database systems software. All processes were conducted at the Office of Scientific Instruments and Testing, PSU, Thailand.

2.6 Statistical analysis

The student's t-test was performed, and the results were statistically analyzed using the Microsoft Excel program. A t-test was used to compare the means of the two groups. Results were presented as the mean ± standard deviation, and the experiments were carried out in triplicate. A p-value of less than 0.05 was considered statistically significant.

3. Results and Discussion.

This study assessed the antiproliferative activity of ethanol extracts from *C. papaya* leaves, explicitly focusing on four cultivars: Cocoa, Holland, Khaek Dam, and Red Lady. Furthermore, this study provided insight into how the bioactive compound affects the extract's effectiveness using GC-MS analysis. These investigations showed naturally occurring bioactive substances associated with various biological functions. The extraction yields of the four papaya varieties (Cocoa, Holland, Khaek Dam, and Red Lady) provide valuable insights into the potential efficiency of ethanol as a solvent for extracting bioactive compounds from dried leaves (10 g). Of the tested varieties, Red Lady showed the highest extraction yield with 37.83 ± 0.95%, followed by Khaek Dam with 34.93 ± 2.73%, Cocoa with 32.87 ± 2.73% and Holland with 31.10 ± 2.62%. The difference in extraction yield observed among the four papaya varieties could be due to a combination of genetic factors, metabolite profiles, moisture content, tissue structure, and environmental conditions, consistent with previous reports [20]. These factors affect the solubility and availability of compounds during the ethanol extraction process. Red Lady, having the highest yield, may contain more ethanol-soluble metabolites than the other varieties.

3.1 Antiproliferative activity

In this research, we investigated the antiproliferative activity of four plant extracts, Red Lady, Khaek Dam, Cocoa, and Holland, against three different breast cancer cell lines (MCF-7), colorectal adenocarcinoma cell lines (SW620) and Vero cell as shown in **Table 1**. The following criteria were used to rank the cytotoxic:

≤ 20 μg/mL is highly cytotoxic, 21-200 μg/mL moderately cytotoxic, 201-500 μg/mL weakly cytotoxic and > 501 μg/mL cytotoxic not inhibit [16].

Table 1. Cytotoxicity of ethanolic extract of papaya leaves against Vero, MCF-7, and SW620 cell lines in parameters of IC_{50} values (mean±SD) and SI

Sample	Vero	MCF-7		SW620	
	IC_{50}	IC_{50}	SI	IC_{50}	SI
Cocoa	not inhibit	not inhibit	ND	not inhibit	ND
Holland	not inhibit	not inhibit	ND	not inhibit	ND
Red lady	301.73 ± 0.73	90.88 ± 0.39	3.32	258.41 ± 2.16	1.17
Khak Dam	337.04 ± 8.28	328.60 ± 2.24	1.03	not inhibit	ND
Doxorubicin	1.02 ± 0.01	0.47 ± 0.00	2.17	0.35 ± 0.00	2.90

Data represented mean±SD from three independent experiments. SI Vero cells are a selectivity index calculated by dividing the IC50 of Vero by IC_{50} cancer cells. Not determined (ND)

Our research revealed that Red Lady exhibited moderate antiproliferative activity against MCF-7 cells with an IC_{50} value of 90.88 ± 0.39 μg/mL. At the same time, it showed weak activity against SW620 and Vero cells with IC_{50} values of 258.45 ± 2.16 and 301.73 ± 0.73 μg/mL, respectively. In contrast, Khaek Dam displayed weak antiproliferative activity against MCF-7 and Vero cells with IC_{50} values of 328.60 ± 2.24 and 337.04 ± 8.28 μg/mL, respectively, and no inhibition against SW620 cells. These findings offer promise for the potential of Red Lady as a potential treatment option for breast cancer. Additionally, the absence of cytotoxic effects of Cocoa and Holland extracts against MCF-7, SW620, and Vero cells suggests that these extracts may not be toxic to normal cells. In our study, the unrefined extract exhibited limited anticancer potential, as evidenced by weak inhibition of cancer cell lines. This observation is probably due to impurities in the extract, which consists of various constituents.

Upon examination, the extracted compound revealed a wide range of cytotoxic effects against the tested cancer cell types. The variations in cytotoxicity observed among different cells can be attributed to differences in their structure, genetic makeup, and origins, all of which influence their susceptibility to chemotherapy. Consequently, the potent cytotoxicity displayed by the investigated compound can be directly attributed to its high concentration of chemicals obtained from the plant extract. Notably, the Red Lady extract demonstrated significant cytotoxic activity against MCF-7 cancer cell lines with a Silective Index (SI) of 3.32, compared to doxorubicin. Achieving an SI greater than 3 indicates a high level of selectivity. The SI, which measures the differential activity of a sample, becomes higher as the selectivity increases. When the SI value surpasses two, it suggests the sample may pose potential risks [17]. In light of this, the Red Lady extract exhibited no cytotoxic activity on normal cells while exhibiting selectivity towards MCF-7 cancer cell lines.

3.2 Antioxidant activity

The antioxidant properties of the extracts were assessed through the DPPH radical scavenging assay. The data in **Table 2** illustrate the differences in the antioxidant activity of the four papaya leaves, reflected in their IC_{50} values. Red Lady has the lowest IC_{50} value of 163.87 ± 6.81 μg/mL, meaning it has the highest antioxidant capacity. Khaek Dam outperformed Cocoa and Holland with a moderate IC_{50} value of 288.16 ± 7.19 μg/mL, although it was still significantly less potent than Red Lady. In comparison, Holland (428.34 ± 23.30 μg/mL) showed the weakest antioxidant activity, closely followed by Cocoa (405.06 ± 6.89 μg/mL). The higher IC_{50} values for these varieties indicate a lower ability to neutralize free radicals, probably due to the bioactive compounds' lower concentration or activity. The differences in antioxidant activity between cultivars may be due to several factors. Genetic variations between papaya varieties significantly impact the synthesis and accumulation of secondary metabolites, which are directly related to antioxidant capacity. In addition, environmental factors such as soil composition, climate, and cultivation methods may also play a role in differences in secondary metabolite content, which is in agreement with previous reports [21-22].

Table 2. Antioxidant activity of ethanolic extract of papaya leaves in parameters of IC_{50} values (mean ± SD)

Sample	IC_{50} (μg/mL)
Cocoa	405.06 ± 6.89
Holland	428.34 ± 23.30
Red lady	163.87 ± 6.81
Khak Dam	288.16 ± 7.19
Trolox	49.86 ± 1.29

3.3 GC–MS analysis

Following a thorough antiproliferative activity of four extracts, Red Lady, Khaek Dam, Cocoa, and Holland, we have identified the potent extracts for antioxidant and antiproliferative activities. Our findings indicate that the ethanolic extracts of Khaek Dam and Red Lady exhibit the most significant activity, as evidenced by their IC_{50} values. Consequently, plant components of two extracts were continually analyzed using gas chromatography-mass spectrometry (GC–MS), a widely used technique for phytoconstituent separation. These chemicals were determined by their retention time on the fused silica capillary column. They were categorized as bioactive components by comparing their mass spectrum fragmentation patterns with those of recognized chemicals from the NIST library (NIST14). The GC–MS chromatogram of the identified compounds is shown in **Figure 1**.

Many biologically active components were observed. The identified chemical constituents are listed in **Table 3** by retention time, peak area (%), molecular formula and match factor (> 80.00), and % of the total (> 0.5). The GC–MS chromatograms of the Red Lady and Khaek Dam extracts show a total of 23 and 22 components, respectively. 11 chemicals are present in both varieties, including acetic acid, 1,1-cyclohexanedimethanol, 4'-methylacetophenone, neophytadiene, dodecanoic acid, n-hexadecanoic acid, palmitoleic acid, *cis*-13-octadecenoic acid, 1-heptadecanecarboxylic acid, loliolide, and linolenic acid.

In this study, the main constituents of the Red Lady leaf extract have been identified. The 9-octadecenamide was the most abundant, accounting for 20.82% of the total peak area. It was followed by n-hexadecanoic acid at 8.26%, palmitoleamide at 5.43%, hexadecanamide at 5.36%, and *cis*-13-octadecenoic at 1.92%. Other chemical components included neophytadiene, 1,1-cyclohexanedimethanol, acetic acid, and tetradecanoic acid. On the other hand, the papaya leaf extract from the Khaek Dam variety showed a different composition. The n-hexadecanoic acid was the main component, constituting 17.53% of the total peak area. It was followed by 1-heptadecanecarboxylic acid at 6.86%, loliolide at 5.58%, and 1,2,3-propanetriol at 3.04%. Other notable components included acetic acid, 4-oxo-pentanoic acid, and myristic acid. Several phytoconstituents were found in ethanol extracts of *C. papaya* leaves (Khaek Dam and Red Lady). These results suggest that the chemical components present in papaya leaf extracts vary depending on the variety. Both varieties contained a diverse range of compounds, including monoterpenes, monoterpenoid lactones, sesquiterpenes, diterpenes, fatty acids, fatty acid amide, phenolics, esters, alcohols, ketones, and aldehydes. This work shows that the main components of the two varieties are very different.

From GC–MS data), many components of the Khaek Dam variety were significant, including loliolide, 1-heptadecanecarboxylic acid, and n-hexadecanoic acid. Compared to Red Lady, Khaek Dam contains less loliolide, 1-heptadecanecarboxylic acid, and n-hexadecanoic acid. According to this research, Red Lady appears to have a higher phytochemistry component abundance or concentration than Khaek Dam. The results of the present study show that extracts from papaya leaves contain a broad spectrum of important and valuable chemicals, with the availability and content of these compounds varying depending on the cultivars.

Previous research has studied the chemical composition of papaya. It was revealed that in 2018, the significant phytocomponents found in Red Lady leaves were benzylnitrile, pyridine-2d,6-methyl-1,1'-dimethyl-2'-propenylbenzoylformate, osmium, methyl-2-(1,1-dimethylethyl)-4,5-dihydroxy-3-xazolidinecarboxylato[2-4,O5]dioxobispyridine[OC644]2R(2α,4β,5β), 6-(N,N-diethylaminomethyl)-2,5-dimethylphenol,

and benzene, isothio-cyanatomethyl [23]. However, In 2021, the main phytochemicals discovered in papaya leaves extract were butyl-9,12,15-octadecatrienoate, dasycarpidan-1-methanol, acetate (ester), n-hexadecanoic acid, neophytadiene, oleic acid, phytol, sitosterol, tocopherol, tetramethyl-2-hexadecen, campesterol, squalene, octadecenoic acid, stigmasterol, and D-limonene [24]. In 2022, Smrati *et al*., reported that 9,12,15-otadecatrienoic acid, (Z,Z,Z)-Linolenic acid, 1,2-benzenedimethanol, 4-methyl-benzaldehyde, nonadecanoic acid, sucrose, d-glycero-dgalacto-heptose, rhodopin, benzyl nitrile, and ethyl-9,12,15-octadecatrienoate from the leaves [25]. Previous reports show that papaya has similar ingredients in research results. However, the main chemical constituents exhibit both similarities and differences. The phytochemical composition of an extract is influenced by factors such as the plant species, plant part used, growth conditions, solvents used, and the extraction method. Different subspecies of a plant species may have genetic variations that result in distinct biochemical pathways and the production of unique secondary metabolites.

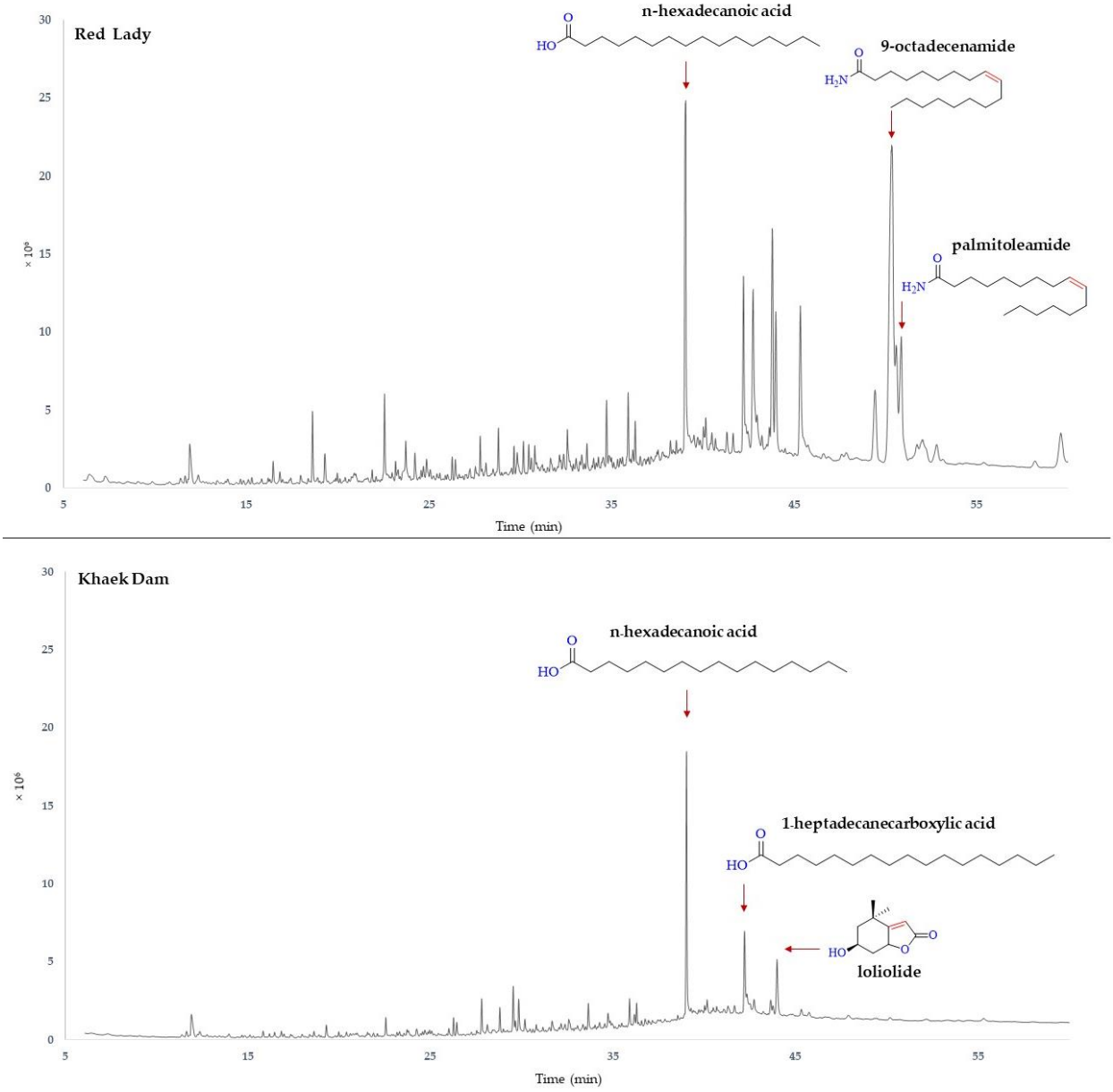

Figure 1. Three main components were displayed on GC–MS analysis of Red Lady and Khaek Dam.

