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ASEAN Journal of Scientific and Technological Reports (AJSTR)

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Editorial note

The ASEAN Journal of Scientific and Technological Reports (AJSTR) Vol. 26 No. 3 (July-September 2023) ISSN 2773-8752 is the fourth issue under AJSTR. This issue features 8 research articles that are highly recommended for readers. Experts from various universities and institutions have diligently reviewed and responded to these exciting research articles. We sincerely hope these research papers will serve as a guide and inspiration for our active researchers, encouraging them to produce more valuable research soon.

The AJSTR has consistently provided outstanding service to our enthusiastic readers and customers internationally. In line with our commitment to excellence, all selected and accepted research articles are meticulously written and organized in English. The AJSTR and its new editorial team are fully prepared to effectively organize, manage, publish, and disseminate high-quality articles written in well-structured English to the global academic community.

This issue covers a diverse range of topics. One article investigates the suitable solvent for extracting active substances from Z. montanum rhizomes for use in cosmetic products. Another article explores phytochemical extraction from three local weeds in Sakon Nakhon Province, demonstrating their potential for medicinal applications and the synthesis of metal particles. Additionally, a study in the Philippines examines various aspects of sweet potato farming practices among small-scale farmers, emphasizing the need for targeted interventions to improve their livelihoods.

Moreover, the issue presents research on the successful accumulation of a copolymer of PHBV by Bacillus megaterium PP-10, with implications for large-scale production. Another article focuses on the efficient microwave-assisted extraction technique for phenolic compound extraction from S. ovalifolia root, highlighting its potential as a source of natural bioactive components. Furthermore, a study investigates the emulsifying property of a biosurfactant produced by B. oceanisediminis PM 08, showcasing its stability and potential applications in various fields.

Lastly, an article explores using municipal solid waste to produce RDF-5 fuel using natural rubber as a binder, addressing environmental concerns and economic viability. Additionally, a research study evaluates the bioactive substances and properties of M. hortensis crude extract, highlighting its potential antibacterial and antiinflammatory activities.

In conclusion, this issue of AJSTR represents a significant contribution to advancing scientific and technological research in the ASEAN region. We thank the authors, reviewers, and readers who have made this issue possible. We encourage researchers to continue their dedication and enthusiasm for producing high-quality research, fostering sustainable development and progress in the scientific community.

We hope you find this issue informative and inspiring, and we look forward to your continued support.

Sincerely,

Sompong O-Thong
International College, Thaksin University
Editor-in-Chief of ASEAN Journal of Scientific and Technological Reports

List of Contents

AJSTR
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Research article

Effect of Extraction Solvents on Antioxidant and Antibacterial Activity of *Zingiber montanum* Rhizomes

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Abstract: The *Zingiber montanum* rhizome has been utilized for its antiviral, immunomodulatory, anti-inflammatory, and antibacterial properties for a long time, particularly in Malaysia, Indonesia, and Thailand. Additionally, the rhizome has been a traditional ingredient in Asian cosmetic products. This study aimed to investigate the impact of different extracting solvents (hexane, dichloromethane, acetone, ethanol, methanol, 50% ethanol, and 75% ethanol) on the phenolic content, as well as the antioxidant and antibacterial activities of *Zingiber montanum*. The antioxidant activity was evaluated using two methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric-reducing antioxidant power (FRAP) assay, while antibacterial activity was tested against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* strains. The methanol extract demonstrated the highest phenolic content, while the ethanol extract exhibited a slightly lower amount. In the DPPH assay, the methanol extract showed an IC_{50} value of 36.89 ± 2.53 μg/mL, whereas the ethanol extract displayed a marginally higher value of 38.89 ± 0.27 μg/mL. In terms of ferric-reducing antioxidant power, the ethanol extract had slightly higher FRAP values (78.65 ± 4.73 mg AAE/g) than the methanol extract (76.09 ± 4.57 mg AAE/g). All extracts exhibited low activity against the three tested bacterial strains. Ethanol extract demonstrated the most antibacterial activity, with a clear zone ranging from 10.50 to 12.00 mm. The results suggest that ethanol is a suitable solvent for extracting *Zingiber montanum* rhizome for value-added materials application for cosmetic products.

Keywords: *Zingiber montanum*, solvent extraction, antioxidant, antibacterial

1. Introduction

Zingiber montanum (J. Koenig) Link ex A. Dietr., also known as Phlai in Thai, is a biennial plant that belongs to the *Zingiber* genus, which comprises around 85 species. Its rhizomes possess a bitter taste and have been traditionally used for culinary, dietary supplement purposes, and medicinal such as relieving dysentery, asthma, bruises, constipation, dyspepsia, gastritis, stomach bloating, and stomach-ache [1]. *Z. montanum* has shown potential for further development due to its wide range of pharmacological properties, such as anti-inflammatory, antibacterial,

antifungal, antioxidant, antihistaminic, anticholinesterase, and smooth muscle relaxant activities [2]. These properties make it an attractive area for further research and development, with the potential for new treatments for various medical conditions and cosmetics applications.

Z. montanum is frequently utilized as an ingredient in cosmetic products [3] available in the market, as the active compounds found in the plant can help address various skin concerns such as reducing acne, moisturizing the skin, minimizing wrinkles, alleviating skin allergies, and enhancing skin radiance. Phytochemical investigation of *Z. montanum* rhizomes revealed the presence of numerous bioactive compounds such as alkaloids, flavonoids, terpenoids, saponins, tannins, phlorotannins, steroids, and glycosides [4]. The important process in isolating active compounds is extraction, which can be influenced by different factors such as chemical composition, extraction technique, sample particle size, duration, and solvent. The solid-liquid extraction method, utilizing various solvents, is commonly employed for extracting active compounds from plants [5]. The ethanol extract of *Z. montanum* exhibited antibacterial properties and antioxidant activity in several studies [6,7]. The complex curcuminoids, cassumunins A, B, and C, isolated from acetone extract of *Z. montanum* rhizomes, exhibited more potent antioxidant and anti-inflammatory properties than curcumin [8]. Methanol extract from *Z. montanum* displayed DPPH radical scavenging activity with a half maximal inhibitory concentration (IC_{50}) value of 0.34 mg/mL [9]. The volatile oils of some *Zingiber* plants displayed multiple biological activities, including antioxidant effects [10]. Indeed, numerous studies have been conducted on extracting active ingredients using a single solvent or a mixture of solvents. The choice of solvent used for extraction can significantly impact the types and quantities of active components extracted [11]. Thus, using different solvents for extraction can yield different sets of active ingredients and, consequently, affect the overall efficacy of the resulting extract. Researchers must consider this when selecting a solvent or a combination of solvents for their extraction process.

Z. montanum is a plant that is found throughout all regions of Thailand. The DPPH antioxidant activity of *Z. montanum* ethanol extracts from various areas in Thailand revealed that the levels of antioxidants present in the plant varied significantly depending on the cultivation area. The percentage of antioxidants was 57.63-80.88, and the sample from the North showed the highest amount of antioxidants [12]. As a result, the researcher aims to explore the use of solvent extraction to obtain substantial amounts of antioxidants and antibacterial agents, as well as analyze the constituents of the extract acquired from southern Thailand. The total phenolic content will be analyzed as a quality control measure to ensure the products used in the production cycle are of high quality. Additionally, the study will assess the antioxidation and antibacterial activity of the extracts, providing essential information for effectively utilizing *Z. montanum* as a cosmetic ingredient.