Table 3. GC–MS analysis of ethanol extract from *C. papaya*; Red Lady and Khaek Dam

No.	Red Lady				Khaek Dam			
	RT (min)	Name	Formula	Component Area (% of total)	RT (min)	Name	Formula	Component Area (% of total)
1	11.8997	acetic acid	$C_2H_4O_2$	17091171.6 (1.09%)	11.9268	acetic acid	$C_2H_4O_2$	10880794.1 (2.89%)
2	18.6307	1,1-cyclohexanedimethanol	$C_8H_{16}O_2$	17101098.3 (1.09%)	18.6308	1,1-cyclohexanedimethanol	$C_8H_{16}O_2$	1918112.3 (0.51%)
3	19.3059	4'-methylacetophenone	$C_9H_{10}O$	8555602.5 (0.54%)	19.3060	4'-methylacetophenone	$C_9H_{10}O$	4016117.2 (1.07%)
4	22.5742	neophytadiene	$C_{20}H_{38}$	20291240.2 (1.29%)	22.5634	neophytadiene	$C_{20}H_{38}$	5038236.6 (1.34%)
5	23.7410	phytol	$C_{20}H_{40}O$	8229725.6 (0.52%)	24.2597	11-decyldocosane	$C_{32}H_{66}$	2923372.1 (0.78%)
6	27.8141	phytol isomer	$C_{20}H_{40}O$	9731994.2 (0.62%)	26.2800	1,2-heptanediol	$C_7H_{16}O_2$	3816053 (1.01%)
7	29.8399	levulinic acid	$C_5H_8O_3$	8921515.7 (0.57%)	26.4529	hexahydrofarnesyl acetone	$C_{18}H_{36}O$	3353178.5 (0.89%)
8	30.1802	dihydroactinidiolide	$C_{11}H_{16}O_2$	8135241.2 (0.52%)	27.8196	1-eicosanol	$C_{20}H_{42}O$	8082527.8 (2.14%)
9	32.5841	dodecanoic acid	$C_{12}H_{24}O_2$	12090855.4 (0.77%)	28.1275	methyl palmitate	$C_{17}H_{34}O_2$	2940370.5 (0.78%)
10	34.7341	phytol	$C_{20}H_{40}O$	15738932.4 (1.00%)	29.5483	1,2,3-propanetriol	$C_3H_8O_3$	11449063.1 (3.04%)
11	35.9172	tetradecanoic acid	$C_{14}H_{28}O_2$	16925273.9 (1.08%)	29.6671	2,4-di-tert-butylphenol	$C_{14}H_{22}O$	2304985.4 (0.61%)
12	36.3007	2-tertbutylcyclohexylpropyl-phospho-nofluoridate	$C_{13}H_{26}FO_2P$	10038392 (0.64%)	29.8400	4-oxo-pentanoic acid	$C_5H_8O_3$	8354470.3 (2.22%)
13	39.0720	n-hexadecanoic acid	$C_{16}H_{32}O_2$	129805907.5 (8.26%)	30.1857	dihydroactinidiolide	$C_{11}H_{16}O_2$	3095708.4 (0.82%)
14	40.1632	palmitoleic acid	$C_{16}H_{30}O_2$	9827461.1 (0.63%)	32.5842	dodecanoic acid	$C_{12}H_{24}O_2$	4237708.4 (1.12%)
15	42.7616	*cis*-13-octadecenoic acid	$C_{18}H_{34}O_2$	64966881.5 (4.13%)	33.3621	3-hydroxy-β-damascone	$C_{13}H_{20}O_2$	2275341 (0.60%)
16	42.2268	1-heptadecanecarboxylic acid	$C_{18}H_{36}O_2$	47671291.2 (3.03%)	35.9173	myristic acid	$C_{14}H_{28}O_2$	5955232.8 (1.58%)
17	42.9777	octadec-9-enoic acid	$C_{18}H_{34}O_2$	10140869.1 (0.65%)	39.0397	n-hexadecanoic acid	$C_{16}H_{32}O_2$	66092962.6 (17.53%)
18	43.8042	hexadecanamide	$C_{16}H_{33}NO$	84254217.1 (5.36%)	40.1633	palmitoleic acid	$C_{16}H_{30}O_2$	4512992.1 (1.20%)
19	43.9986	loliolide	$C_{11}H_{16}O_3$	54058360.8 (3.44%)	42.2161	1-heptadecanecarboxylic acid	$C_{18}H_{36}O_2$	25852532.1 (6.86%)
20	45.3437	linolenic acid	$C_{18}H_{30}O_2$	58907641.5 (3.75%)	42.7347	*cis*-13-octadecenoic acid	$C_{18}H_{34}O_2$	3421784.5 (0.91%)
21	49.4385	octadecanamide	$C_{18}H_{37}NO$	48439157.5 (3.08%)	43.9879	loliolide	$C_{11}H_{16}O_3$	21049801.5 (5.58%)
22	50.3406	9-octadecenamide	$C_{18}H_{35}NO$	327220486 (20.82%)	45.3222	linolenic acid	$C_{18}H_{30}O_2$	2799327.2 (0.74%)
23	50.8700	palmitoleamide	$C_{16}H_{31}NO$	85354814.6 (5.43%)				

3.3 Biological activity of major compounds of Red Lady and Khaek Dam variety

In this research on papaya leaf extracts, it has been discovered that the prominent phytochemicals present are n- hexadecanoic acid and 9- octadecenamide. Red Lady extracts rich sources of 9-octadecenamide has proven effective in anti-inflammatory, antimicrobial, and antioxidant activities [26–28]. The 9-octadecenamide, known as oleamide, was isolated from *Moringa oleifera*. It was shown to have anticancer activity on human myelogenous leukemia cells (K562), human squamous cell carcinoma (SCC-15), and breast cancer cells (MDA-MB-231) [29]. Khaek Dam extract enriched n-hexadecanoic acid. It also exhibited against breast cancer and Vero cells, which corresponded with the previous report. The n-hexadecanoic acid-enriched extracts of *Ulva intestinalis*, *Sargassum ilicifolium*, *Halimeda macroloba*, and *Halymenia durvillei* have cytotoxic activity against human breast and hepatocellular carcinoma cells [30-31]. Moreover, it has been demonstrated to be effective against human colorectal carcinoma (HCT-116) cells [32]. It also promoted human leukemia cells [33] and colon cancer cells (HT-29) [34]. Notably, n-hexadecanoic acid showcases its value through its ability as an antibacterial, antioxidant, and anti-inflammatory activity [35–37]. Additionally, other components of the extracts, such as palmitoleamide and *cis*-13-octadecenoic, exhibit anti-inflammatory properties [38], while hexadecanamide has anti-inflammatory, antifungal, and anti-nociceptive [39]. Moreover, 1-heptadecanecarboxylic acid displays antimicrobial properties [40], and loliolide shows antioxidant, antifungal, antibacterial, and anti-cancer effects [41–43].

4. Conclusions

The study assessed the antiproliferative activity of the ethanol extracts from *C. papaya* leaves (Cocoa, Holland, Khaek Dam, and Red Lady). Among these, the Red Lady variety showed the most promising results. It exhibited moderate antiproliferative activity against breast cancer (MCF-7) cells with an IC_{50} value of 90.88 + 0.39 μg/mL, coupled with a Selectivity Index (SI) of 3.32, indicating a high level of selectivity towards cancer cells without affecting normal cells. Antioxidant analysis revealed that Red Lady again stood out, with the highest antioxidant capacity (IC_{50} = 163.87 μg/mL). The extracts of Khaek Dam and Red Lady show various components. However, further investigations are needed to purify and identify the active compounds to confirm the antiproliferative activity.

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Research article

Biodegrading Lignocellulosic Agricultural Waste Using *Phanerochaete chrysosporium* and Electrical Current Stimulation

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Abstract: White-rot fungi (WRF), such as *Phanerochaete chrysosporium*, play a significant role in the lignin degradation (LD) of biomass, an essential process in the carbon recycling of terrestrial ecosystems. However, the rapid development of the agroindustry has imposed a daunting task on biomass waste management. One green initiative focuses on enhancing the bioremediation of lignin since it forms a resistant barrier to chemical and biological LD. This work demonstrated that electric current stimulation (ECS) can markedly enhance LD by *P. chrysosporium*. Palm oil empty fruit bunches (EFBs) were utilized as a lignin-rich substrate for *P. chrysosporium*. These were placed in a 250-ml enclosure filled with unbuffered potato dextrose broth (PDB) as the electrolyte. The ECS was supplied in situ in two ways: (1) by inserting a zinc anode/air electrode redox couple into the enclosure to produce a self-sustaining discharge current (DC), and (2) by connecting the enclosure to an external current (EC). The lignin content (LC) of the EFBs was assessed after 30 days of exposure to fungal microbes in an uncontrolled environment. The fungal LD rate was highest at 3 mA and even doubled under the influence of the EC, enhancing the lignin removal by 74.6%. The proposed method is much simpler and cheaper than the electrocatalytic reactions produced by the electro-Fenton method.

Keywords: Lignin degradation; microbial zinc/air cell; electric current stimulation; white rot fungi

1. Introduction

The agroindustry generates a huge quantity of biomass wastes and residues. Without serious efforts and mitigation measures, this will ultimately lead to significant environmental pollution. Growing environmental concerns have pushed the industry's sustainable development to center stage. Bioremediation of biotransformation has been identified as a potential secondary technology for converting biomass wastes into valuable byproducts or their reproduction into environmentally benign byproducts. Empty fruit bunches (EFBs) are a solid biomass the palm oil industry produces. On average, 50-70 tonnes of biomass are produced from a hectare of oil palm plantation. Empty fruit bunches (EFBs) contribute 20-23% of solid waste [1]. Unfortunately, most of this biomass is either incinerated, disposed of as waste in landfills, or dumped on plantation grounds

as mulch [1]. Although these disposal methods are cost-effective, they can lead to greenhouse gas emissions and create homes for pests [2].

Using white-rot basidiomycetes for wood's lignin degradation (LD) is a central step of carbon recycling in terrestrial ecosystems. As such, they have been extensively studied for the bioremediation of wastes and residues from the agroindustry. White-rot fungi (WRF), such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus, Trametes versicolor*, and *Xylobolus frustulatum*, have a unique ability to break down wood lignin [3,4]. Lignin is a recalcitrant material that forms the protective structural matrix surrounding the hemicellulose and cellulose within cell walls [5,6]. It is not only abundant in nature but is also a potential source of renewable chemicals and energy. However, the main challenge lies in its intrinsic mechanical and chemical resistance. *P. chrysosporium* has been widely researched for this purpose, given its ability to produce a more complete LD enzyme complex than most other strains [7].

Earlier, we reported on a self-sustaining microbial zinc/air cell (MZAC) employing *P. chrysosporium* fed with oil palm EFBs [8]. This lignocellulosic biomass was mainly composed of three main components: cellulose (44.2%), hemicellulose (33.5%), and lignin (20.4%) [9]. The anodic zinc oxidation was believed to provide a continuous supply of free electrons for the LD of the EFBs. It was thought that such a condition would most likely alter the metabolism pathways of the microbes and eventually influence the LD rates of the biomass. Therefore, this work assessed *P. chrysosporium* and electric current stimulation (ECS) for LD in EFBs. First, a discharge current was generated by the electrochemical reactions of the MZAC fed with the fungus-cultivated EFBs. Second, an external current was used to pass an ECS through the enclosure containing the fungus-cultivated EFBs.

Microbes' metabolic activities, bacteria, and fungi have been reported to be strongly influenced by an ECS. This is of particular interest for enhancing the bioremediation of environmental pollutants. Vasileva et al. observed that the symbiotic *Bradyrhizobium japonicum* bacterium's phenolic degradation capability was enhanced by applying an electric field [10]. Since EFBs consist of lignin with a highly-branched phenolic polymer, it would also be interesting to observe the influence of an applied electric field on LD. Shen et al. used an advanced electro-Fenton method to promote LD using the WRF, *T. versicolor* [4]. In the electro-Fenton method, the electro-oxidation of the iron-based composite cathode generates hydroxyl radicals. These strong oxidants attack various functional groups in lignin, thereby accelerating the LD using *T. versicolor* [4]. The present study proposed a much simpler method of enhancing LD using another WRF, *P. chrysosporium*, and a small ECS.

2. Materials and Methods

2.1 Selecting the raw materials and microorganisms

Empty fruit bunches (EFBs) were collected from a palm oil plantation. The raw EFBs were manually shredded and sieved into small sizes of around 0.5 cm. These were soaked for 24 hours, rinsed with distilled water, and dried under ultraviolet (UV) light. The WRF, *P. chrysosporium*, was used as the organic waste-degrader and cathodic catalyst source in the MZAC. *P. chrysosporium* secretes several ligninolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. Of these three enzymes, laccase plays a crucial role in MZACs due to its ability to reduce oxygen (O_2) to water (H_2O). *P. chrysosporium* was first cultivated in potato dextrose agar (PDA) for six days and was then transferred into a potato dextrose broth (PDB) for re-cultivation for 14 days. The specimen was filtered out from the PDB and dried until its moisture content was < 15%.

2.2 Preparing the microbial zinc/air cell (MZAC)

The cell was a membrane-less, single-chamber cylindrical jar with a capacity of 250 ml that was filled with 24 g/L of unbuffered PDB electrolyte. A zinc anode strip (30 x 30 mm) was paired with a commercial E4/E4A-EFL air cathode of the same dimensions. The fungus-cultivated EFBs were prepared from 5 g of dried *P. chrysosporium* and 2 g of oil palm-derived EFBs and incubated for six days. In the MZAC, the fungal microbes were left freely suspended in the electrolyte. They eventually grew and attached themselves to the EFBs at the bottom of the cell enclosure. Refer to our previous publication for details [8]. The electrolyte was not buffered; no additives, such as an electron transfer mediator, microbe growth enhancer, or particular nutrients, were added. The MZAC was left to operate in an uncontrolled environment.

2.3 Operating principles of the microbial zinc/air cell (MZAC)

When supplied with lignin-rich EFBs, *P. chrysosporium* fungal microbes will secrete ligninolytic enzymes that predominantly contain laccase, which has a specific affinity for O_2 molecules as its electron acceptor. Therefore, a bio-catalyzed electrochemical MZAC can be produced by pairing a zinc anode with an air cathode in a medium rich with *P. chrysosporium* hyphae. While the fungal microbes degrade the lignocellulosic wall, the laccase will catalyze the reduction of molecular O_2 [11].

2.4 Influence of electric current stimulation (ECS) on the lignin degradation (LD) of empty fruit bunches (EFBs)

The influence of ECS on the LD of EFBs was observed under two conditions:

(a) Discharge current from MZAC
ECS was generated from the discharge current of the MZAC. A discharge current of 1, 2, and 3 mA was produced continuously for 30 days.

(b) External current
The same MZAC enclosure was utilized, but both electrodes were replaced by nickel mesh. A constant external current of 1, 2, 3, 5, and 6 mA was applied continuously for 30 days.

The current was regulated using a NEWARE® BTS4000 battery tester for both conditions. After 30 days of discharge, the EFBs were retrieved, rinsed, and dried for characterization.

2.5 Characterising the lignin degradation (LD) of the empty fruit bunches (EFBs)

The LD of the EFBs was characterized according to their surface morphology and quantitative lignin content (LC). Changes to the surface morphology were observed using a JEOL® JSM-6700F scanning electron microscope (SEM). The quantitative LC was determined using the acid-chlorite method. In this method, 0.15 g of EFBs was first boiled twice with 75 ml of H_2O for 1 hour to remove the hot-water-soluble materials before being dried at 60 ℃ for 15 hours and weighed (W_1) [12]. The samples were treated with 30 ml of H_2O, 2 ml of 10% acetic acid, and 0.6 g of sodium chlorite at 75 ℃ for 1 hour. Then, they were treated further with 10% acetic acid (2 ml) and 0.6 g of sodium chlorite at 75 ℃ for 2 hours. Finally, the samples were rinsed with H_2O (five times), acetone (twice), and ether (once) and then dried at 105 ℃ for 90 minutes and weighed (W_2). All samples were tested in triplicate. The LC was determined using:

$$Lignin\ content\ (\%) = \frac{W_1 - W_2}{0.15}, \quad (1)$$

3. Results and Discussion

Empty fruit bunches (EFBs) are a lignocellulose waste of lignin, cellulose, and hemicellulose. Lignin is the most chemically resistant to decomposition among the three components due to its complex structure. It is formed by the oxidative coupling of three distinct phenylpropane building blocks: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, connected by carbon-carbon and ether bonds [13]. Due to the heterogeneity of the bonds, instead of hydrolytic enzymes, ligninolytic enzymes are required for delignification, and basidiomycetes WRF has been proven to be the most effective.

The basidiomycetes WRF, *P. chrysosporium*, is highly capable of degrading lignocellulosic biomass by secreting enzymes such as laccase, MnP, LiP, xylenes, cellulase, glucose-1-oxidase, and glucose-2-oxidase [14]. Compared to other WRFs, *P. chrysosporium* possesses the advantages of a high growth rate, adaptability under harsh environments, simultaneous decay of lignocellulosic materials, and excellent oxidation abilities [13,15].

It was observed that upon exposing the EFBs to *P. chrysosporium*, the outer protective lignin layer showed signs of degradation starting on Day 20. By Day 30, the cell walls had almost wholly collapsed [8]. The efficacy of the LD of the EFBs under ECS was also characterized based on the estimation of the LC after 30 days of exposure to *P. chrysosporium*. Changes to the morphology of the EFB substrate were also observed to support the quantitative data. The chemical structure of lignin and its abundance, even from the same plant, vary with its source. These characteristics contribute to the lignin-breaking behavior as well [16]. However, the structural characteristics of lignin are often overlooked when discussing LD.

As anticipated, the ECS strongly influenced the LD of the EFBs using *P. chrysosporium*. The lignin composition in EFB was estimated at around 34.0%. In the absence of the ECS, after 30 days of exposure to *P. chrysosporium*, the EFBs' LC was reduced from 34.0% to 28.4%. At a discharge current of 1 mA, the LC was 24.2% (~14.8% decrease); at a discharge current of 2 mA, the LC was 20.3% (~28.5% decrease); and at a discharge current of 3 mA, the LC was the lowest, i.e. 17.9% (~37% decrease) (Table 1). Therefore, the discharge current promoted the LD of the EFBs using *P. chrysosporium,* and the influence increased as the DC increased up to 3 mA. The cell could not sustain a constant discharge current greater than 3 mA.

Table 1. The LC is a function of the ECS (MZAC's discharge current).

LC (%)						
Control	**Post-30 Days Exposure to a Constant DC**					
No Current	1 mA	Δ (%)	2 mA	Δ (%)	3 mA	Δ (%)
28.4 ± 0.2	24.2 ± 0.2	14.8	20.3 ± 0.02	28.5	17.9 ± 1.0	37.0

Δ - Decrement in LC
Each value is a mean of three replicates ± standard errors.

The presumption that the availability of free electrons promotes LD by *P. chrysosporium* was tested by applying an external current to an enclosure containing the EFBs and *P. chrysosporium*. Both the zinc anode and air cathode were replaced with nickel mesh. An electric current stimulation (ECS) was externally supplied to pass through the enclosure. The percentage of remaining LC as a function of the external current is presented in Table 2. The results substantiated the role of an external current in promoting LD using *P. chrysosporium*. Interestingly, the external current had a more substantial influence on LD as the LC of the EFBs was lower than that of the MZAC discharge current. It was thought that in the MZAC, a portion of the discharge current participated in the electrochemical redox reactions, while the remaining was utilized in the fungal metabolic activities. On the other hand, when an external current was applied in the absence of the anode-cathode couple, the entire external current could be utilized in the fungal metabolic activities, thus contributing towards a higher LD. When the external current was 3 mA, the fungal LD rate was the highest and even doubled under its influence. However, the fungal LD rate did not increase when the external current increased. When the external current was 5 and 6 mA, the LC was roughly similar to when the external current was 1 mA. Figure 1 summarises the variations in the LC of the EFBs as a function of the ECS.

Table 2. The LC is a function of the ECS (external current).

LC (%)										
Control	**Post-30 Days Exposure to a Constant DC**									
No Current	1 mA	Δ (%)	2 mA	Δ (%)	3 mA	Δ (%)	5 mA	Δ (%)	6 mA	Δ (%)
28.4 ± 0.2	22.8 ± 0.1	19.7	17.1 ± 0.8	39.8	7.5 ± 0.01	74.6	22.1 ± 0.5	22.2	22.2 ± 4.0	21.8

Δ - Decrement in LC
Each value is a mean of three replicates ± standard errors.

Beyond 3 mA, the supply of free electrons no longer increased the LD. There are two possible explanations. First, the excess charge supplied might have disrupted or inhibited the fungal metabolic activities, and hence, they were no longer at an optimum rate [4]. Second, with an increasing supply of free electrons, more reactive oxygen species would have been generated, which, in turn, would have produced more phenoxyl-free radicals. However, when too many phenoxyl-free radicals are generated, they can react with other organic compounds, such as cellulose and hemicellulose. This probably contributed to the high standard error in determining the LC at 6 mA. Brenelli et al. [17] found that in a laccase-mediated system, electrons from the delignification of lignin can be used to activate lytic polysaccharide monooxygenases and, ultimately, enhance the enzymatic hydrolysis of cellulose.

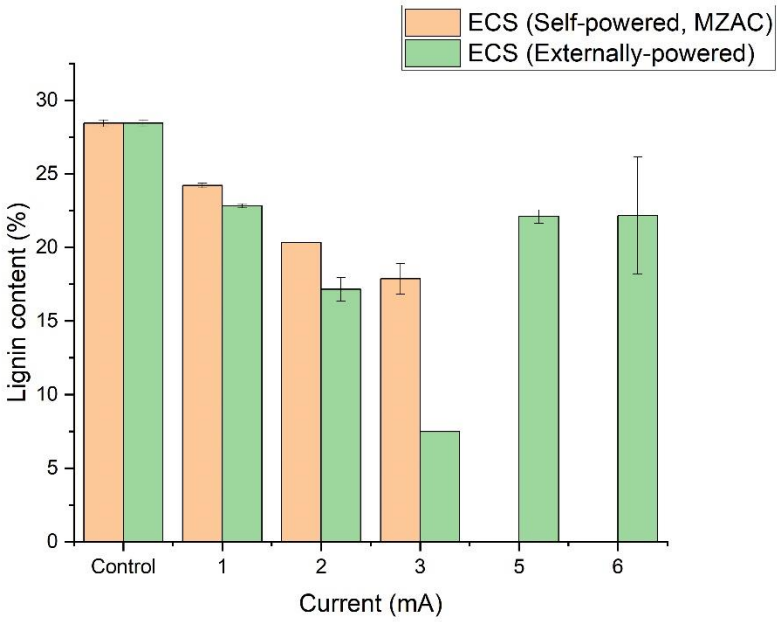
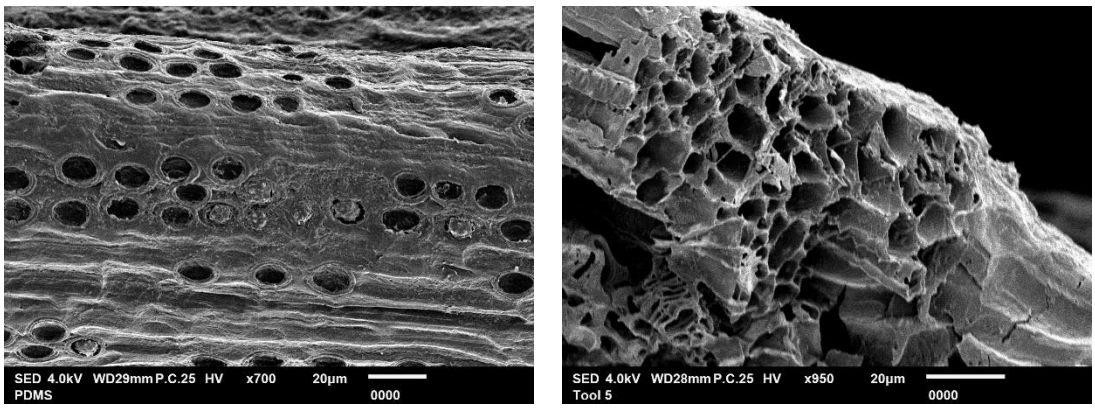


Figure 1. The influence of ECS on the LD of the EFBs using *P. chrysosporium*. Each value is a mean of three replicates ± standard errors.

The LD of EFBs by *P. chrysosporium* under ECS was also characterized by observing the changes to their morphology. Figure 2 shows the scanning electron microscope (SEM) images of an EFB strand. It had a rigid and rough surface with craters and silica bodies attached (Figure 2a), preventing fungal hyphae from penetrating the cellulose and hemicellulose matrix. Once the silica bodies are removed from the surface, it will be easier for fungal hyphae to penetrate through for delignification. Figure 2b suggests that each EFB strand comprised rod-shaped microfibrils lumped with cavities in the middle. Each rod-shaped microfibre was surrounded by cell walls containing most of the lignin [3,18].

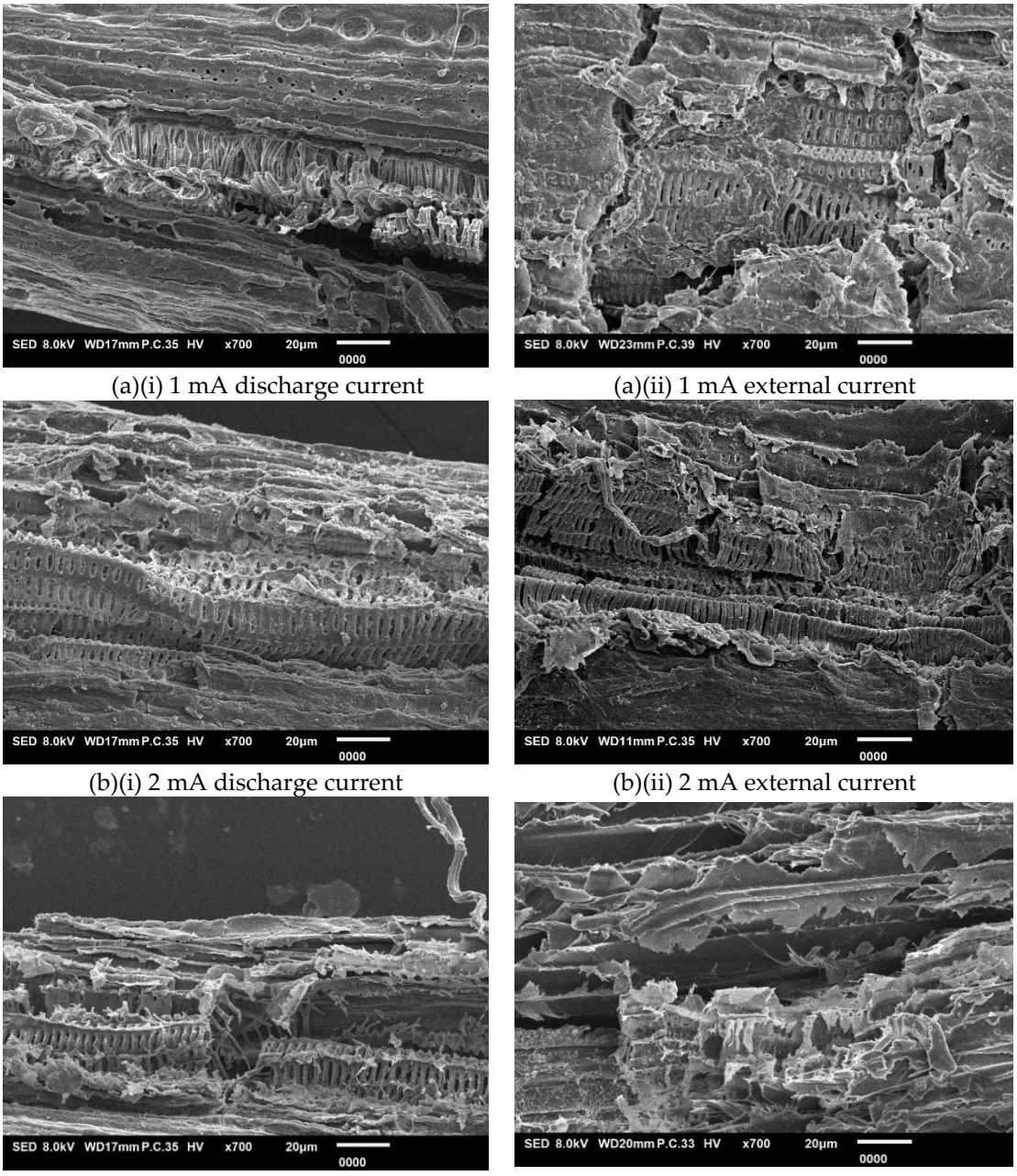


(a) Longitudinal view (b) Cross-sectional view

Figure 2. SEM images of the morphology of the EFB strand pre-exposure to P. chrysosporium.

Figure 3 shows the morphology of the LD of the EFBs using *P. chrysosporium* and after 30 days of exposure to varying ECS. The observations clearly showed that the ECS enhanced the LD of the EFBs using *P. chrysosporium*, as substantiated earlier by the determination of the LC. At ECS = 1 mA (Figure 3a), the outermost cell wall had almost wholly degraded, showing a pitted vessel and helical/matrix-like structure attributed to cellulose [19]. More pitted vessels were visible at ECS = 2 mA, exposing a more matrix-like structure and allowing the *P. chrysosporium* mycelium to penetrate the innermost cell wall (Figure 3b). Finally, at ECS = 3 mA, most of the outermost cell wall and the outermost matrix-like structure had almost completely

collapsed, revealing the inner vessels and matrix-like structure. From the SEM images, the external current yielded more a significant LD in the EFBs, as proven by the lower LC.



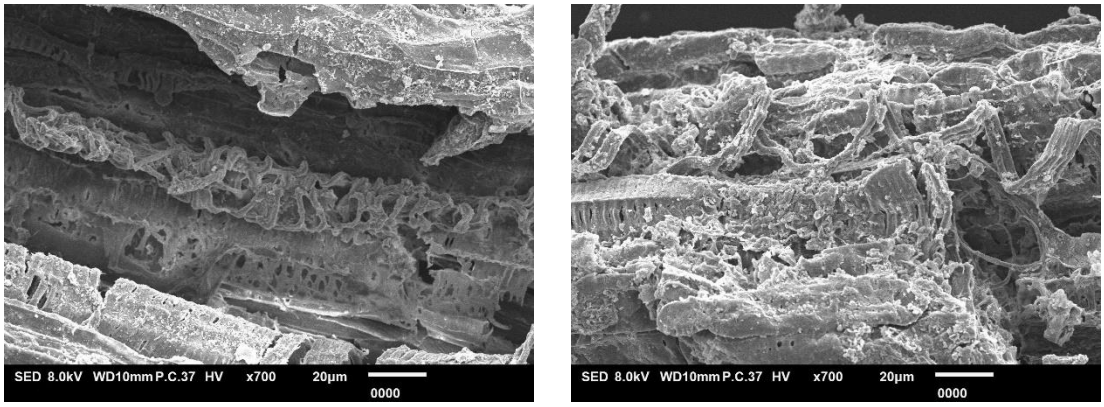
(a)(i) 1 mA discharge current (a)(ii) 1 mA external current

(b)(i) 2 mA discharge current (b)(ii) 2 mA external current

(c)(i) 3 mA discharge current (c)(ii) 3 mA external current

Figure 3. SEM images of an EFB strand morphology post-30 days of exposure to ECS: (i) MZAC discharge current and (ii) external current.

Figure 4 shows the SEM micrographs of the LD of the EFBs using *P. chrysosporium* when the external current was 5 and 6 mA. At first glance, the LD appeared to be advanced. However, this was not supported by the acid-chlorite LC data. Upon careful observation, the inner vessels and matrix-like structure, which corresponded to the cellulose and hemicellulose, collapsed faster than the protective outermost structure, i.e., the lignin layer. This was, most likely, due to a reaction of the excess phenoxyl-free radicals.



(a) 5mA external current (b) 6mA external current

Figure 4. SEM images of the LD of EFBs using *P. chrysosporium* and external current of (a) 5 mA and (b) 6 mA.