2. Materials and Methods

2.1 Plant materials

Fresh *Zingiber montanum* rhizomes were purchased from a local market in Phattalung province, Thailand, in November 2022. Dr. Paveena Kaewubon, a plant taxonomist at the Department of Biology, Thaksin University, Thailand, identified the rhizomes.

2.2 Chemicals and reagents

Gallic acid was purchased from Sigma-Aldrich Chemicals. Folin-Ciolcateu reagent was obtained from Fisher Scientific. Fluka Chemie GmbH supplied 2,2-Diphenyl-1-picrylhydrazyl (DPPH), while ascorbic acid and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were acquired from Merck. All other reagents used in this study were of analytical grade. Mueller Hinton Agar was obtained from Hi-media, and Nutrient Agar was purchased from Difco.

2.3 Preparation of crude extracts

The fresh rhizomes of *Z. montanum* were washed with tap water to remove dirt, sliced into small pieces, and then dried in an oven at 50°C for three days. The dried rhizomes were ground into coarse powder by a grinding machine. 100 g of the powdered samples were soaked in 300 mL of seven different solvents (hexane, dichloromethane, acetone, ethanol, methanol, 50% ethanol, and 75% ethanol) at room temperature for thirty minutes and then subjected to ultrasound-assisted extraction at 45 KHz (35°C) for thirty minutes. The solutions were filtered through a Whatman filter No.1. The extracted solutions were evaporated below

40°C using a rotary evaporator and then freeze-dried. Seven brown extracts were obtained and stored at 4°C until further investigation.

2.4 Determination of total phenolic content

The total phenolic content of *Z. montanum* extracts was determined using the Folin-Ciocalteu colorimetric method, based on the procedure described by Iqbal and coworkers [13], with some modifications. In brief, 0.2 mL of each extract solution (10 mg/mL) was mixed with 1.0 mL of 10% Folin-Ciocalteu reagent. After 5 min, 0.8 mL of 20% Na_2CO_3 solution was added. The solution was vortexed for 15s and left in the dark for 90 min at 25°C for color development. The absorbance was measured using a UV–Vis spectrophotometer (SHIMADZU UV-1700, USA) at 725 nm against a blank. The experiment was repeated three times at each concentration, and the total phenolic contents were calculated based on the calibration curve of gallic acid (0.01-0.07 mg/mL). The results were expressed in milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g extract).

2.5 DPPH radical scavenging assay

The DPPH radical scavenging activity of *Z. montanum* extracts was evaluated using the method of Vichit and Saewan (2015) [14] with slight modifications. The DPPH assay measures the electron transfer of antioxidants towards the stable DPPH radical at 517 nm. In brief, a reaction mixture containing 0.2 mL of the sample and 1.8 mL of 0.1 mM DPPH solution was vortexed ultimately and incubated for 30 min in the dark at room temperature. A UV-Vis spectrophotometer was used to measure the absorbance at 517 nm, and each sample was tested in triplicate using ascorbic acid as a positive standard. The scavenging activity was calculated as the percentage of inhibition and derived using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

$A_{control}$ is the absorbance of DPPH radical with ethanol (without extract), and A_{sample} is the absorbance in the presence of the DPPH radical and test sample. The scavenging activity of *Z. montanum* extract was expressed as the IC_{50} value, which represents the concentration (mg/mL) of the extract required to scavenge 50% of the DPPH radical.

2.6 Ferric reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power (FRAP) was determined according to the method described by Benzie and Strain (1996) [15] with some modifications. Briefly, 0.2 mL of the sample at a concentration of 0.5 mg/mL was mixed with 1.8 mL of the FRAP reagent and incubated at room temperature for 5 min in the dark. The absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Samples were measured in triplicates, and ascorbic acid was used as the standard. The results were expressed in milligrams equivalent of ascorbic acid per gram of dry extract (mg AAE/g extract).

2.7 Antibacterial testing

An agar well diffusion assay was performed with some modifications based on the method described by Shimanuki and Knox (2000) [16] to evaluate the antibacterial activity of *Z. montanum* extracts against gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria. The cell cultures were initially grown on nutrient agar (NA) medium and incubated for 24 h at 37 °C. The final turbidity of the cultures was adjusted to a 0.5 Mcfarland standard (1.5×10^8 CFU/mL) using 0.85% w/v NaCl. The Mueller Hinton agar (MHA) surface was inoculated by spreading a volume of the microbial inoculum over it. A sterile cork borer was then used to aseptically punch a hole with a diameter of 6 mm in the agar plate surface. In each well, 60 µL of the test sample (0.1 mg/mL) was added using a sterile pipette. The plates were then incubated at 37°C for 24 h. After the incubation period, the diameters of the inhibition zones were measured in mm. The experiment was conducted in triplicate, with ampicillin as a positive control for *S. aureus* and ciprofloxacin as a positive control for *E. coli* and *P. aeruginosa*. 10% DMSO was used as the negative control.

2.8 Gas chromatography-Mass spectrometry (GC-MS) analysis

The ethanol extract of *Z. montanum* was analyzed using a gas chromatography-mass spectrometry (GC-MS) system equipped with an Agilent 19091S-433UI GC and a mass spectrometer, which was fitted with an HP-5ms capillary column (5% phenyl methyl polysiloxane, 30 m length, 250 µm diameter, 0.25 µm film

thickness, and temperature range of -60 to 350°C). The GC-MS system was also interfaced with a flame ionization detector (FID). In a gas chromatography analysis, helium was used as the carrier gas with a flow rate of 3.0 mL/min in split mode with a ratio of 20:1. A 1 µL sample was injected into the column through an injector with a temperature set to 250°C. The column temperature was initially set at 50°C for 5 min, then gradually increased at a rate of 5°C /min until reaching 250°C without any holding period. The temperature was then held at 280°C for 3 min at a 5°C/min program rate. The total elution time was 49 min. The relative percent amount of each component was calculated by comparing its average peak area to the total area. The ion source temperature was maintained at 230°C. The MS Spectrum was obtained using electron ionization at 70 eV. After separating the column, the components were identified and further analyzed by FID in scan mode from 50-1000 amu. The total running time was 650 min. To determine the compounds, the spectrum of the unknown compound was compared to known compounds in the NIST MS 1.4 structural library, which allowed for the determination of the compound names, molecular weights, and structures.

2.9 Statistical analysis

The experiments were conducted in triplicate. Values were reported as the mean ± standard deviation (SD). ANOVA tests were performed using GenStat software to determine significant differences in extracts. Differences with a probability (p) value of ≤ 0.05, indicating a 95% confidence level, were considered statistically significant. Simple linear regression analysis was used in the data analysis.