Shen et al. [4] reported the enhancement of LD by *T. versicolor* upon inducing an electrocatalytic electro-Fenton reaction for 96 hours. The study utilized a commercial de-alkaline lignin. They reported an increase in LD from 25 to 85.4% when the current was increased from 50 to 300 mA. The electro-Fenton method is an electrocatalytic reaction based on the in-situ generation of hydroxyl radicals on an iron-based composite cathode (Fe^0 and Fe_3O_4/graphite felt). A huge amount of hydroxyl radicals are produced from the reaction between hydrogen peroxide (H_2O_2) and Fe^{2+} ions. The strong hydroxyl oxidant is primarily thought to degrade the complex lignin structures and functional groups. Consequently, this will promote the acceleration of LD by LiP and MnP.

Beschkov et al. [10] and Vasileva et al. [20] studied the effect of a constant EC on phenol degradation using the bacteria *Pseudomonas putida* and *B. japonicum 273*. The enhanced phenol degradation (3 - 4 times) was attributed to the ECS generated by the activities of the excreted enzymes (phenol hydroxylase and catechol dioxygenase) rather than the electrochemical oxidation at the anode. They applied an anodic potential of 0.8 - 1.0 V (SHE) to a fed-batch mode fermenter with an initial concentration of 0.06 g/L of phenol. They reported that the highest phenol removal efficiency was observed at 0.8 V using *P. putida* and 1.0 V using *B. japonicum 273*. For most of the experiment, the discharge current was < 10 µA [10]. They conjectured that the applied external current induced some changes at the site of the active enzymes and stimulated the activation energies of phenol oxidation and benzene ring cleavage [10].

The present study examined the LD of EFBs using *P. chrysosporium* and a constant ECS. *P. chrysosporium* secretes ligninolytic enzymes such as laccase and H_2O_2-dependent ligninase (i.e., LiP and MnP). All these enzymes are thought to act synergistically to degrade lignin and other biopolymers [21]. Given the aerobic nature of the fungal degradation of wood, the enzymatic processes are directly or indirectly (via intermediatory substrates) oxidative [16]. There is no general agreement on the enzymatic LD mechanisms due to two key variables: the heterogeneous lignin source and unpredictable lignin structure [16], let alone the type of fungal species involved. A typical laccase-based LD is commonly described as follows: Laccase catalyzes the one-electron oxidation of lignin phenolic units into phenoxyl-free radicals and electrons. These free electrons are then transferred to the side chain, leading to the cleavage of bonds (C_α-C_β cleavage, alkyl-aryl cleavage, and C_α oxidation) [22]. Manganese peroxidase (MnP) and LiP, on the other hand, act by producing mediators of oxidative LD (i.e., chelated Mn^{3+} and veratryl alcohol radical cations) [16,23]. Laccase, in turn, also possesses the ability to act as a mediator of the reactive products formed above [16].

Regardless of the proposed LD pathways using fungal microbes, they are characterized largely by electron transfers and radical formations [24]. One of the roles of the produced radicals is to serve as electron acceptors/mediators [24,25]. Thus, the present study posits that if free electrons are abundant during the LD of fungal microbes, this can lead to the cleavage of more bonds and eventually promote LD. Enhancing the rate of LD is a key step in the economic and green decomposition of lignocellulosic agricultural waste. This study was able to successfully develop an LD method that is much cheaper, significantly less complicated, and requires significantly less current than the electro-Fenton method.

4. Conclusions

The present study has substantiated that LD can be enhanced by nearly 74.6% by merely supplying free electrons to *P. chrysosporium*-cultivated EFBs. The electron supply can be generated in situ by the insertion of redox couple electrodes. This approach is much cheaper than the electrocatalytic reactions of the electro-Fenton method.

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Research article

Production of Red Palm Oil and Red Palm Fats by Vacuum Frying Sterilization and Multi-step Fractionation

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Abstract: The question of the health impact of refining palm oil has driven the oil palm producers in Thailand toward developing wellness products that are in high demand in the future. Red palm oil, rich in carotenoids and vitamin E, catches attention. However, the production of red palm oil using the current steam sterilization method harms the environment and requires a high capital investment. In contrast, the cost-saving dry sterilization method delivers a low-quality product. The vacuum frying method was studied to develop an effective red palm oil processing method suitable for small and medium-size enterprises (SMEs). The production steps include vacuum frying sterilization, acid degumming, neutralization, and multi-step fractionation. The steam sterilization method was compared using the same arrangement step except for the sterilizing procedure. Sterilization by the vacuum fryer was controlled at 80 °C, -400 to -720 mmHg. Results showed that the optimal vacuum frying time was 90 min. The fractionation temperatures of the vacuum frying method were 34 °C, 27 °C and 17 °C, whereas those of the steam sterilized method were 34 °C, 25 °C and 15 °C. The quality of the red palm oils produced from both methods was within the edible oil standard. The vacuum frying method produced red palm oil with 0.17% free fatty acids, 6.70 meq.O_2/kg peroxide value, 620 mg/kg carotenoids, 835 mg/kg vitamin E, and 61.31% yield. The main advantage of the vacuum frying method's quality lies in improving yield and oil odor, which is a sensory characteristic that plays an important role in consumer acceptance of the product. The red palm fats obtained from the multi-step fractionation provided a wide range that is suitable for different applications. Therefore, vacuum frying could be considered a sustainable technology that is appropriate for SMEs.

Keywords: Vacuum frying sterilization; Multi-step Fractionation; Red palm oil; Red palm fat; Crude palm oil

1. Introduction

Red palm oil is a less refined palm oil containing several phytonutrients important for good health, such as carotenoids, vitamin E, sterols, phospholipids, glycolipids, and squalene [1]. The distinctive characteristic of red palm oil is its red color due to its high content of carotenoids [2]. Production of red palm oil consists of two major steps: crude palm oil preparation and fractionation [2]. Preparing crude palm oil for red palm oil production should not undergo bleaching and deodorizing processes that destroy phytonutrients but mainly

involve bunch reception, fruit sterilization, and oil extraction [3]. The fractionation step, where the solid phase is separated from the liquid phase, controls the selectivity of crystallization and separation [4].

Fruit sterilization is an important step that inactivates the lipase enzyme to prevent the formation of free fatty acids [5]. This treatment also softens and loosens the palm fruit to facilitate fruit digestion and oil extraction. Steam sterilization, where the palm bunches are sterilized in a sterilizer using steam generated by a boiler, has been widely used in large-scale operations. This process achieves high productivity but requires high capital investments. It also discharges large amounts of wastewater. In addition, the extracted oil is likely to develop off-flavor and discoloration [6]. For small-scale operations in Thailand, dry sterilization has been widely used. This process involves heating palm fruit in a utensil fired by fiber or palm shells. After sterilization, the fruits are extracted through a process called pressing, which involves the application of mechanical pressure to the whole fruits without separating the palm kernel from the mesocarp. This process is cost-saving in terms of machinery and maintenance, but it is difficult to control the heating parameters of the process, which leads to low-quality crude palm oil. Besides, the obtained crude palm oil is likely to have a smoky odor and contain residual substances from the burnt fiber.

Since low-quality crude palm oil would deliver low-quality red palm oil, a new method for producing high-quality red palm oil, namely the vacuum frying method, was developed in this study to overcome the limitations of the steam and dry sterilization methods. The important steps in the vacuum frying method consist of vacuum frying sterilization, acid degumming, neutralization, and multi-step fractionation. The vacuum frying sterilization is a green process where palm fruits are fried under vacuum conditions to rapidly remove water from the palm fruits without wastewater discharge. By controlling the vacuum level, frying temperature, and length of frying time, vacuum frying sterilization is expected to preserve most phytonutrients and improve the odor quality of red palm oil [7]. Good-quality red palm oil is expected when combining vacuum frying sterilization with the post-treatment degumming process, neutralization, deodorization, and fractionation.

Three fractionation techniques have been used in the palm oil industry, including detergent fractionation, solvent fractionation, and dry fractionation. The detergent fractionation has high separation efficiency, but it is expensive and has been found to cause the products to become contaminated with the detergent. In solvent fractionation, it provides a high yield and purity of the product, but its production cost and capital investment are high. Dry fractionation is the simplest and cheapest approach. There is no effluent, no chemicals, and less losses. However, the viscosity of the fat could be the problem when a single fractionation is operated in bulk. Multi-step fractionation can overcome the viscosity problem [8].

The vacuum fryer used in this study was invented at the Chaipattana Foundation in Cha-am District, Phetchaburi Province, Thailand. Crude palm oil was used as a frying medium because it is a stable, cheap, and readily available oil. However, the frying medium crude palm oil contains existing carotenoids, which may affect the results of this study's extracted crude palm oil. Thus, the migrations of carotenoids and the frying medium crude palm oil were calculated to validate the study's data. The frying parameters for palm fruits were designed at a temperature of 80 °C and pressure of -400 to -720 mmHg, whereas a certain frying time has never been reported. The low temperature used would reduce the adverse effects on the oil quality. The operating pressure should be low enough to cause a significant decrease in the boiling point of water, allowing frying to be performed at low temperature. In this study, the lowest pressure that could be operated was -720 mmHg, which enabled the frying temperature to be 80 °C. However, there was an increase in pressure towards the evaporation of water during vacuum frying. The highest pressure was detected at -420 mmHg. Regardless of the frying pressure, the palm fruit experienced the desired temperature of 80 °C.

After frying, oil was extracted from the palm fruits with a single screw press, which is normally used by Thailand's small-scale palm oil industry. The important factor influencing the single screw press performance is the fruits' moisture content. The higher moisture content promoted plasticity decreased the degree of compression, and resulted in poor oil yield. In addition, moisture acted as a lubricant in the barrel; thus, higher moisture content contributed to inadequate friction during pressing. However, too low moisture in the fruit caused high oil sediment [9]. Our preliminary study found that 7% moisture was optimal in single screw-pressing of palm fruit.

Therefore, the current study aimed to determine the vacuum frying time that reduces the water content of the palm fruit to 7%. Meanwhile, the appearance of the fried palm fruits taken from different frying times was evaluated to ensure the quality of the end products. Though the exact frying time cannot be applied to fruits with different sizes and initial water contents, the finding on the effect of frying time would be of fundamental importance for researchers or producers who want to implement this method. At the multi-step fractionation, the fractionation temperatures were examined. The properties of the red palm oils and red palm fats were compared using vacuum frying sterilization and steam sterilization. The dry sterilization method was not included in this study because our preliminary experiment suggested that the smoky smell of the crude palm oil sterilized by this method is too strong and unacceptable by most panelists.

2. Materials and Methods

2.1 Materials

Fresh palm fruits were obtained as bunches or loose fruits from the field of the Chaipattana Foundation Project, Phetchaburi Province, Thailand. The fruit bunch consists of palm fruits embedded in spikelets growing on a bunch stem. Manual threshing was achieved by cutting the fruit spikelets from the bunch stem with an axe. Then, the fruits were separated from the spikelets by the slitting machine. The steam-sterilized crude palm oil was processed at 120-140 °C for 45 min and obtained from Srisuk Palm Co., Ltd., Prachuap Khiri Khan, Thailand. The refined palm oil is purchased from the local market. The chemicals used were either analytical or HPLC grade.

2.2 Methods

2.2.1 Red palm oil production

2.2.1.1 Vacuum frying sterilization

The vacuum fryer used in this experiment is located at the Chaipattana Foundation Project, Phetchaburi Province, Thailand. The major parts of the vacuum fryer are shown in Figure 1. Fresh palm fruits with a water content of 35.05 ± 4.49% were previously removed from the bunches, then a batch of 700 kg palm fruits was placed into the vacuum fryer filled with 400 kg crude palm oil. The palm fruits were vacuum fried at 80 °C and a pressure of -400 to -720 mmHg. The palm fruits were collected after vacuum-fried for 15 min and examined for water content, yield, and appearance. This experiment was repeated at 30, 45, and 60 minutes, frying until the water content of the fruits was about 7%. At each frying time, migration of the frying medium crude palm oil into the palm fruits was calculated to obtain the actual oil yield. The yield and appearance of the fruits identified the optimal vacuum frying time. After that, the palm fruits were vacuum fried under the optimal condition and extracted by the screw press at a rate of 120 kg fruits/hr. The extracted oil was kept at 25 °C in a dark brown glass container and analyzed for carotenoid content [10], free fatty acids [11], iodine value [11], peroxide value [11], phosphorus content [12], iron [12], copper [12], vitamin E content [13], melting point (DSC8000, Perkin Elmer, USA) and solid fat content (Pluse Nuclear Magnetic Resonance Spectrometers, Bruker, Germany). The GC method analyzed the free fatty acids with a capillary column and hydrogen flame ionization detector. The analyzing conditions were an inlet temperature of 225 °C, a split ratio of 200:1, a flow rate of 0.75 ml/min, and helium as carrier gas [11]. The carotenoid contents of the crude palm oil before and after use as the frying medium were analyzed and used to determine the actual carotenoid contents in the extracted oil. Similar tests were performed on the crude palm oil prepared by steam sterilization.

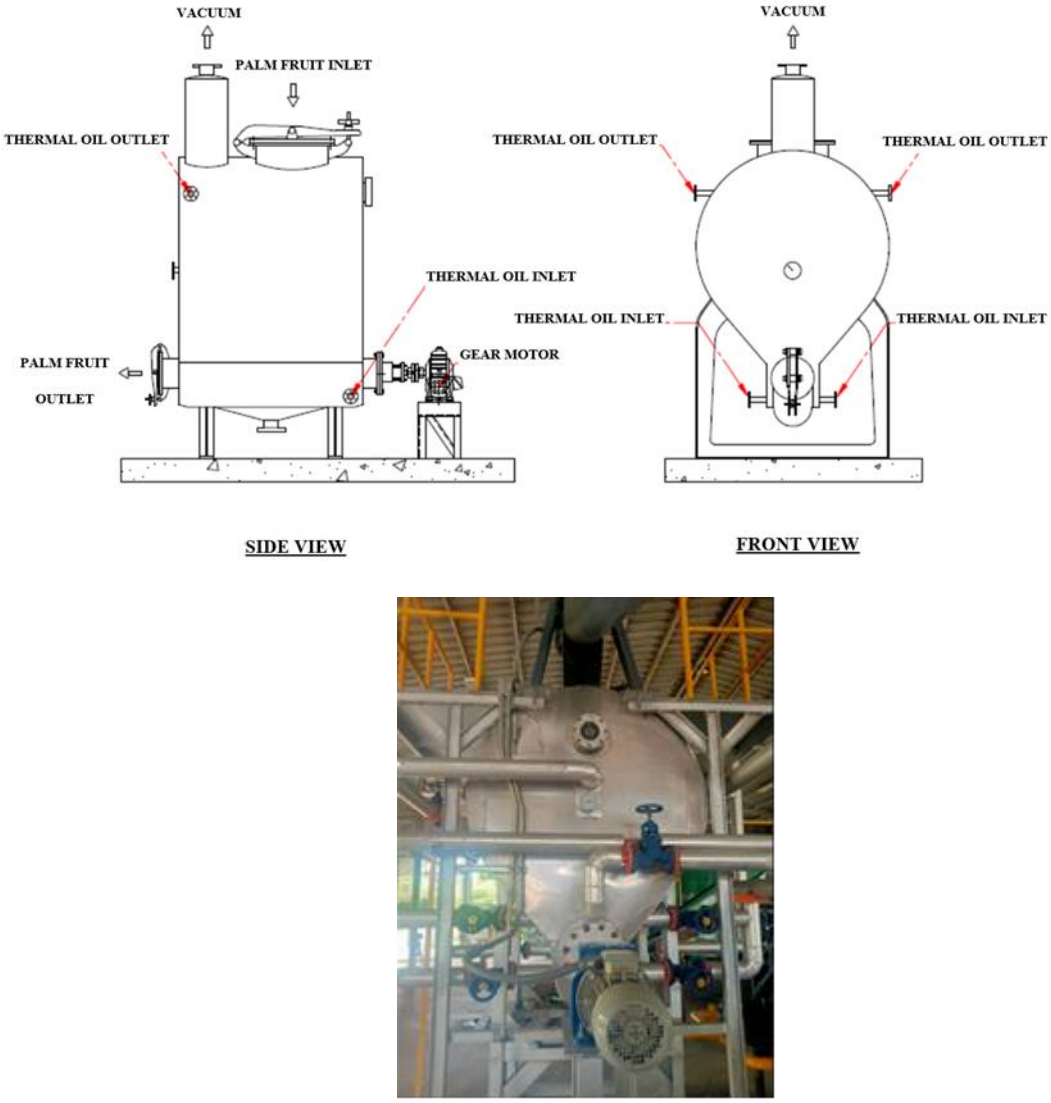

Figure 1. Components of the vacuum fryer (above) and appearance of the vacuum fryer (below)

2.2.1.2 Degumming and neutralization

The crude palm oil samples obtained from the vacuum frying and steam sterilizations were degummed and neutralized using the procedures described by Rakprasoot et al. [14]. Briefly, two hundred grams of the sterilized oil sample were degummed at 90 °C with vigorous stirring for 20 min. A mixture of 0.06% phosphoric acid and 0.04% citric acid per oil weight was diluted 9 times with distilled water before mixing with the crude palm oil. The sedimented gum was separated from the oil by washing the oil with warm water at 60 °C. Then, the oil was evaporated at 80 °C under vacuum for 30 min to reduce the water content to less than 7%. The excess free fatty acids and phosphorus were removed by neutralization with 20% NaOH at 80 °C for 30 min. Soap and excess NaOH were washed with warm water at about 60 °C. The oil samples were vacuum-dried at 50 °C until the water content was less than 0.1%. The oil samples were analyzed for yield [10], carotenoid content [10], free fatty acids [11], phosphorus [12], iron [12], and copper [12].

2.2.1.3 Multi-step fractionation

After degumming and neutralization, the multi-step fractionation was performed using the predetermined temperatures and the predetermined cooling rate (0.17 °C/min). The fractionation was operated in a 3,000 ml glass reactor (model JGR1L, Yuchem brand, China) equipped with a cooling system (model SDC-6, Drywell brand, China). The procedure started by adjusting the oil temperature to 60 °C and

then reducing the oil temperature to 5 different levels starting from 35 °C. When the oil temperature reached each predetermined level, the temperature was maintained for 30 min. The sample was then vacuum-filtered to separate the red palm fats from the oil fraction. The major unit operations in red palm oil and fat processing are shown in Figure 2. The obtained red palm fats and the red palm oil were analyzed for fatty acids composition [12], yield [10], iodine value [11], free fatty acids [11], peroxide value [11], melting point (DSC, Perkin Elmer, USA), carotenoids content [10], vitamin E content [13] and solid fat content (Pulse Nuclear Magnetic Resonance Spectrometers, Bruker, Germany).

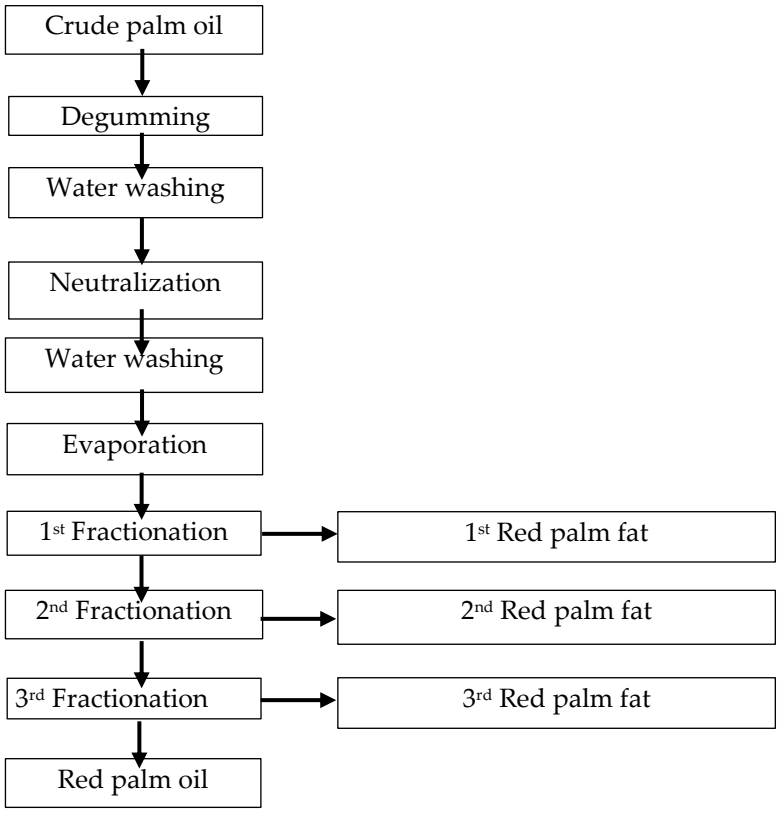

Figure 2. The major unit operations in red palm oil and red palm fats processing.

2.2.2 Consumer acceptance test

2.2.2.1 The red palm oils

The commercial refined palm oil (control) and the red palm oil derived from the vacuum frying and the steam sterilization methods were presented to the healthy, trained 50 panelists aged between 20-25 years. The test included evaluation of appearance, clearness, color, odor, and overall acceptability using the 9-point hedonic scale, ranging from "dislike extremely" =1 to "like extremely" =9 [15]. The experimental design was a randomized complete block design. The panelists were advised to clean the leftover previous aroma in their nasal cavity by sniffing roasted coffee beans between evaluations of each sample.

2.2.2.2 The deep-fried potato sticks

The experiment followed the procedure of Chompoo et al. [15] with minor modifications. The potato sticks were prepared and deep-fried in refined palm oil (control), and the red palm oils were derived from the vacuum frying and steam sterilization methods. Acceptances of the deep-fried potatoes or the French fries were evaluated using a 9-point hedonic scale with specific attributes, including appearance, odor, crispness, flavor, crispness aftertaste, and overall acceptability (N=50). The experiment was designed using a randomized complete block design.

2.2.3 Statistical analysis

All the determinations mentioned above were carried out in triplicates and were reported as mean ± standard deviation. Analysis of variance (ANOVA) and Duncan's multiple range tests ($P \leq 0.05$) were used to test the differences.

3. Results and Discussion

3.1 Vacuum frying sterilization

The optimal vacuum frying time was selected because of the high oil yield and the desirable appearance of the fried palm fruits. The proper frying improved the oil yield by making pores in the oil micelles, facilitating oil extraction at the subsequent step [16]. It also affects the content of phytonutrients and the sensory characteristics of the oil. Therefore, monitoring and controlling the frying condition is important. Table 1. presented that oil yield increased with frying time. An oil yield of more than 30% was achieved when the frying times reached 90 and 105 min. However, it was observed that increasing the frying time beyond 90 min negatively impacted the characteristics of palm fruits. Excessive frying (105 min) leads to a burning crust, which may contribute to unpleasant flavor, dark color, and toxic compounds. Though further research is required to assess the nutritional value and safety of the oil, this study revealed the frying time of 90 min was optimal for the vacuum sterilization of the palm fruit at a temperature of 80 °C and pressure of -400 to -720 mmHg.

After frying, the fried palm fruits were screw-pressed to extract the oil. The properties of the extracted oils from vacuum fried and steam sterilizations are presented in Table 2. When comparing properties to the specification issued by the Department of Malaysian Standard [17], which we have adopted as our national standard for premium quality crude palm oil, it was revealed that both extracted oils complied with the requirements for melting points, iodine value, and carotenoid contents. Technically, melting point is defined as the temperature at which it changes state from solid fat to liquid oil. Generally, most materials' melting and freezing points are approximately equal but not always the same as the freezing point because nucleating substances are significantly affected [18]. Nevertheless, it was predicted from the similar melting point that both extracted oils tend to have similar fractionation temperatures. However, a precise measurement of their exact fractionation temperatures will be confirmed in the fractionation study.

The iodine value not only reflects fatty acid composition and degree of unsaturation of the oil but can be used to predict some aspects of the crystallization behavior of the oil during the fractionation process. However, the crystallization behavior may be shifted due to the multiple polymorphic transitions that occur during melting and crystallization [19]. In this study, the iodine values of both extracted oils were high. They were close to that of the olein [20], which means there was higher unsaturated triacylglyceride concentrate in the solid fraction, so the lower fractionation temperature was expected.

The steam-sterilized oil contained higher amounts of carotenoids and vitamin E contents. The reason was that the oil obtained from the steam sterilized process was extracted from the mesocarp of the palm fruits, which are rich in carotenoids and vitamin E. In contrast, the vacuum fried oil was derived from whole fruits, including the kernel, which does not contain carotenoids and has a lower level of vitamin E [21].

It was obvious that the phosphorus contents were out of the range specified in the Malaysian standard [17]. Considering that soils in Southeast Asia naturally contain deficient amounts of phosphorus to support palm tree growth, inappropriate fertilizers have been applied, leaving behind a high amount of phosphorus in palm oil [22]. However, the amounts of iron and copper in the extracted oil derived from the vacuum frying sterilization were considerably lower than those obtained from the commercial steam sterilization process. Szydłowska-Czerniak et al. [23] noted that heavy metals in commercial steam sterilized oil could come from equipment, industrial wastes, vehicle exhaust, and chemical treatments.

The oil derived from the vacuum frying sterilization is also of better quality in terms of free fatty acids and peroxide value. The lower level of free fatty acids in the vacuum fried oil was mainly attributed to the better quality of palm fruits. In this case, the accumulation of free fatty acids in the fruits was investigated,

and it found that the vacuum frying sterilization plant encountered fewer problems that typically occurred during post-harvesting the palm fruits, such as damaged fruits, delayed delivery, and prolonged storage, as the Chaipattana foundation project well manages the plant. The lower free fatty acids level resulted in a lower neutralizing cost [2, 26] and less destruction of carotenoids during the neutralization process [1]. The result of the peroxide test, which represents the oil's oxidative stability, showed that a higher peroxide value corresponded with a higher content of free fatty acids. In the case of vacuum frying sterilization, the lower peroxide value is contributed by two factors; using good quality palm fruits and frying under vacuum condition that permits a sucking of volatile compounds from the oil [8].

Table 1. Effect of frying time on water content, oil yield, and appearance of palm fruits during vacuum frying at a temperature of 80 °C and pressure of -400 to -720 mmHg.

Frying time (min)	Water content (%)	Oil yield (%)	Appearance of palm fruits
15	14.08 ± 2.52^{a}	19.63 ± 1.29^{d}	
30	11.87 ± 0.90^{ab}	22.22 ± 0.87^{c}	
45	10.42 ± 0.80^{b}	24.40 ± 0.54^{b}	
60	9.31 ± 1.83^{bc}	25.67 ± 0.95^{b}	
75	6.63 ± 1.43^{c}	27.15 ± 0.64^{b}	
90	5.15 ± 1.46^{d}	30.35 ± 0.57^{a}	
105	4.34 ± 1.21^{d}	30.63 ± 0.63^{a}	

Note: Mean values within each column followed by different superscript letters (a, b, c, d) were significantly different ($P \leq 0.05$).

Table 2. Properties of extracted oils derived from vacuum frying sterilization and steam sterilization.

Properties	Extracted oil derived from vacuum frying sterilization (80 °C , -400 to -720 mmHg, 90 min)	Extracted oil derived from steam sterilization (120-140 °C, 45 min)	Malaysian standard [17]
Melting point (°C)	33.57 ± 0.83	33.75 ± 0.63	33.8-39.2
Iodine value (g I_2/100g)	51.37 ± 1.74	52.29 ± 2.17	50.4-53.7
Carotenoid (mg/kg)	538.30 ± 10.75	639.90 ± 5.3	474-689
Free fatty acids (%)	3.28 ± 0.07	4.10 ± 0.14	NA
Peroxide value (meq.O_2/kg)	6.83 ± 0.13	8.31 ± 0.08	NA
Phosphorus (mg/kg)	68.74 ± 0.23	111 ± 0.13	NA
Iron (mg/kg)	8.29 ± 0.02	20.27 ± 0.10	NA
Copper (mg/kg)	< 0.1	55.56 ± 0.19	NA
α-TP (mg/kg)	244.85 ± 60.49	279.46 ± 11.46	NA
δ-TP (mg/kg)	ND	ND	NA
γ-TP (mg/kg)	ND	ND	NA
α-TT (mg/kg)	193.37 ± 29.70	133.68 ± 11.47	NA
δ-TT (mg/kg)	46.37 ± 6.56	59.45 ± 4.85	NA
γ-TT (mg/kg)	249.72 ± 86.38	290.54 ± 21.66	NA
Total Vitamin E (mg/kg)	734.31 ± 9.90	763.25 ± 21.75	NA

Note: TP-Tocopherol, TT Tocotrienol, ND-Not detected, NA-Not analyzed

3.2 Degumming and neutralization

The extracted oils derived from vacuum frying and steam sterilization processes were degummed and then neutralized afterward. Degumming is intended to eliminate gums, phosphatides, and other sticky substances from the oil to improve oil stability and facilitate further processing. This study used acid to dissociate the nonhydratable phosphatides into phosphatidic acid and calcium or magnesium bi-phosphate salt [25]. After that, the sedimented gum was separated from the oil. Therefore, the degumming process led to yield loss and lower residual phosphorous (Table 3).

The main purpose of neutralization is to reduce the free fatty acids accumulated in oils by saponifying acids with an alkaline solution. However, based on the analysis results presented in Table 3, it was revealed that the neutralization procedure used in this study could eliminate free fatty acids, phosphorus, and iron that can function as pro-oxidants. Removing phosphorus and iron from the oil occurred when the oil was washed with warm water to eliminate soap [28, 29]. Similar results were discovered in the oil processed under steam sterilization, as reported by Rakprasoot et al. [26]. It can be stated that the selected degumming and neutralization processes used in this study are by far the most suitable processes as they can be used with the extracted oils of all quality, that is, oil from steam sterilization, which is high in free fatty acids and peroxide value. Besides that, neither process had a high deterioration influence on carotenoids, as shown in Table 3.

Table 3. Properties of vacuum-fried crude palm oil after degumming and neutralization processes.

Properties	Vacuum fried oil	Oil after degumming	Oil after neutralization
Oil yield (%)	100.00	95.78 ± 4.06[a]	82.77 ± 3.43[b]
Carotenoids (mg/kg)	538.30 ± 10.75[a]	518.34 ± 11.77[b]	533.98 ± 12.76[a]
Free fatty acid (%)	3.28 ± 0.07[a]	3.38 ± 0.18[a]	0.17 ± 0.07[b]
Phosphorus (mg/kg)	68.74 ± 0.23[a]	62.9 ± 0.03[b]	ND
Iron (mg/kg)	8.29 ± 0.02[a]	7.41 ± 0.02[b]	< 0.1
Copper (mg/kg)	< 0.1	< 0.1	< 0.1

Note: Mean values within each row followed by different superscript letters (a, b) were significantly different (P≤0.05), ND-Not detected

3.3 Fractionation

Dry fractionation was used in this study as it is the simplest and cheapest fractional technique [24]. However, when crystallization operates in bulk, the viscosity problem limits crystallization efficiency in one step [27], so multi-step fractionation was employed. Another advantage of the multi-step technique is that it delivers a wide range of red palm fats suitable for different applications. The available crystallization temperatures of the commercial refined palm oil cannot be applied to our oil samples because different components in the solid state may affect crystallization behavior even at the same conditions of cooling. Generally, there are three crystalline points in which oil can solidify [24], and results showed that the crystallization temperatures of the neutralized vacuum fried oil occurred at 34 °C, 27 °C, and 17 °C, respectively. In contrast, those of the steam sterilized oil appeared at 34 °C, 25 °C, and 15 °C, respectively. The shifting was mainly due to the difference in composition of the oil samples. As previously mentioned, the steam-sterilized oil was extracted from the fruit's mesocarp. In contrast, the vacuum-fried oil was extracted from the palm fruit, including palm kernel, with the prominent saturated fatty acid being lauric acid [21]. As a result, the second and the third fat fractions of the vacuum-fried oil contained higher levels of saturated fatty acids. The fat fractions' higher proportion of lauric oil resulted in higher crystallization temperatures (Tables 4 and 5). This study confirmed that the crystallization temperatures of the oil samples depend on the oil's origin.