3. Results and Discussion

3.1 Percentage yield

The rhizomes of *Z. montanum* were extracted by maceration with various solvents, including hexane, dichloromethane, acetone, ethanol, methanol, 75% ethanol, and 50% ethanol, at a ratio of 1:3 w/v for 3 h, followed by ultrasound-assisted extraction for 30 min. After removing the solvent, seven different extracts were obtained. The percentage yield for each extract is shown in Table 1. The results demonstrated that 50% ethanol and methanol were the most effective solvents, producing the highest weight of crude extracts, with yields of 5.78% and 5.37%, respectively. The substantial mass of the polar solvent extract implies that it predominantly contains the polar components found in *Z. montanum*. However, the percentage yield in this study was lower than that reported by Rungruang and coworkers [17], who achieved a yield of 5.57% by extraction with 50% ethanol using an orbital shaker at 150 rpm for 24 h. This difference in yield may be due to the shorter extraction time used in this study with an ultrasonic bath. The results suggested that the primary constituents in the rhizomes of *Z. montanum* were likely highly polar.

Table 1. The percentage yield of crude extract and total phenolic content of *Z. montanum* extract

Extract[1]	% Yield of crude extract	Total phenolic content (mg GAE/g extract)
PH	2.30	24.83 ± 0.31^{f}
PD	3.98	53.74 ± 0.84^{d}
PA	3.73	57.53 ± 2.92^{c}
PE	3.72	62.22 ± 0.30^{b}
PM	5.37	71.45 ± 1.45^{a}
PE75	2.15	50.30 ± 0.91^{e}
PE50	5.78	49.52 ± 0.32^{e}

[1] PH: hexane extract, PD: dichloromethane extract, PA: acetone extract, PE: ethanol extract, PM: methanol extract, PE75: 75% ethanol extract, and PE50: 50% ethanol extract

3.2 Total phenolic content

Phenolic compounds are a diverse group of secondary metabolites produced by plants, and their concentrations and compositions depend on various factors such as plant species, growth conditions, and extraction techniques. Phenolics are polar substances reported to possess various beneficial properties, including antioxidant, anti-inflammatory, and anticancer activities. Therefore, measuring the total phenolic content of a plant extract can serve as an initial screening method for antioxidant activity. The total phenolic

content of *Z. montanum* extracts was determined using the Folin-Ciocalteu reagent. The reagent is reduced in the presence of phenolic compounds, producing a blue-colored complex. The intensity of the blue color is directly proportional to the number of phenolic compounds in the extract [18].

The total phenolic content of *Z. montanum* extracts was expressed as milligram equivalents of gallic acid per gram of extract (mg GAE/g extract), which was calculated using the gallic acid standard curve $y = 11.441x + 0.0146$ ($R^2 = 0.9985$), as shown in Table 1. The total phenolic content ranged from 24.83 ± 0.31 to 71.45 ± 1.45 mg GAE/g extract, with the methanol extract exhibiting the highest phenolic content while the hexane extract showed the lowest. A polar solvent can extract a broader range of phenolic compounds, both polar and nonpolar, leading to a higher total phenolic content. Suggests that methanol is a suitable solvent for phenolic extraction from *Z. montanum*, while higher mixes of polar solvents like 50% ethanol and 75% ethanol are not ideal for this study. These results are different from those of previous studies that reported an ethanol-water mixture (40–60% v/v) as being more effective for extracting phenolic compounds from plants than a mono-solvent system [19-21], due to the wide range of phenols that the ethanol-water mixtures can dissolve [22]. Additionally, a study by Rungruang and coworkers, which used 50% ethanol as a solvent, found a phenolic content value of 213.16 mg/g extract [17], which differs from this study's result of 49.52 ± 0.32 mg GAE/g extract. These discrepancies may be due to differences in plant ages and extraction processes.

3.3 DPPH radical scavenging activity

The ability of *Z. montanum* extracts to scavenge free radicals was evaluated using the DPPH radical scavenging method. The reaction involves substances that donate radical hydrogen species, which convert the DPPH radical to its non-radical form, DPPH-H. A significant reduction of DPPH indicates a large amount of hydrogen radical in the reaction. The results revealed that the methanol extract exhibited the highest DPPH radical scavenging activity, followed by ethanol extract and acetone extract, with IC_{50} values of 36.89 ± 2.53, 38.89 ± 0.27, and 40.63 ± 1.23 μg/mL, respectively. The results were consistent with the total phenolic content of the extract, as the high range of phenolic compounds in the methanol extract resulted in a significant decrease in the DPPH content. The extracts showed good antioxidant efficiency. However, the standard ascorbic acid still exhibited a lower IC_{50} value of 22.82 ± 0.20 μg/mL, as shown in Table 2.

Table 2. The antioxidant activity of *Z. montanum* extract

Extract[1]	DPPH radical scavenging activity (IC_{50}, μg/mL)	Ferric-reducing antioxidant power activity (FRAP value, mg AAE/g extract)
PH	264.34 ± 0.51^{f}	48.85 ± 4.57^{e}
PD	68.36 ± 0.15^{d}	73.57 ± 0.78^{b}
PA	40.63 ± 1.23^{b}	73.81 ± 3.23^{b}
PE	38.89 ± 0.27^{a,b}	76.09 ± 4.57^{a,b}
PM	36.89 ± 2.53^{a}	78.65 ± 4.73^{a}
PE75	64.70 ± 3.23^{c}	67.51 ± 1.89^{c}
PE50	151.14 ± 3.12^{e}	62.87 ± 0.11^{d}
ascorbic acid	22.82 ± 0.20	-

[1] PH: hexane extract, PD: dichloromethane extract, PA: acetone extract, PE: ethanol extract, PM: methanol extract, PE75: 75% ethanol extract, and PE50: 50% ethanol extract

Our study found a significant difference in IC_{50} values between the ethanol extract (38.89 μg/mL) and that reported by Rungruang and coworkers in 2021 (213.16 μg/mL). However, our results revealed that the efficacy of the ethanol extract was inferior to that of ascorbic acid by a factor of 1.7. Likewise, Rungruang and coworkers found the extract less effective than ascorbic acid by a factor of 1.2. The difference in the scavenging activity of the ethanol extract and the other report could be attributed to the age of the plant and the drying technique employed [17]. Specifically, using a freeze-drying approach in our study may have resulted in a lower residual solvent or water content than the method used by Rungruang coworkers in 2021. Nonetheless, it is worth noting that this drying process may potentially lead to the loss of some antioxidants. Cassumunins A-C are the phenolic antioxidants in *Z. montanum*, as previously reported by Masuda and Jitoe in 1994 [8] and Nagano and coworkers in 1997 [23].

3.4 Ferric-reducing antioxidant power (FRAP) activity

Ferric-reducing antioxidant power activity of the extract involved the transfer of a single electron with an antioxidant compound. The activity of the extract was reported as FRAP value in mg AAE/g extract. The high FRAP value corresponds to the good reducing ability of the extract. The study of *Z. montanum* extracts on reducing ferric iron was conducted at a 0.5 mg/mL concentration. The FRAP values were calculated from the ascorbic acid calibration curve: y= 0.0254x – 0.0504 (R^2 = 0.9935), and the result is shown in Table 2. Although the polarity of methanol was close to 75% ethanol, the phenolic content and antioxidant properties differed. The ethanol and methanol extracts had relatively the most effective in reducing ferric ions with FRAP values of 78.65 ± 4.73 and 76.09 ± 4.57 mg AAE/g extract, respectively. The activity trend was slightly different from the DPPH assay, but the ethanol and methanol extract values were not significantly different.