Table 4. Fatty acids are composed of red palm fats and red palm oil produced from the oil processed by vacuum frying sterilization.

Fatty acids (g/100g)	Red palm fats			Red palm oil
	First fractionation (34 °C)	Second fractionation (27 °C)	Third fractionation (17 °C)	
C6:0	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.00
C8:0	0.27 ± 0.03	0.33 ± 0.07	0.37 ± 0.04	0.48 ± 0.08
C10:0	0.27 ± 0.04	0.31 ± 0.01	0.38 ± 0.03	0.44 ± 0.05
C12:0	3.63 ± 0.53	4.57 ± 0.25	4.31 ± 0.28	5.67 ± 0.11
C14:0	2.60 ± 0.12	3.05 ± 0.09	3.10 ± 0.28	2.71 ± 0.29
C15:0	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
C16:0	54.98 ± 1.19	44.28 ± 0.83	44.28 ± 1.28	35.23 ± 0.25
C16:1	0.10 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.15 ± 0.01
C17:0	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.10 ± 0.01
C18:0	4.44 ± 0.17	4.84 ± 0.10	4.78 ± 0.39	3.97 ± 0.07
C18:1	26.42 ± 0.43	34.12 ± 0.90	34.01 ± 1.27	40.19 ± 0.29

Table 4. Fatty acids are composed of red palm fats and red palm oil produced from the oil processed by vacuum frying sterilization. (Continue)

Fatty acids (g/100g)	Red palm fats			Red palm oil
	First fractionation (34 °C)	Second fractionation (27 °C)	Third fractionation (17 °C)	
C18:1*trans*	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
C18:2	6.32 ± 0.10	7.26 ± 0.08	7.52 ± 0.61	9.97 ± 0.33
C18:3	0.18 ± 0.04	0.25 ± 0.03	0.22 ± 0.03	0.32 ± 0.04
C20:0	0.31 ± 0.10	0.39 ± 0.05	0.35 ± 0.10	0.33 ± 0.02
C20:1	0.10 ± 0.05	0.11 ± 0.01	0.13 ± 0.05	0.14 ± 0.01
C20:2	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
C22:0	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.01
C24:0	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	0.08 ± 0.01
Unsaturated fatty acids	33.16 ± 0.55	41.92 ± 0.95	42.08 ±0 .41	50.84 ± 0.50
Saturated fatty acids	66.84 ± 0.61	58.08 ± 0.91	57.92 ± 0.39	49.16 ± 0.54

Table 5. Fatty acid compositions of the red palm fats and red palm oil produced from the oil processed by steam sterilization.

Fatty acids (g/100g)	Red palm fats			Red palm oil
	First fractionation (34 °C)	Second fractionation (25 °C)	Third fractionation (15 °C)	
C8:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C10:0	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
C12:0	0.12 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
C14:0	1.03 ± 0.07	1.08 ± 0.13	1.04 ± 0.01	0.91 ± 0.02
C15:0	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
C16:0	50.97 ± 0.44	45.11 ± 0.23	44.32 ± 0.47	38.27 ± 0.45
C16:1	0.11 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
C17:0	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.10 ± 0.02
C18:0	5.25 ± 0.38	4.84 ± 0.49	4.97 ± 0.11	4.49 ± 0.06
C18:1	32.82 ± 0.75	37.96 ± 0.55	38.62 ± 0.37	43.54 ± 0.68
C18:1*trans*	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
C18:2	8.45 ± 0.23	9.54 ± 0.12	9.53 ± 0.21	11.18 ± 0.17
C18:3	0.24 ± 0.05	0.24 ± 0.03	0.25 ± 0.01	0.32 ± 0.02
C20:0	0.39 ± 0.02	0.38 ± 0.04	0.38 ± 0.01	0.37 ± 0.01
C20:1	0.12 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.16 ± 0.01
C20:2	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
C22:0	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.00	0.06 ± 0.01
C23:0	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
C24:0	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.01
Unsaturated fatty acids	41.84 ± 0.87	48.11 ± 0.44	48.78 ± 0.53	55.46 ± 0.56
Saturated fatty acids	58.16 ± 0.82	51.90 ± 0.40	51.23 ± 0.58	44.54 ± 0.51

3.4 Properties of red palm oil and red palm fats produced from the vacuum frying process

As the main purpose of this research was to develop an effective red palm oil processing method suitable for SMEs, properties analysis of the end products was performed only on those derived from the vacuum frying process. Analysis results are shown in Tables 6 and 7. Yield is important when developing a new process, as yield can significantly impact the business's profit. The yield of red palm oil obtained from the vacuum frying process was 61%, more than that from the steam sterilization process (53.45%) [14]. As important as yield, concentrations of carotenoids and vitamin E in the red palm oil obtained from the triple fractionation increased by about 15% and 14%, respectively, compared to the contents in the extracted oils (Table 2). The main form of vitamin E in red palm oil is tocotrienol, especially in the form of γ-tocotrienol, which has potent antioxidant, anti-inflammatory and neuroprotective activities [28]. The higher content of these phytonutrients would favor positive claims on the nutritional label. Greater concentrations of carotenoids derived from the multi-step fractionation compared to the non-fractionated oil have been reported [18]. The result also showed that the increase of carotenoids and vitamin E concentrations positively related to increased red palm fats and oil iodine values. Previous work reported that the dissolving of both compounds is based on the principle of similar phase solubility [29]. In this case, the temperature at the end of the fractionation process was 15 °C. Carotenoids and vitamin E that have freezing points of less than -20 °C [30] and -27.5 °C [31], respectively, still appeared in the liquid form. Thus, the solubility of carotenoids and vitamin E increased with the degree of unsaturation of the liquid fraction (red palm oil >first red palm fat >second red palm fat >third red palm fat). Comparing parameter quality (Table 6) with other research (Table 7) [36-38] showed that the developed vacuum-fried method could be used to produce good-quality red palm oil.

For red palm fats, differences in properties such as iodine value, melting point, and solid fat content (Table 6, 8) enable red palm fats to be tailor-made for many applications. According to the solid fat content and melting point, the important information for characterizing fats used in the bakery, confectionery, and margarine industries, the first red palm fat with low iodine value might be used as a confectionery ingredient [24]. Meanwhile, the second and third red palm fats with medium and high iodine values can produce margarine and cacao butter substitutes, respectively [18]. Another main characteristic of red palm fats is their nutritional and functional properties due to their fair carotenoids and vitamin E contents. This property certainly responds to the demand for healthy fat products.

Table 6. Properties of red palm fats and red palm oil produced from the vacuum fried oil.

Properties	First red palm fat	Second red palm fat	Third red palm fat	Red palm oil
Yield (%)	14.54 ± 2.70^{b}	13.94 ± 2.36^{bc}	10.21 ± 0.93^{c}	61.31 ± 1.82^{a}
Iodine value (g I_2/100g)	36.90 ± 3.03^{d}	41.94 ± 2.27^{c}	49.27 ± 0.05^{b}	62.06 ± 0.14^{a}
Free fatty acids (%)	0.16 ± 0.02^{a}	0.17 ± 0.02^{a}	0.17 ± 0.02^{a}	0.17 ± 0.03^{a}
Peroxide value (meq. O_2/kg)	6.40 ± 0.14^{a}	6.54 ± 0.07^{a}	6.65 ± 0.07^{a}	6.70 ± 0.13^{a}
Melting point (°C)	53.2 ± 0.3^{a}	43.5 ± 0.1^{b}	37.1 ± 0.3^{c}	NA
Carotenoids (mg/kg)	321.90 ± 34.81^{d}	382.18 ± 37.31^{c}	417.69 ± 19.64^{c}	620.34 ± 9.90^{a}
α-TP (mg/kg)	200.75 ± 16.90^{b}	205.07 ± 16.60^{b}	209.14 ± 18.29^{b}	263.31 ± 42.57^{a}
δ-TP (mg/kg)	ND	ND	ND	ND
γ-TP (mg/kg)	ND	ND	ND	ND
Total TP (mg/kg)	200.75 ± 16.90^{c}	205.07 ± 16.60^{cd}	209.14 ± 18.29^{b}	263.31 ± 42.57^{a}
α-TT (mg/kg)	154.29 ± 7.81^{c}	155.85 ± 13.63^{c}	186.69 ± 11.40^{b}	221.48 ± 10.44^{a}
δ-TT (mg/kg)	16.31 ± 2.29^{c}	19.00 ± 3.57^{c}	26.00 ± 2.95^{b}	49.95 ± 5.67^{a}
γ-TT (mg/kg)	64.37 ± 12.23^{d}	84.70 ± 16.27^{c}	117.54 ± 28.50^{b}	300.30 ± 56.06^{a}
Total TT (mg/kg)	234.97 ± 6.55^{c}	259.55 ± 13.06^{c}	330.23 ± 26.03^{b}	571.73 ± 50.77^{a}
Total Vitamin E (mg/kg)	435.71 ± 10.40^{d}	464.62 ± 8.55^{c}	539.37 ± 12.10^{b}	835.04 ± 23.06^{a}

Note: Mean values within each row followed by different superscript letters (a, b, c, d) were significantly different ($P \leq 0.05$), TP-Tocopherol, TT-Tocotrienol, ND-Not detected, NA-Not analyzed

Table 7. Fatty acid contents and properties of vacuum-fried red palm oil and commercial red palm oils

Properties and fatty acids (g/100g)	Red palm oil derived from the vacuum frying process	Commercial red palm oils		
		Boon et al., [32]	El-Hadad et al., [33]	Bonnie & Choo., [34]
Free fatty acids (%)	0.17 ± 0.03	NA	0.12 ± 0.02	NA
Peroxide value (meq. O_2/kg)	6.70 ± 0.13	NA	1.5 ± 0.22	NA
Iodine value (g I_2/100g)	62.06 ± 0.1	NA	56.7 ± 0.42	NA
C12:0	0.15 ± 0.01	0.0	0.2	0.25
C14:0	0.91 ± 0.02	1.2	0.9	1.07
C16:0	38.27 ± 0.45	39.5	39.3	36.6
C16:1	0.16 ± 0.01	0.0	0.2	NA
C17:0	0.10 ± 0.02	NA	0.1	NA
C18:0	4.49 ± 0.06	3.7	4.2	3.7
C18:1	43.54 ± 0.68	43.5	43.7	46.7
C18:2	11.18 ± 0.17	12.2	10.5	12.8
C18:3	0.32 ± 0.02	0.0	0.5	NA
C20:0	0.37 ± 0.01	0.0	0.4	NA
Unsaturated fatty acids	55.46 ± 0.56	55.6	54.9	59.5
Saturated fatty acids	44.54 ± 0.51	44.4	45.1	41.62
Carotenoids (mg/kg)	NA	564	580	665
Vitamin E (mg/kg)	835.04 ± 23.06	1,141	820	717–863

Note: NA-Not analyzed

Table 8. Solid fat contents of the red palm fats.

Temperature (°C)	Solid fat contents		
	First, red palm fat	Second, red palm fat	Third, red palm fat
10	74.48 ± 0.33	62.85 ± 0.65	60.33 ± 0.49
15	66.90 ± 0.15	51.78 ± 0.11	50.37 ± 0.34
20	57.42 ± 0.08	38.03 ± 0.15	37.22 ± 0.17
25	46.83 ± 0.04	25.69 ± 0.18	22.94 ± 0.17
30	37.31 ± 0.05	17.17 ± 0.07	13.28 ± 0.05
35	29.30 ± 0.24	11.48 ± 0.16	6.96 ± 0.10
40	22.87 ± 0.16	6.81 ± 0.18	2.00 ± 0.03
45	16.54 ± 0.76	3.23 ± 0.19	0.22 ± 0.10

3.4 Consumer acceptance test

3.4.1 The red palm oils

The refined palm oil had the highest score in all sensory attributes, followed by the red palm oils produced from the vacuum sterilization and the steamed sterilization processes, respectively ($P \leq 0.05$) (Table 9). This study suggested that consumers prefer the colorless and odorless oil more than the oil with a red color and distinctive nutty flavor. Nevertheless, red palm oil may move from a niche to a widespread commercial oil depending on validation of the health benefits and its various applications. Nevertheless, the

panelists prefered vacuum-fried red palm oil for all attributes over steam-sterilized red palm oil. Thus, the vacuum frying method could overcome problems regarding off-flavor and off-odor commonly existing in steam-sterilized oil. This conclusion agreed with the previous study that vacuum frying could effectively remove the volatile compounds from the oil [35].

Table 9. Sensory scores of oil samples.

Attributes	Commercial refined palm oil (Control)	Red palm oils	
		Vacuum frying sterilization	Steam sterilization
Appearance	7.78 ± 0.83[a]	6.94 ± 1.08[b]	6.82 ± 1.00[b]
Clearness	8.12 ± 0.74[a]	7.46 ± 1.07[b]	7.30 ± 1.29[b]
Color	7.88 ± 0.79[a]	6.82 ± 1.27[b]	6.72 ± 1.18[b]
Odor	7.43 ± 1.17[a]	6.20 ± 0.95[b]	5.64 ± 1.04[c]
Overall acceptability	7.73 ± 0.80[a]	6.60 ± 0.92[b]	6.26 ± 0.96[b]

Note: Mean values within each row followed by different superscript letters (a, b c) were significantly different (P≤0.05), and the scores were the nine-point hedonic scales ranging from 1 = Dislike extremely and 9 = Like extremely.

3.4.2 The deep-fried potato sticks

This study aimed to comprehensively examine the application of red palm oils in deep frying. The appearances of deep-fried potato sticks or French fries were shown in Figure 3, and sensory scores were presented in Table 10. The panelists rated all the sensory characteristics of the control, and the sample was fried in vacuum-fried red palm oil from slightly to moderately. Both samples had the highest scores for their odors. This result suggested that the vacuum condition applied during the sterilization step effectively eliminates the off-odor compounds. For color attribute, it was revealed that carotenoid pigment in red palm oils was practically stable throughout the frying period. It was shown to absorb in the fried samples, leading to lower appearance scores. Compared with others, the potato fried in the steam-sterilized red palm oil obtained less acceptance in all attributes and had the lowest score for crispness. According to Kita et al. [36], the composition of frying oils' fatty acids affected the crispiness of the fried products. In this case, the higher unsaturated fatty acids of the steam-sterilized red palm oil led to a greasy texture, reducing the crispiness of the French fries. In conclusion, the overall acceptance scores indicated the potential efficacy of using red palm oil derived from the vacuum frying sterilization method as an alternative for replacing oil.

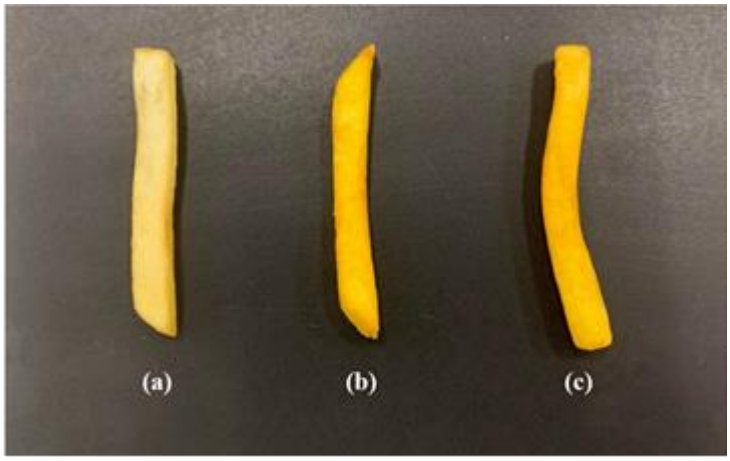

Figure 3. Appearances of potato sticks fried in refined palm oil (a), vacuum-fried red palm oil (b), steam sterilized red palm oil (c).

Table 10. Sensory scores of the potato sticks deep-fried in refined palm oil and red palm oils

Attributes	Refined palm oil (Control)	Red palm oil	
		Vacuum frying sterilization	Steam sterilization
Appearance	7.10 ± 1.18[a]	6.74 ± 0.96[a]	6.74 ± 1.17[a]
Odor	7.44 ± 1.05[a]	7.02 ± 1.12[ab]	6.78 ± 1.27[b]
Flavor	7.22 ± 1.11[a]	6.30 ± 0.84[b]	6.12 ± 1.21[b]
Crispiness	6.42 ± 0.67[a]	6.16 ± 0.76[a]	5.64 ± 0.78[b]
After taste	6.70 ± 1.16[a]	6.22 ± 0.91[b]	5.86 ± 0.99[b]
Overall acceptability	6.90 ± 0.97[a]	6.24 ± 0.89[b]	5.90 ± 0.93[b]

Note: Mean values within each row followed by different superscript letters (a, b c) were significantly different (P≤0.05).

4. Conclusion

The overall results of this study indicated that combined vacuum frying sterilization and multi-step fractionation is a practical approach for producing red palm oil and red palm fats. Vacuum frying sterilization contributes to red palm oil's high-quality and pleasant odor. At the same time, the multi-step fractionation allows the production of a wide range of red palm fats that have many potential applications in the food industry. The presence of carotenoids and vitamin E in red palm oil and red palm fats would promote palm oil as an attractive option for health-conscious consumers. Unlike conventional steam sterilization, this cost-saving and environmentally friendly process suits small to medium-sized operations.

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Research article

Isolation and Selection of Probiotic Bacteria from Nile Tilapia *(Oreochromis niloticus)* as Probiotics for Promoting Fish Growth

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Abstract: *Bacillus* spp. and lactic acid bacteria (LAB) were isolated from samples of Nile tilapia body (gastrointestinal tract, mucus, and fish scales) and fishpond water in Songkhla province, Thailand. Fifty-one bacterial isolates were obtained, and only 44 Gram-positive isolates were tested for their probiotic properties. These isolates were selected based on the ability to inhibit serious pathogens in tilapia, namely *Streptococcus agalactiae* and *Aeromonas hydrophila*; only isolated bacteria that can inhibit both fish pathogens were selected. Hence, 6 selected bacteria were further tested for their nutrient digestion, adhesion, and tolerance to acids and bile salts. It was found that only 5 isolates passed those tests. There were three isolates of bacilli and two isolates of LAB. The five isolates were identified using the 16s rRNA gene method and API test kits, and only two isolates (*Bacillus subtilis* HW3B and *Lactiplantibacillus plantarum* DW5L) that could be safe for fish and humans were selected for further studies as probiotics for fish cultivation.

Keywords: Nile tilapia; Fish pathogens; Probiotics

1. Introduction

The aquaculture system and the food used for raising aquatic animals have developed greatly due to the increasing demand for aquatic animals. However, in raising aquatic animals, there is waste from the remaining food that the animals cannot eat, including excreta. The waste will accumulate in the water and the environment. For example, ammonia produced from the digestion of protein in food, primarily in fish hepatocytes, is excreted through the gills [1]. The excess ammonia causes poor water quality, leading to fish weakness and high sensitivity to infection by pathogens. Due to such problems, probiotics have a greater role in aquaculture. For the above reasons, microorganisms with probiotic properties have been selected to solve such problems. Especially the ability to promote the growth of aquatic animals and inhibit pathogens. Probiotics used in aquaculture are mainly in the lactic acid bacteria (LAB) and *Bacillus* spp. group [2].

The first evidence of the application of probiotics in aquatic animals was in 1986 when Kokasa used *Bacillus toyoi* spores in the diet of yellowtail fish (*Seriola quinqueradiata*) to promote growth [3]. Following that, many more studies

were aimed at selecting bacteria for use as probiotics, such as Worananthakij's study. Worananthakij [4] selected probiotics from the gastrointestinal tract of tilapia, which mainly included *Bacillus* spp. and LAB. Several studies also use *Bacillus* spp. and LAB to inhibit pathogenic bacteria in aquaculture. For example, bacteria that can inhibit and resist *Aeromonas hydrophila* include *Lactococcus lactis*, *Lactobacillus plantarum*, and *Lactobacillus fermentum* [5], as well as *Bacillus subtilis* [6] and *Bacillus pumilus* [7]. Additionally, *Bacillus subtilis* has been shown to inhibit *Streptococcus agalactiae* [8].

Currently, in aquaculture, probiotics serve multiple functions, including facilitating the decomposition of organic matter in aquaculture ponds, reducing concentrations of nitrogen and phosphorus by transforming them into non-toxic forms for aquatic animals, and promoting the growth of aquatic plants and algae through the conversion of organic matter into inorganic matter. Additionally, probiotics enhance the growth of aquatic animals and provide resistance against pathogens, stimulate the immune system in aquatic organisms, and aid digestion by producing beneficial nutrients such as amino acids, fatty acids, and vitamins [9].

Hence, in this study, we isolated LAB and *Bacillus* spp. from tilapia bodies and water in tilapia ponds to select them based on probiotic properties. The main requirement of probiotics focused on the ability to inhibit serious fish pathogens and digest waste in rearing water. Then, the selected bacterial strains were identified before being used as probiotics to search for safe strains. The outcome of this study may benefit fish farmers in the future.

2. Materials and Methods

2.1 Sample collection and isolation of potential probiotic bacterial strains

Ten Nile tilapia (*Oreochromis niloticus*) samples were collected from various locations in Songkhla province, including 3 samples from the Aquatic Science and Innovative Management Division (AQ), 2 samples from Klong Rian Market (RN), and 5 samples from Songkhla Inland Fishery Research and Development Center (KH). Additionally, 5 samples of fishpond water were collected from various locations (AQ, 3 samples, and KH, 2 samples) in Songkhla Province.

Bacterial strains were collected from Nile tilapia samples weighing 500-800 g by swapping the mucus around the fish's body with a sterile loop. The fish were euthanized by placing them in a sterile bag and submerging them in ice water before surgical procedures to collect fish scales. Then, a cloth was used to wipe the fish's body. Ethanol at 70% (v/v) was applied to clean the fish's abdomen before surgery to remove the gut. The gut was weighed and placed in 0.85% (w/v) normal saline solution (NSS) before being shaken in the stomacher at 230 rpm for 30 s. The liquid portion from this shaking was collected and used to isolate LAB and *Bacillus* spp.

2.2 Isolation of lactic acid bacteria (LAB)

One ml liquid portion was added into 9 ml NSS to dilute 10 times and with a ten-fold serial dilution to reach a 10^{-5} dilution. Subsequently, the pour plate technique was used to isolate LAB by adding one ml of every appropriate diluent into melted de Man, Rogosa, and Sharpe (MRS) agar, containing 0.04% (w/v) bromocresol purple and 0.02% (w/v) sodium azide. This agar plate was incubated at 30 °C for 1-2 days. Yellow colonies appeared after incubation, and these colonies were investigated for LAB characteristics by Gram staining and catalase test, following the procedure of Axelsson (1998)[10]. LAB appeared as Gram-positive and non-catalase-producing characteristics.

2.3 Isolation of *Bacillus* spp.

The diluents in Section 2.2 were used to isolate *Bacillus* spp. by a modified method from Phianpak *et al*. [11]. One ml of diluent was added into 9 ml of melted tryptic soy agar (TSA) medium in the test tubes, and the mixture was immersed in a water bath at 80 °C for 10 min. After heating, the mixture was thoroughly mixed and poured into sterile petri dishes to solidify. After 1-2 days of incubation, colonies were isolated and

examined for characteristics by Gram staining and microscopy. *Bacillus* spp. was identified as Gram-positive, rod-shaped, and endospores formation.

2.4 Investigation of probiotic properties

2.4.1 Antagonistic effect of potential probiotics against Aeromonas hydrophila and Streptococcus agalactiae

Isolated LAB and *Bacillus* spp. were tested for their ability to inhibit serious pathogens in Nile tilapia, specifically *A. hydrophila* and *S. agalactiae*, using an agar well diffusion method modified from Aslim *et al*. (2005) [12]. These two pathogens were obtained from the Songkhla Aquatic Animal Health Research and Development Center. A cell density of 10^5 CFU/ml of each indicator pathogenic bacterium was poured over the TSA medium with 0.75% agar, and the mixture was allowed to dry. Once the agar solidified, a sterile borer with a diameter of 5.8 mm was used to drill holes in the agar plate. LAB isolates were cultivated in MRS broth, while *Bacillus* spp. isolates were cultivated in tryptic soy broth. All cultures were incubated at 35 °C for 18 h. Each culture broth was centrifuged at 8,000 × *g* for 5 min to obtain supernatant. Subsequently, eighty microliters of the LAB and *Bacillus* spp. supernatants were added to the agar wells. The plates were then incubated at 30 °C for 24 h. The inhibition zone was measured to evaluate the antagonistic effect.

2.4.2 Nutrient digestion test

Both LAB and *Bacillus* spp. isolates with the antagonistic effect were tested for nutrient digestion properties, including protein, carbohydrate, and fat. LAB isolates were cultivated in MRS medium and *Bacillus* spp. isolates were cultivated in tryptic soy broth medium, with both groups incubated at 35 °C for 18 h. After cultivation, the culture was washed twice with 0.85% NSS, and the cell was suspended in NSS. One loopful of each cell suspension was dropped on gelatin, starch, and tributyrin agars[13]. After incubation at 30 °C for 48-72 h, protein and fat digestion tests were observed as clear zones around colonies. The digestion of starch was examined by adding an iodine solution. No blue color around colonies indicated the positive result of starch digestion.

2.4.3 Cell surface hydrophobicity test

The adhesion properties were modified using the method of Taheri *et al.*[14]. LAB isolates were cultivated in MRS medium and *Bacillus* spp. isolates were cultivated in tryptic soy broth medium, with both groups incubated at 35 °C for 18 h. After cultivation, the culture was washed twice with 0.85% NSS. Subsequently, cells were suspended in NSS and adjusted with 0.85% (w/v) NSS to turbidity at OD600 = 0.5. Three milliliters of cell suspension were placed before adding 1 ml of xylene. The mixture was vortexed for 90 seconds and then set for 15 min for solvent separation. The cell suspension measured the turbidity at OD600 and calculated the surface cell as the following equation.

$$\text{Cell surface hydrophobicity } (\%) = ((OD_0 - OD_t)/OD_0) \times 100$$

OD_0 and OD_t are the turbidity of the cell suspension before xylene mixing and the turbidity of the aqueous layer after mixing.

2.4.4 Acid and bile salts resistance test

Isolated bacteria that passed the inhibition of fish pathogens, nutrient digestion, and the cell surface hydrophobicity tests were tested for acid and bile salt resistance. The resistance of the acid and bile salts test was according to the method applied previously by Ratanaburee *et al.* [15]. Firstly, one loopful of LAB and *Bacillus* spp. were transferred into 4 ml of MRS broth and TSB, incubated at 37 °C for 24 h to use as inoculums. A 10% culture was added to 10 ml of MRS broth and TSB medium, again incubated at 37 °C for 24 h, then pipette 1 ml of culture broth into a sterile 1.5 ml microtube. Culture broths were centrifuged at 10,000 rpm at 4 °C for 10 min, rinse the cells with 0.85% saline twice, and then resuspend the prepared cells in 0.85% saline adjusted to pH 2.5 containing pepsin 3 mg/ml, 1 ml volume, and then incubated at 37 °C for 2 h in a water bath. The surviving organisms were then counted using the drop plate method on MRS agar containing 0.004% bromocresol purple and TSA and incubated at 37 °C for 24 h. The acid-incubated suspension for 2 h was

centrifuged at 10,000 rpm at 4 °C for 10 min. The cells were washed twice with 0.85% saline solution. The cell suspension was prepared with phosphate buffer pH 8 and 1 mg/ml of pancreatin enzyme, and 1 ml of tilapia bile of 3% concentration was incubated at 37 °C for 6 h in a water bath. Surviving bacteria were also counted using the drop plate method. Bacterial strains with constant residual cells were selected for further study (adapted from Madureira *et al*. [16]).

2.5 Identification of selected bacterial strains

Five bacterial strains from morphological inspections were all in Gram-positive bacteria. Phenotypic characterization of bacterial isolates on biochemical property was conducted using commercial test kits, API 50 CHL (Ref 50410, BioMérieux, France) and 50 CHB/E (Ref 50430, BioMérieux, France) for *Bacillus* spp., respectively. The procedure was tested according to manufacturing instructions. More specifically, molecular identification was applied [17]. In brief, 3 ml of bacterial suspension (OD660 = 1.0) extracted the DNA by following the protocol (TIANamp bacteria DNA kit, Tiangen, PR China) to obtain 30 µl of genomic DNA solution finally. 16s rRNA region was applied for amplification with both specific primers. 70 µl of amplified cocktail (TopTaq Master Mix Kit, Qiagen, Germany) was included each 1.4 µl of 10 µM of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') as a forward and a reverse primer, 35 µl of 2x master mix, 3 µl of DNA template, and 29.2 µl of RNase-free water, and then applied for 3 min at 94 °C of initial activation, 30 s at 94 °C of denaturation, 30 s at 46.4 °C of annealing, 1 min at 72 °C of extension, and 10 min at 72 °C of final extension with 35 cycle amplification. The PCR product was purified by kit protocol (TIANquick Mini Purification Kit, Tiangen, PR China) to obtain a final volume of 25 µl. The purified PCR product was sequenced using the Sanger sequencing method and submitted to 1st Base Company (Malaysia). After receiving the results, the sequences were analyzed by combining the forward and reverse sequences using BioEdit version 7.6.2.1. software. The sequences were identified with Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to receive the most similar species [18]. After identification, the Submission Portal submitted the sequences to the NCBI database (https://submit.ncbi.nlm.nih.gov/). The accession number of isolated bacteria was composed of OL365735 for strain AQ1/1B, OL365736 for HW3B, OL365737 for RN3B, OL365738 for DW5L, and OL365739 for LS7L. A phylogenetic tree was constructed to estimate the relationship between strains by the Bootstrap method of phylogeny with 1,000 replication tests in the Jukes-Cantor nucleotide model in a neighbor-joining statistical method by MEGA11 software. *Clostridium perfringens* DSM 756^T (NR_121697.2) was used as the out-group in the phylogenetic tree.

2.6 Statistical analysis

Data on the probiotic properties from *in vitro* tests were analyzed for statistical differences in variables by One Way Analysis of Variances (ANOVA) and comparing differences in means of variables using Duncan's multiple range test at a significance level of $p < 0.05$. Results are given as mean of three replicates ± standard deviation (S.D.).

2.7 Permission to use animals

The experimental procedures in this study followed the Institute of Animals for Scientific Purposes Development (IAD) National Research Council of Thailand (NRCT), permit's date: 26-2/2018, and license number: U1-07693-2561.

3. Results and Discussion

3.1 Sample collection and isolation of potential probiotic strains

Bacteria with different colony characteristics were isolated using TSA and MRS media for *Bacillus* and LAB, respectively (Table 1). 14 LAB isolates were found on MRS medium and 30 *Bacillus* spp. isolates were found on the TSA medium. All LAB isolates were obtained from the tilapia body, including the digestive tract, fish scale, and mucus, while *Bacillus* spp. isolates were obtained from fish pond water and the fish body (digestive tract, fish scale, and mucus). The detection of isolated strains in this study is correlated with

Worananthakij [4], who selected probiotics from the gastrointestinal tract of tilapia, which mostly found *Bacillus* spp. and LAB. Based on this data, 44 bacterial isolates were further studied for their probiotic properties.