The results of both methods indicated that methanol and ethanol extracts were similar constituents. They also showed higher antioxidant activity than the other *Z. montanum* extracts. The results confirmed that extraction with different solvents led to various constituents and activities. In addition, the antioxidant activity of crude extracts was inversely proportional to the phenolic content. An extract that contains a large amount of total phenolic compounds also showed good antioxidant activity, while the extract with low total phenolic content displayed poor antioxidant activity. Although the ethanol and methanol extracts had very similar antioxidant activity, in terms of safety and utilization, ethanol is the most suitable extraction solvent for cosmetic products.

3.5 Antibacterial activity

The antibacterial activity against gram-positive bacteria (*S. aureus*) and two gram-negative bacteria (*E. coli* and *P. aeruginosa*) of all extracts was determined using an agar well diffusion assay. The gram-positive and gram-negative bacteria were tested with ampicillin and ciprofloxacin as the positive controls. The result showed that all *Z. montanum* extracts gave a quite low efficiency against *S. aureus*, *E. coli*, and *P. aeruginosa*, as shown in Table 3. All the extracts did not inhibit the growth of *S. aureus* at 0.1 mg/mL. Ethanol and 75% ethanol extracts displayed poor inhibition against *E.coli* with a clear zone of 10.5 ± 1.53 and 12.0 ± 0.72 mm and also exhibited insufficient activity against *P. aeruginosa* with a clear zone of 12.0 ± 1.76 and 11.0 ± 0.58 mm.

Table 3. Antibacterial activity of each *Z. montanum* extract

Extract[1]	Zone of Inhibition (in mm diameter)		
	S. aureus	***E. coli***	***P. aeruginosa***
PH	NI	NI	NI
PD	NI	NI	NI
PA	NI	NI	NI
PE	NI	10.5 ± 1.53	12.0 ± 1.76
PM	NI	NI	NI
PE75	NI	12.0 ± 0.72	11.0 ± 0.58
PE50	NI	NI	NI
ampicillin	34 ± 1.82		
ciprofloxacin		35.0 ± 1.14	34.0 ± 1.48

[1] PH: hexane extract, PD: dichloromethane extract, PA: acetone extract, PE: ethanol extract, PM: methanol extract, PE75: 75% ethanol extract, and PE50: 50% ethanol extract, NI: No inhibition zone was observed.

The result indicated that the polar extracts were more active against gram-negative than gram-positive bacteria. The antibacterial properties of *Z. montanum* extracts were comparable to those reported by Aji and colleagues in 2022 [24], who also found 70% ethanol to be the optimal solvent for extraction. However, the difference in the type of bacteria tested was noted. Jena and colleagues reported that the chloroform extract showed significant antimicrobial effects against various pathogens compared to the methanolic extract [25]. However, the age differences, planting locations, and drying methods of the extract led to distinct chemical components and biological activities, differentiating it from this study. The freeze-drying process may potentially remove volatile compounds, such as small terpenoids, which could have antibacterial properties.

The decomposition or evaporation of these terpenoid compounds may result in the remaining substances within the extract having good antioxidant properties but limited antibacterial activity.

3.6 Gas chromatography-Mass spectrometry (GC-MS) analysis

The ethanol extract of *Z. montanum* exhibited high DPPH scavenging activity and strong reducing power. A combined gas chromatograph system and mass spectrophotometer were used to identify its chemical constituents. The analysis of the ethanol extract revealed that it contained a total of 43 volatile compounds, out of which 10 were identified by name at the indicated retention time: 4-methyl-1-(1-methyl ethyl)-bicyclo[3.1.0]hex-2-ene (4.424, 0.42%), terpinene-4-ol (9.285, 0.41%), 4-hydroxy-2-methyl acetophenone (12.905, 0.24%), vanillin (15.125, 0.84%), 3,4-dimethoxybenzaldehyde (17.145, 1.24%), 1,4-dimethoxy-2-methyl-5-(prop-1-en-2-yl)benzene (19.720, 1.64%), 1,4-bis(methoxy)triquinacene (20.733, 19.73%), 2,4,5-trimethoxy-benzaldehyde (22.560, 0.21%), 3-(3,4-dimethoxy phenyl)-2-propenal (23.817, 0.97%), (*E*)-4-(3,4-dimethoxy-phenyl)but-3-en-1-yl acetate (28.172, 11.92%). The major volatile compound is 1,4-bis(methoxy)triquinacene, representing 19.73% of the extract, as illustrated in Figure 1.

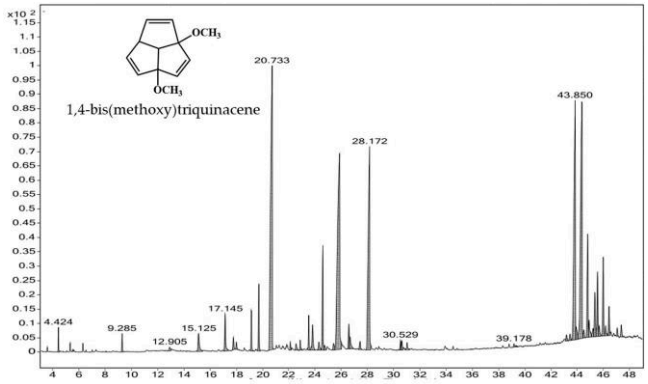

Figure 1. GC-MS analysis of the ethanol *Z. montanum* extract.

The presence of an allylic proton at the fused ring of 1,4-bis(methoxy)triquinacene contributes to its antioxidant properties. This proton can be readily abstracted by the DPPH radical, resulting in the formation of a stable free radical and the generation of DPPH-H, as shown in Figure 2.

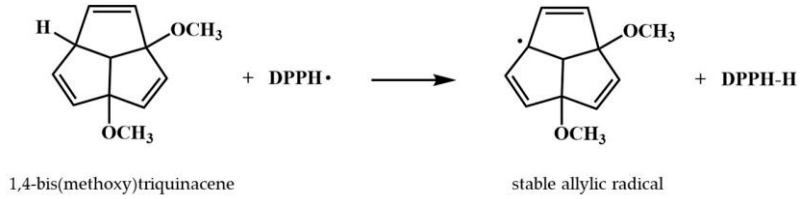

Figure 2. Antioxidant mechanism of 1,4-bis(methoxy)triquinacene with DPPH radical.

4. Conclusions

The suitable solvent for extracting active substances from *Z. montanum* rhizomes for use in cosmetic products was investigated. The rhizomes of *Z. montanum* from Phattalung province, southern Thailand, were extracted using seven types of solvent (hexane, dichloromethane, acetone, ethanol, methanol, 50% ethanol, and 75% ethanol) and freeze-drying. Methanol extract showed the highest phenolic content corresponding to the DPPH radical scavenging activity. At the same time, ethanol extract showed a slightly higher ferric-reducing antioxidant power than methanol extract. All extracts showed poor activity against three tested bacteria strains. Ethanol extract showed the most increased activity against *P. aeruginosa*, whereas 75% ethanol extract showed the highest activity against *E. coli*. The result indicated that ethanol is a suitable solvent for

extracting *Z. montanum* for application in cosmetic products. The major volatile compound present in the ethanol extract is 1,4-bis(methoxy)triquinacene.

5. Acknowledgements

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Research article

Extraction and Phytochemical Profile of Three Herbal Weeds: *Chromolaena odorata* L., *Amaranthys virdis* L., and *Cyperus Rotudus* L. for Green Synthesis of Silver Nanoparticles

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Abstract: Phytochemical screening and silver nanoparticle synthesis of extract of 3 local weeds from Sakon Nakhon Province, namely, *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L., were carried out. The preliminary testing of chemical constituents revealed secondary metabolites with pharmacological activity. The weed extracts possessed bioactive compounds and were potent for silver nanoparticle synthesis. The weed extracts demonstrated a lower inhibition percentage (DPPH inhibition) than ascorbic acid, with significant differences at $p<0.05$. Reducing agent analysis revealed that the weed extracts contained reducing compounds such as phenol and carboxylic acid suitable for AgNPs. The XRD patterns of silver nanoparticles synthesized from the extracts demonstrated the efficacy of the weed extract for medical applications (the production of AgNPs). In the future, the size and morphology of silver particles should be investigated. The current results are expected to be a guideline for further applications of local weeds.

Keywords: Herbal weed; green synthesis; biological approach; Ag nanoparticle

1. Introduction

"Principles and Practices of Weed Management" proposed the meaning of weed as the type of plant which are unwanted, useless, and harmful. There are a variety of weed definitions, such as "a plant out of place or growing where it is not wanted" [1]. Weeds cause various problems, harming the global economy, particularly in agriculture, where they compete for nutrients, water, and light with the crop. Herbicides have been used to reduce the amount of weed in agricultural areas [2]. Nevertheless, weeds have important chemical constituents with pharmacological potential. The common weeds observed in the region of Sakon Nakhon province are *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L.

Chrmolaena odorata L. or Siam weed is commonly found in tropical Africa, North America, and South and Southeast Asia, as reported by biological action and the efficiency of antimicrobials [3]. *C. odorata* is a plant belonging to

the Asteraceae family (Aster family), Chromolaena genus (Thoroughwort), and the species of *Chromolaena odorata* L [4]. According to Srisuda *et al*. [3], the leaf, stem, and root of *Chromolaena odorata* were extracted using various solvents (water, ethanol, methanol, and hexane). That showed a correlation between total phenolic content and total flavonoid content. *Amaranthus virdis* L. or Amaranthaceae is a herbal that rapidly spreads in Asia, Africa, and Latin America. The extracts of amaranth leaves revealed total phenolic content of 1.03 to 3.64 GAE, g/100 g, total flavonoid content of 18.4 – 5.42 QE, g/100 g, and radical scavenging activity of IC_{50}:14.25 - 83.43 µg/ml [5]. *Cyperus Rotudus* L. is a plant [6] with the local name of Saed, Sajal, Seil in Arabic and nut grass, purple nutsedge in English, and Xiang Fu in Nagarmotha and China [7]. *C. rotundus* was used in traditional medicine recipes for its beneficial compounds, such as phenols, flavonoids, tannins, and glycosides, which have a medicinal effect [8].

Silver nanoparticles (AgNPs) are increasingly used in many fields due to their physical and chemical properties (in medicine, food, health care, consumer, and industrial applications). AgNPs, which have many benefits, can be used for antibacterial, food, household, health care, medical device products, optical sensors, and cosmetic products [9]. The AgNPs properties are based on aspects of the crystal (size of crystalline, density, and structure). The distribution of AgNPs nanoparticles inhomogeneous crystalline demonstrated high chemical and physical properties [10]. That can be synthesized using both physical and chemical processes. Physical procedures include laser radiation and condensation processes, while the chemical approach, which provides for hydrazine, sodium borohydride, and green synthesis, is the most widely used method [11]. Nanotechnology has been used in industrial agriculture to increase agricultural products and decrease postharvest waste. AgNPs have been applied to extend the life of farm products as ethylene inhibitors and antimicrobial agents [12].

Green synthesis is a process that can produce silver nanoparticles in cost-effective, environmentally friendly, and direct ways. Silver nanoparticles have been improved by using the silver solution and plant extracts with insoluble organic compounds [13]. The plant extracts converted the $AgNO_3$ solution to AgNPs, which improved their size, morphology, and optical properties [14]. MOSHFEGH *et al*. [15] synthesized nanoparticles of gold (Au), silver (Ag), and gold-silver (Au-Ag) by using biological synthesis. The results demonstrated maximum absorption at 530 nm for AuNPs, 440 nm for AgNPs, and 458 nm for Au/AgNPs. The laser light scattering method revealed the particle sizes of Au, Ag, and Au-Ag as 89 nm, 37 nm, and 63, respectively. The literature has reported advances in nanoscience and nanotechnology, both of which have medical applications. AgNPs are reported to possess therapeutic activities, such as antifungal, antiviral, antiinflammatory, and anticancer [9]. Siddhant and Mohan [16] synthesized AgNPs from *Ocimum Sanctum* (Tulsi) and its derivative quercetin (flavonoid was present in Tulsi). Various physicochemical conditions synthesized the AgNPs regarding pH, temperature, reaction time, and reactants concentration. The silver nanoparticles separately produced from leaf extract and neat quercetin demonstrated the same optical properties, morphology, and antimicrobial action.

According to the information on the properties of weeds, the following are *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L. That is pharmaceutical properties and the typical local plant. The biosynthesis process of the plant extracts can be used to synthesize silver nanoparticles. The nanoscale has multiple medicinal properties. This research proposed to use the local plant extracts of *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L. for AgNPs synthesis. That is cost-saving, reduces chemical waste, and is environmentally friendly. According to the results, to guide the weed usable for more applications.

2. Materials and Methods

2.1 Identification and Collection of The Three Herbal Weeds

The leaves of *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L. were obtained from Sakon Nakhon, Thailand. The specimens of the plants were identified and confirmed by three experts in botany, Thai pharmacy instructors, and plant science professors.

2.2 Preparation and Extraction of The Three Herbal Weeds

The fresh leaves of *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L. were cleaned with distilled water and desiccated. The plant samples' crude extracts were extracted using the decoction method (T_b = 100°C) [17]. A hundred grams of each sample of fresh weed leaves were chopped into smaller pieces. This was then boiled with 500 ml of distilled water (5 minutes) and filtrated with a filter paper (Whatman No.1). The crude solution was kept at 4°C for 7 days for sedimentation, after which the upper layer was collected. The filtrated solution was used for analysis and the synthesis of AgNPs.

2.3 Silver Nanoparticle Synthesis

The reaction for AgNPs synthesis was carried out with 100 mL of the sample with 900 mL of $AgNO_3$ (1mM) and treated with sodium hydroxide solution at pH 8 (1M). The mixture was heated at 70 °C for 1 h and then centrifuged at 11,000 rpm for 5 minutes. The obtained black powder was kept for analysis [17-18]. The plant extracts presented a phytochemical capable of donating electrons to reduce Ag^+ ions to Ag^0 [19]. Figure 1. shows the silver ion reduction, agglomeration, and stabilization to form a particle of nano size.