Table 1. Isolation of *Bacillus* and lactic acid bacteria (LAB) with Gram staining from Nile tilapia and fish pond water

Location	Aquatic Science and Innovative Management Division (AQ)	Klong Rian Market (RN)	Songkhla Inland Fishery Research and Development Center (KH)	Water from AQ pond	Water from KH pond	Total
Bacillus Isolates (TSA)	4	7	6	10	3	30
LAB Isolates (MRS)	6	4	4	0	0	14

Data are the number of different colony forms.

3.2 Probiotic properties test

It was found that from the 44 isolates of isolated bacteria, there were only 14 isolates that were able to inhibit fish pathogens. Four bacterial isolates could inhibit only *Aeromonas hydrophila*, and only 4 isolates inhibited *Streptococcus agalactiae*. Furthermore, only 6 bacterial isolates inhibited both types of pathogens, consisting of DW5L, LS7L, AQ1/1B, RN3B, RX1B, and HW3B. They were separated into 2 LAB (DW5L, LS7L) isolates and 4 *Bacillus* spp. isolates (AQ1/1B, RN3B, RX1B and HW3B). Table 2 shows their inhibition zones of potential probiotics against the growth of fish pathogens. This is consistent with many studies reporting that *Bacillus* spp. and LAB can inhibit both pathogens. For example, bacteria that can inhibit and resist *A. hydrophila*, including *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus fermentum* [5], *B. subtilis* [6], and *B. pumilus* [7], whereas *B. subtilis* inhibited *S. agalactiae* [8]. This was due to the probiotics producing bioactive compounds, i.e., hydrogen peroxide, bacteriocins, lysozymes, siderophores, and proteases, to inhibit pathogens in fish [19, 20]. Some bacteria can also produce organic acids (acetic, butyric, lactic, and propionic acids) to lower the pH, making it an unsuitable condition for pathogenic growth [20].

Then, all 6 isolates of bacteria were tested for their ability to digest nutrients, including protein (gelatin), carbohydrates (starch), and fat (tributyrin agar). It was found that 3 isolates could digest protein, namely bacilli strains (RN3B, A/1/1B, and HW3B). Some isolates could digest carbohydrates, namely *Bacillus* strain AQ1/1B and *Bacillus* strain HW3B, and there were no isolates that could digest the medium mixed with tributyrin in the fat digestion test. This means that they have only enzymes to digest protein and carbohydrates. They can secrete the enzyme protease to cut protein peptide bonds into amino acids. As a result, probiotics can be absorbed and used for growth by probiotics, which benefits aquatic animals by allowing them to use nutrients better [21, 22]. Both bacilli (AQ1/1B and HW3B) also digested starch. Consistent with the study of Hayashida *et al.* [23], it was found that the bacterium *B. subtilis* produced the enzyme alpha-amylase to be used in the digestion of raw starch. *Bacillus* strain RN3B and two lactobacilli strains showed no digestion on the nutrients test. However, both LAB could inhibit both serious fish pathogens at a high level and were included for further study.

Table 2. Inhibitory effect of potential probiotic bacteria against the growth of *Aeromonas hydrophila* and *Streptococcus agalactiae*

Bacterial isolate	Inhibition zone (mm)		Bacterial isolate	Inhibition zone (mm)	
	A. hydrophila	*S. agalactiae*		*A. hydrophila*	*S. agalactiae*
B5L	12.5 ± 0.4	-	RX3L	-	13.6 ± 0.3
DW5L	15.5 ± 0.3	17.2 ± 0.3	RX4L	-	14.4 ± 0.2
B9L	-	13.3 ± 0.3	NN12L	-	12.7 ± 0.3
LS7L	10.1 ± 0.3	12.6 ± 0.2	AQ1/1B	10.5 ± 0.3	12.7 ± 0.3
RN1B	11.6 ± 0.3	-	AQ1/3B	10.5 ± 0.4	-
RN3B	12.4 ± 0.5	14.9 ± 0.3	HW2B	14.9 ± 0.2	-
RX1B	10.6 ± 0.2	12.0 ± 0.2	HW3B	15.8 ± 0.2	14.2 ± 0.3

The values presented are expressed as Mean of triplicate ± SD. "**-**" stands for no inhibition.

Hence, both LAB strains and 3 bacilli strains were selected to test probiotic properties *in vitro*, consisting of an acid tolerance test, bile salt tolerance test, and adhesion test by cell surface hydrophobicity. Table 3 presents the results of the characteristics of probiotics, including an acid tolerance test, bile salt tolerance test, and adhesion test by cell surface hydrophobicity. They all survived in simulated gastric juice (pH 2.0) and simulated intestinal juice (pH 8.0 + tilapia bile salt). Both strains of LAB (DW5L and LS7L) survived in simulated gastric juice at 0 and 2 h incubation more than the group of *Bacillus* spp. However, the bacilli (AQ1/1B, HW3B, and RN3B) survived in the simulated intestinal juice and grew at a longer incubation time than LAB (at 6 h). In adhesion tested by cell surface hydrophobicity method using xylene droplets, the isolates DW5L and RN3B were the best to adhere at 69.50 ± 12.41% and 18.08 ± 1.74%, respectively (Table 3). This suggests that both bacterial strains have the potential to be used as probiotics.

All of them survived in acidic pH. Normally, the pH of the tilapia's digestive system is between 2.5-8, and the physiological concentration of bile in the tilapia's digestive system is between 0.4 and 1.3% [5]. In acidic conditions, lactobacilli (DW5L and LS7L) grew better than the bacilli group because the former group already produces acid and grows in low-pH environments. This agrees with the experiment of Worananthakij [4], *Lactobacillus* sp. strain M202 could grow well in the test medium with a pH of 1-4. Moreover, Chowdhury *et al.* [24] reported the acid tolerance of *L. plantarum* as it grew well at pH 4-8. On the other hand, *Bacillus* spp. (AQ1/1B, HW3B, and RN3B) had a higher ability to survive and grow in simulated intestinal juice (pH 8.0 + tilapia bile salt). This might be because they can form endospores in unsuitable environments. There are also reports that *Bacillus* spp. can develop biofilms to increase resistance to acid and bile in the digestive tract of fish [25].

3.3 Identification of isolated bacterial strains

Five isolated bacteria were identified by phenotypic characterization using API test kits and molecular techniques using 16s rRNA genes. Based on phenotypic results, a high percentage (99.24-100) of close similarity was received from this comparison to type strains (Table 4). The relationship amongst five strains was illustrated in Figure 1. by a phylogenetic tree which explained that three strains were arranged in *Bacillus* genus which included AQ1/1B (OL365735), HW3B (OL365736), RN3B (OL365737) strains, and identified to *Bacillus pumilus* DSM 27^T (NR_112637.1), *Bacillus subtilis* DSM 10^T (NR_027552.1), *Bacillus cereus* DSM 31^T (NR_115526.1). Meantime, the rest strains named DW5L (OL365738) and LS7L (OL365739) were categorized

into LAB as *Lactiplantibacillus plantarum* DSM 20174^T (NR_115605.1) and *Limosilactobacillus fermentum* DSM 20052^T (NR_104927.1). Both phenotypic and phylogenetic methods gave the same result for each bacterial strain.

Table 3. Probiotic properties of LAB (DW5L, LS7L) and *Bacillus* spp. (AQ1/1B, HW3B, and RN3B)

Isolated bacteria	DW5L	LS7L	AQ1/1B	HW3B	RN3B
Acid tolerance test /Survival (CFU/ml)					
Simulated gastric juice pH 2.0 (0 h incubation)	$4.0x10^{3a}$	$1.5x10^{4b}$	$1.2x10^{3a}$	$3.0x10^{2c}$	$5.0x10^{2c}$
Simulated gastric juice pH 2.0 (2 h incubation)	$1.1x10^{4a}$	$2.9x10^{4a}$	$8.5x10^{2b}$	$1.5x10^{2b}$	$3.5x10^{2b}$
Bile salt tolerance test /Survival (CFU/ml)					
Simulated intestinal juice pH 8.0 (0 h incubation)	$7.0x10^{3a}$	$3.3x10^{4b}$	$3.5x10^{4b}$	$4.2x10^{4b}$	$3.5x10^{4b}$
Simulated intestinal juice pH 8.0 (3 h incubation)	$4.0x10^{3a}$	$1.8x10^{4b}$	$5.6x10^{4b}$	$3.2x10^{4b}$	$3.6x10^{4b}$
Simulated intestinal juice pH 8.0 (6 h incubation)	$4.5x10^{3a}$	$1.9x10^{3a}$	$2.2x10^{6b}$	$1.0x10^{4c}$	$1.2x10^{5d}$
Adhesion test					
Cell surface hydrophobicity (%)	69.50 ± 12.41^{a}	7.11 ± 0.75^{bc}	6.36 ± 0.62^{b}	7.64 ± 1.85^{bc}	18.08 ± 1.74^{c}

Means sharing the same superscript are not significantly different ($p > 0.05$).

Table 4. Identification of 5 probiotic bacterial isolates by apiweb™ identification software with database (V5.1)

Isolate	Bacterial identification	% Similarity
AQ1/1B	*Bacillus pumilus* DSM 27^T	99.72
HW3B	*Bacillus subtilis* DSM 10^T	99.93
RN3B	*Bacillus cereus* DSM 31^T	100.00
DW5L	*Lactiplantibacillus plantarum* DSM 20174^T	99.58
LS7L	*Limosilactobacillus fermentum* DSM 20052^T	99.24

Fig. 1 shows the relationship amongst five strains of all Gram's positive isolated bacteria, which can be divided into three strains for *the Bacillus* genus and two strains for LAB, and an out-group that also listed in Gram's positive bacteria was used to distinguish between *Bacillus* and LAB sections. All strains were more significant than 97% similarity, which is used as the threshold percent for the novel species [26]. Strains RN3B, DW5L, and LS7L were isolated from the intestine of Nile tilapia, while strains AQ1/1B and HW3B were found in natural fish ponds close to their habitats. Therefore, 5 bacterial strains were considered to be applied for promoting fish. However, based on their probiotic properties and identification, results suggested that *B. subtilis* HW3B and *LB. plantarum* DW5L should be potential probiotics for application in fish cultivation. This is because the former strain could digest protein and starch, while the latter showed the highest cell surface

hydrophobicity. It should be noted that *B. cereus* strain RN3B showed the highest cell surface hydrophobicity among tested bacilli strains. However, this strain could digest only protein, and some strains of *B. cereus* are usually foodborne pathogens. *B. pumilus* strain AQ1/1B was not considered due to its lower ability to inhibit both fish pathogens than *B. subtilis* strain HW3B. It has long been known that both *Bacillus subtilis* and *Lactiplantibacillus plantarum* have been applied as probiotics and food additives, indicating their well-established safety profile in humans and fish [2, 8, 17, 27, 28, 29]. However, further experiments should be conducted to confirm the safety and effectiveness of both isolated probiotic bacteria in fish. Therefore, the experiments for this purpose were carried out to use both probiotics for promoting fish growth and maintaining water quality in tilapia cultivation.

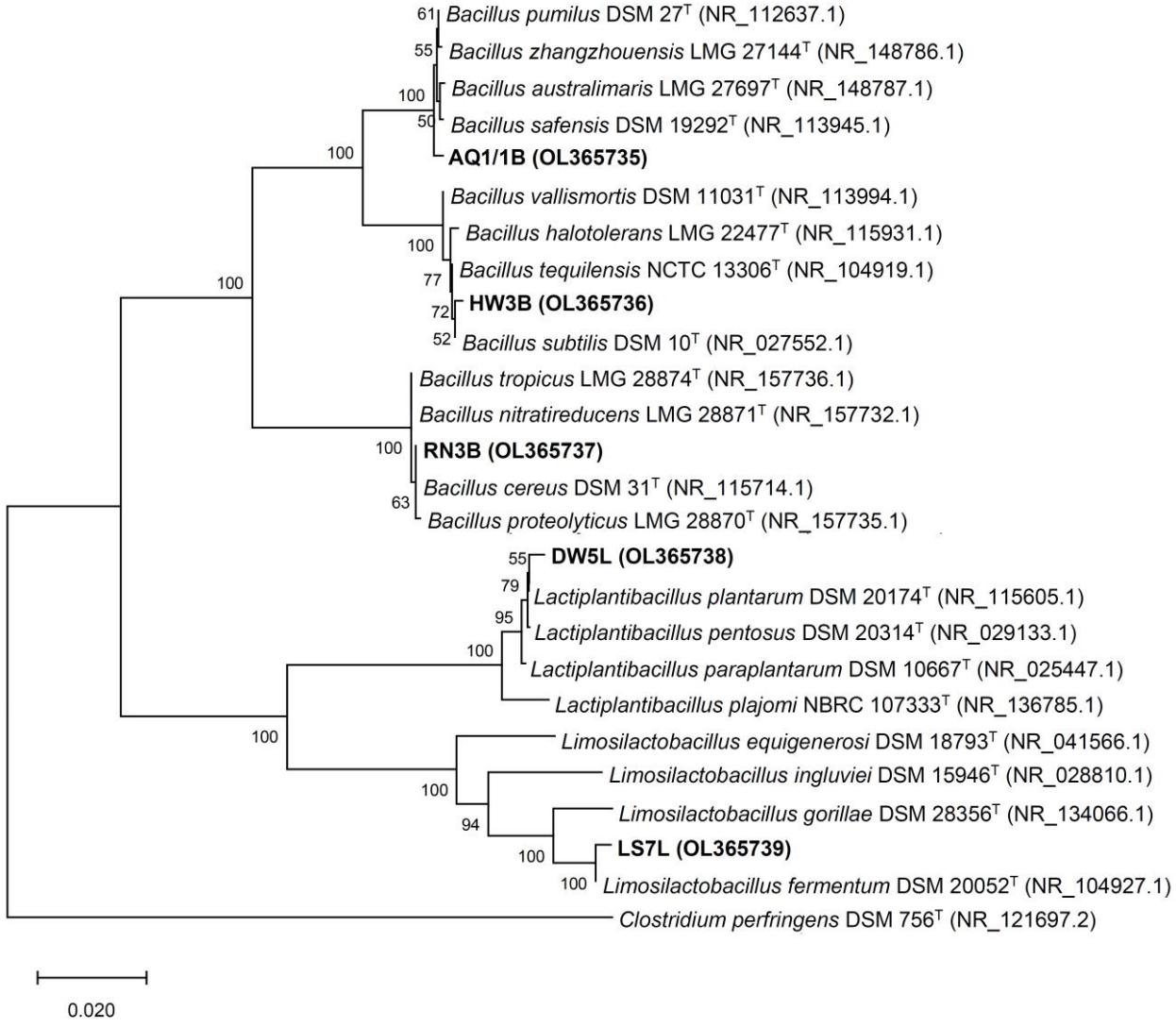

Figure 1. The relationship amongst five bacterial strains, including AQ1/1B (OL365735), HW3B (OL365736), RN3B (OL365737), DW5L (OL365738), and LS7L (OL365739) described by a phylogenetic tree of 16s rRNA gene identification using Jukes-Cantor nucleotide model in the neighbor-joining statistical analysis. The figure's annotation comprised taxonomical name, strain code, and accession number in the parentheses. *Clostridium perfringens* DSM 756T (NR_121697.2) was used as an out-group strain, and evolutionary distance appeared by the bar = 0.020.

4. Conclusions

Focus on isolating and selecting probiotic bacteria (*Bacillus* and LAB) against serious fish pathogens (*Streptococcus agalactiae* and *Aeromonas hydrophila*) from tilapia in Songkhla province of Thailand, 5 bacterial isolates had the desirable probiotic properties. Nevertheless, once 5 isolates were selected and consideration of potential safety for both tilapia and humans, only two isolates, *Bacillus subtilis* HW3B and *Lactiplantibacillus plantarum* DW5L, were promising probiotics for further studies.

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