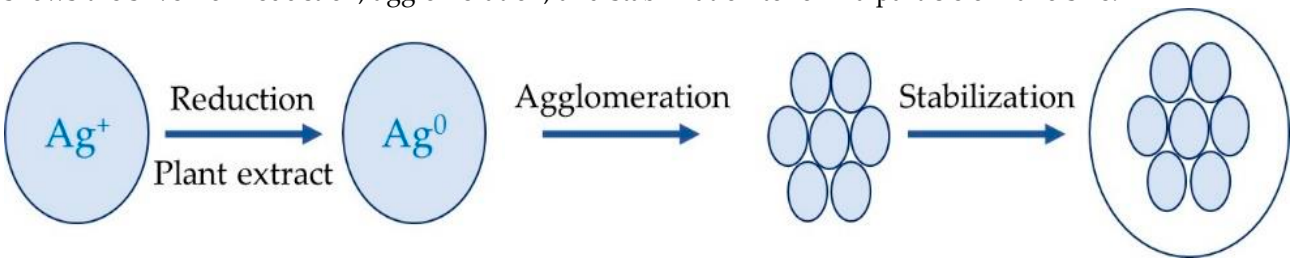

Figure 1. The mechanism of AgNPs synthesis.

2.4 Analysis of the three Herbal Weed Extracts and AgNPs

2.4.1. Preliminary Phytochemical Phytochemical Testing

Preliminary phytochemical testing was used to analyze the secondary metabolite samples of the weed extracts. The phytochemical screening focused on cardiac glycosides, flavonoids, saponins, tannins, terpenoids, anthraquinones, coumarins, phlorotannins, steroids, alkaloids, and glycoside (three replications) [20-22].

2.4.2 Gas Chromatography-mass spectrometry (GC-MS)

The screening of chemical substances in the weed extracts was carried out using the GC-MS technique, Shimadzu GCMS-QP2020 Ultra mass selective detector coupled with Shimadzu QP2020 Ultra gas chromatograph, equipped SH-Rtx-5MS capillary column (30 m x 0.32 µm, film thickness 0.25 µm). The GC-MS method was improved by Keshab *et al*. [23]. The sample was 0.2mg/mL, and helium gas was used as a carrier (flow rate: 3.0 mL/min). The initial column temperature was 50°C and then was programmed to increase at a rate of 5°C/min to 140°C (constant for 5 min), 140-200°C (3°C/min), and 200-260°C (15°C/min). The temperature was adjusted at 250°C for the injector and 280°C for the detector with a split ratio of 1:50. The electron impact voltage was set at 70 ev. The chemical constituents were identified by comparing their retention index with Library NIST17.

2.4.3 Determination of reducing agent

Metal nanoparticle synthesis requires a reducing agent. Most plants contain free radical scavenging molecules as reducing agents, which can be used to synthesize metal nanoparticles. [24]. The reducing agent was determined following the conventional method (Carboxylic acid testing and Amine testing) [25-26] and the modern method (FTIR spectroscopy analysis) [27]. The reducing agents were measured using FTIR (ATR-FTIR; Bruker TENSOR II) following the method reported by Piotr *et al*. [28]. The extracts were dried at 40°C for 7 days and placed on a sample holder. Four scans of dried extracts were measured from 600 – 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.4.4 Scavenging activity percentage

The antioxidant percentage of the sample weed extracts was determined compared to standard free radicals (1,1 diphenyl-2-picryl-hydrazyl: DPPH). The DPPH radical scavenging activity was

evaluated following the research of Eugenio *et al.* [29] and was compared with standard antioxidants (ascorbic acid). The reaction for %AA determination consisted of 0.5 mL of sample, 3 mL of ethanol, and 0.3 mL of DPPH (0.5mM DPPH ethanolic solution). The antioxidant compound provided the hydrogen, which changed in the dark purple DPPH solution to yellow (after incubation for 30 minutes). The light absorption of the reaction was performed at 517 nm. A blank solution obtained from ethanol (3.3mL) and sample (0.5 mL) and ethanol (3.5 mL) mixed with DPPH radical solution (0.3 mL) was used as control. Mensor *et al*. [30] were used for scavenging activity percentage (%AA). The calculation formula is as follows equation (1)

$$\%AA = 100 - [(\frac{Abs_{sample-}\ Abs_{blank}}{Abs_{control}}) \times 100] \quad (1)$$

2.4.5 X-ray Diffractometry (XRD)

The AgNPs powders were identified using XRD (Philips X'Pert-MDP), and the target was Cu and K with a wavelength of 1.54060 Å. The generator was operated at 40kV with a 30 mA current. The scanning range was selected between 10.00 and 90.00, and the scan speed was 2.00 [31].

2.5 Statistical Analysis

The data were expressed as mean values ± standard deviation for each measurement and analyzed by means of the analysis of variance (One-Way ANOVA). A probability of $P<0.05$ indicates that the values are considered statistically significant.

3. Results and Discussion

3.1. Phytochemical Screening

Preliminary phytochemical testing revealed the active substances for the pharmacological activity evaluation of the sample weeds *(Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L.). The extracts possessed a variety of different secondary metabolites (Table 1).

Table 1. Phytochemical screening of Chromolaena odorata L. aqueous extract, *Amaranthus virdis* L., and *Cyperus Rotudus* L.

Class of Compounds	Aqueous Extract		
	***Chromolaena odorata* L.**	***Amaranthus virdis* L.**	***Cyperus Rotudus* L.**
Cardiac glycoside	+	+	-
Flavonoids	+	+	+
Saponins	-	+	-
Tannins	+	-	+
Terpenoids	+	+	-
Anthraquinones	-	-	-
Coumarin	+	+	+
Phlobatannins	-	-	-
Steroids	-	-	-
Alkaloids	-	-	-
Glycoside	-	-	-

Note: (+) Present, (-) Absent

Table 1 demonstrates the active compounds of *Chromolaena odorata* L leaf extracts: cardiac glycoside, flavonoids, tannins, terpenoids, and coumarin. The analysis results were consistent with the Kavitha et al. [33]. The analysis of ethanolic and aqueous extracts of *Chromolaena odorata* L. leaf and *Annona squamosa* seed showed

free radical scavenging, antimicrobial, and antimicrobials, which prevent infections, indicating phytochemical compounds and pharmacology actives. The aqueous extracts of *Chromolaena odorata* L. leaf were found to have secondary substances with antioxidant and therapeutic potential. The secondary substances analyzed were obtained from Amaranthus virdis L. leaf extracts containing cardiac glycoside, flavonoids, terpenoids, and coumarin. Saud *et al*. [34] proposed the properties of *Amaranthus virdis* L. extracts based on leaf and seed (solvent: 80% methanol). *Amaranthus virdis* L. extracts possessed active compounds such as cardiac glycoside, tannins, and flavonoids, which revealed an antioxidant activity of IC50 83.45 - 75.95 µg/mL and a minimal inhibitory concentration (MIC) of extracts ranging from 178 -645 µg/mL. The pharmacological and phytochemical profile of crude extracts of *Amaranthus spinosus* L. leaf was investigated. The results revealed the presence of carbohydrates, phenolic compounds, phytosterol, alkaloids, and flavonoids, indicating the pharmacological potential of the leaves [35]. Tannins (antiinflammatory and antimicrobial), saponins (antibacterial), flavonoids (treat heart disease), alkaloids (treat fever and headache), steroids (regulate hormonal system), phenolic compounds, proteins (help in the growth of the organism) and anthraquinones (used in the dye industry) were reported [36]. Therefore, *Amaranthus spinosus* L. is classified as a medicinal plant with a wide pharmacological application [37].

The phytochemical analysis results of *Cyperus Rotudus* L. leaf extracts revealed flavonoids, tannins, and coumarin. Eman et al. [38] studied the phytochemical, antimicrobial, and GC-MS analysis methanolic (85%) extract of Cyperus Rotudus, which showed tannins, carbohydrates, phytosterols, and alkaloids. This indicates that the plant is biologically active and can be used as antibacterial and antifungal medicine. Khalid et al. [39] described the extraction of dried *Cyperus Rotudus* L. with water and 70% ethanol. The aqueous extract's phytochemicals were alkaloids, flavonoids, phenols, phlorotannins, saponins, tannins, and terpenoids, which showed antibacterial and antifungal activity. Preliminary phytochemical analysis results in this study showed that some substances were linked to flavonoids and coumarin. Cardiac glycoside and terpenoids present in *Chromolaena odorata* L. and *Amaranthus virdis* L., and tannins found in *Chromolaena odorata* L. and *Cyperus Rotudus* L, demonstrated the pharmacological activity of the sample weeds with the property of synthesizing the silver nanoparticles based on green synthesis [9].

3.2 Chemical Constituents Characterization

GC-MS carried out the active compounds analysis of sample weed extracts (Chromolaena odorata L., Amaranthus virdis L. and Cyperus Rotudus L.). The phytochemical analysis results are shown in Tables 2-4.

Table 2. Compounds Identification of *Chromolaena odorata* L. extracts.

Phytochemical compound	R.Time	Area%	Formula	Pharmacological activity	Reference
Terpinen-4-ol	12.215	2.79	$C_{10}H_{18}O$	Antibacterial, Antioxidant Antibiofilm	Laísa *et al*, (2020)
1,6-anhydro-beta-D-Glucopyranose	21.056	1.84	$C_6H_{10}O_5$	NR	NR
2,2,4-Trimethyl-1,3-pentanediol di isobutyrate	25.112	3.26	$C_{16}H_{30}O_4$	NR	NR
n-Hexa-decanoic acid	39.715	5.42	$C_{16}H_{32}O_2$	Antioxidant, pesticide	Hema *et al*, (2011)
Hexa-decanoic acid, ethyl ester	40.735	8.74	$C_{18}H_{36}O_2$	Antioxidant, pesticide	
Phytol	44.109	8.19	$C_{20}H_{40}O$	Anticancer	
cis-Vaccenic acid	44.677	5.01	$C_{18}H_{34}O_2$	Bioactive compound	Okereke *et al*, (2017)

Note: NR (not reported)

The analysis revealed the compounds with pharmacological effects and unspecified compounds**.** The chemical analysis of *Chromolaena odorata* L. leaves extracts showed seven compounds. Terpinen-4-ol compound is responsible for antibacterial and antibiofilm properties, as was found by Laísa *et al.* [40], who described the inhibition study of *Staphylococcus aureus*. Terpinen-4-ol demonstrated a minimal inhibitory (MIC = 0.25% (v/v)) and minimal bactericidal concentrations (MBC= 0.5% (v/v). N-hexa-decanoic n-hexadecanoic acid, hexadecanoic acid, and ethyl ester were found as antioxidants and pesticides. Phytol from *Chromolaena odorata* L. leaves extracts were analyzed and found to have anticancer properties [41]. As a bioactive constituent, the compound cis-Vaccenic acid corresponded to Okereke *et al*. [42], which evaluated *Tithonia diversifolia* gray leaf extracts. Bioactive compounds of the extracts possessed anti-infective action and treatment of endemic diseases. The pharmacology potential of two compounds, 1,6-anhydro-beta-D-Glucopyranose and 2,2,4-Trimethyl-1,3-pentanediol di isobutyrate, could not be identified. l-(+)-Ascorbic acid 2,6-dihexadecanoate and eicosanoic acid, ethyl ester were found in *Amaranthus virdis* L. extracts as reported by Igwe and Okwunodulu [43]. A study analyzed the phytochemical profiles of *Phyllanthus amarus* leaf extracts by GC-MS and found bioactive compounds used for treatment and medicinal herbs. Oleic acid compound with allergenic, anti-alopecia, anti-androgenic, anti-inflammatory, and reduction of hypocholesterolemia properties was also found [41]. The analysis results of *Cyperus Rotudus* L. extracts revealed t (+)-2-bornanone (antibacterial) [44], terpinene-4-ol (antibacterial and antibiofilm) [40].

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The analysis results of *Cyperus Rotudus* L. extracts revealed t (+)-2-bornanone (antibacterial) [44], terpinene-4-ol (antibacterial and antibiofilm) [40]. Copaene is a tricyclic sesquiterpene found in the essential oils of medicinal plants. Hasan *et al*. [45] reported copaene as an antioxidant likely to possess an anticancer effect. Copaene can be used in functional foods for pharmacological purposes. The analysis results revealed four compounds without pharmacological properties (Cyclohexanol, 4-(1,1-dimethyl ethyl)-, acetate, cis-, 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-, 2,4,6-Tris(1,1-dimethyl ethyl)-4-methylcyclohexa-2,5-dien-1-one and 2,2,4-Trimethyl-1,3-pentanediol di-isobutyrate).

3.3 Reducing Agent Analysis

For reducing agent qualification testing, both litmus paper and organic solvent testing methods were used, which revealed the identity of the properties suitable for use as a precursor of the biological synthesis of silver nanoparticles. The results of the experiment are shown in Table 5.

Table 3. Compounds Identification of *Amaranthus virdis* L. extracts.

Phytochemical compound	R.Time	Area%	Formula	Pharmacological activity	Reference
l-(+)-Ascorbic acid 2,6-dihexadecanoate	39.724	24.55	$C_{38}H_{68}O_8$	Antioxidant	Igwe & Okwunodulu, (2014)
Eicosanoic acid, ethyl ester	40.743	7.30	$C_{22}H_{44}O_2$	Antioxidant	
Oleic Acid	44.682	13.24	$C_{18}H_{34}O_2$	Allergenic, Anti-alopecia, Anti-androgenic, Antiinflammatory, Hypocholesterolemic	Hema *et al*, (2011)

Note: NR (not reported)

Table 4. Compounds Identification of *Cyperus Rotudus* L. extracts.

Phytochemical compound	R.Time	Area%	Formula	Pharmacological activity	Reference
(+)-2-Bornanone	11.262	2.86	$C_{10}H_{16}O$	Antibacterial	Somrithai & Narongrit (2019)
Terpinen-4-ol	12.216	4.91	$C_{10}H_{18}O$	Antibacterial, Antioxidant	Laísa *et al*. (2020)
Cyclohexanol, 4-(1,1-dimethylethyl)-, acetate, cis-	17.272	1.25	$C_{12}H_{22}O_2$	NR	NR
Copaene	17.535	1.85	$C_{15}H_{24}$	Antioxidant Anticancer	Hasan *et al*. (2014)
1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	19.225	29.19	$C_4H_9NO_5$	NR	NR
2,4,6-Tris(1,1-dimethylethyl)-4-methylcyclohexa-2,5-dien-1-one	21.449	1.74	$C_{19}H_{32}O$	NR	NR
2,2,4-Trimethyl-1,3-pentanediol di isobutyrate	25.115	5.99	$C_{16}H_{30}O_4$	NR	NR
n-Hexa-decanoic acid	39.725	13.20	$C_{16}H_{32}O_2$	Antioxidant, pesticide	Hema *et al*. (2011)
Hexa-decanoic acid, ethyl ester	40.740	5.21	$C_{18}H_{36}O_2$	Antioxidant, pesticide	

Note: NR (not reported)

Table 5. Reducing agent testing of aqueous extract of *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L.

Method	Results		
	***Chromolaena odorata* L.**	***Amaranthus virdis* L.**	***Cyperus Rotudus* L.**
Carboxylic acid			
1. Litmus Test	-	-	-
2. H_2SO_4	-	-	-
3. NaOH	+	-	+
4.NaOH+$NaHCO_3$	-	+	-
5. $AgNO_3$	-	-	+
Amine			
1. Litmus Test	-	+	+
2. HCL	+	-	-

Note: (+) Present, (-) Absent

Functional group analysis of the sample weed extracts was done using the FTIR spectroscopy technique, which evaluated the composition of the action group with reducing properties. The middle infrared region of 550 - 4,000 cm^{-1} was used. The molecules light absorption resonance is expressed as a frequency relation or wave number. Each functional group represented a wavelength specific to the bond oscillation [46]. The weed extracts were evaluated the reducing moiety, as shown in Figure 2.

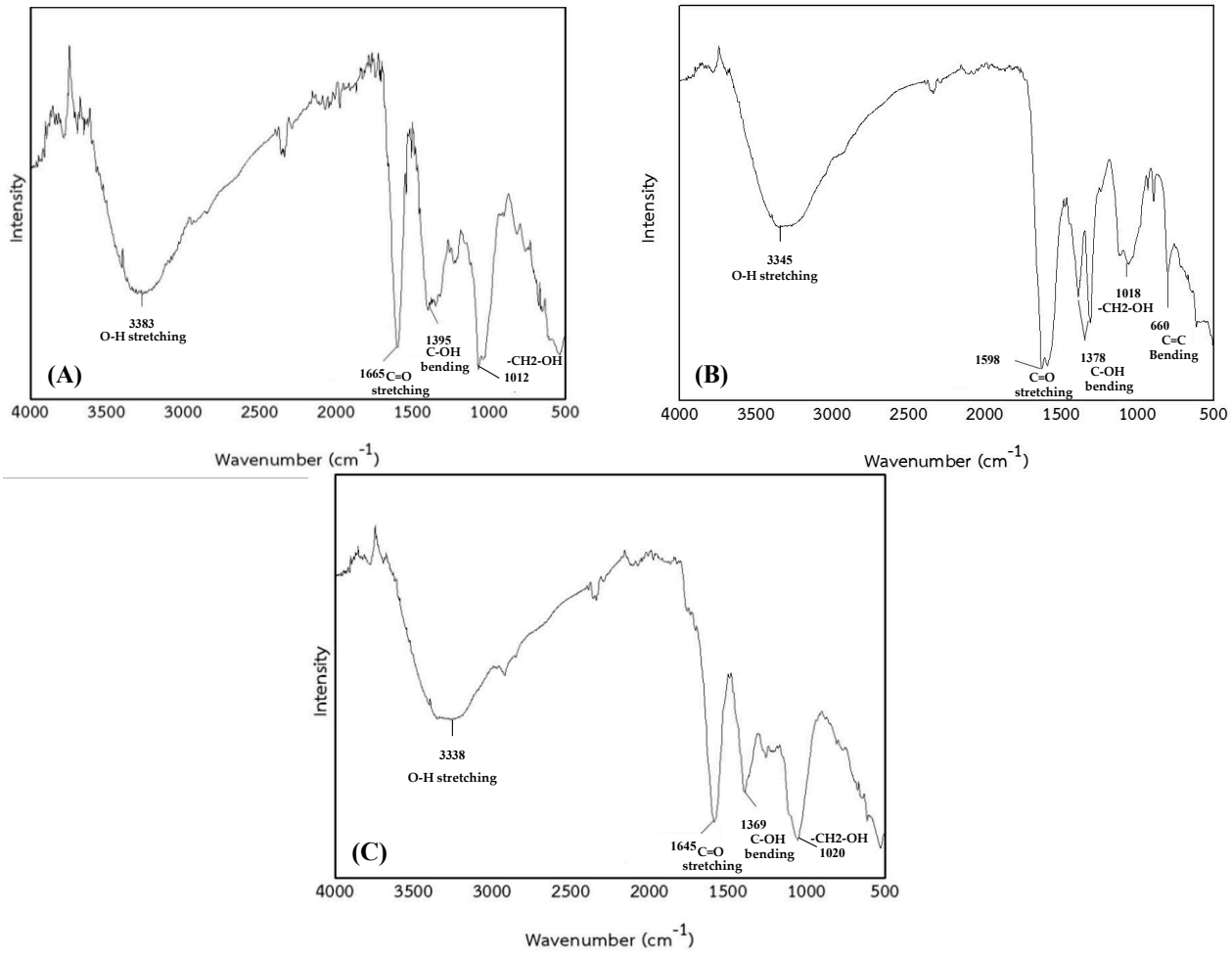

Figure 2. FTIR spectrum of crude extracts of (A) *Chromolaena odorata* L., (B) *Amaranthus virdis* L., and (C) *Cyperus Rotudus* L.

Table 6. Functional group of reducing agent testing of aqueous extracts of *Chromolaena odorata* L., *Amaranthus virdis* L. and *Cyperus Rotudus* L.

Extracts	Band range (standard) (cm^{-1})	Band range (experiment) (cm^{-1})	Assignments (interaction)	Possible compounds
Chromolaena odorata L.	1250-1000	1012	-CH_2-OH	carbohydrate
	1440-1339	1395	C-O-H (Bending)	phenol, alcohol
	1650-1550	1635	C=O (Stretching)	conjugated acid
	3400-3300	3385	O-H (Stretching)	Alcohols, Phenols
Amaranthus virdis L.	900-660	660	C=C (Bending)	alkene
	1250-1000	1018	-CH_2-OH	carbohydrate
	1440-1339	1378	C-O-H (Bending)	phenol, alcohol
	1650-1550	1598	C=O (Stretching)	conjugated acid
	3400-3300	3345	O-H (Stretching)	Alcohols, Phenols
Cyperus Rotudus L.	1250-1000	1020	-CH_2-OH	carbohydrate
	1440-1339	1369	C-O-H (Bending)	phenol, alcohol
	1650-1550	1645	C=O (Stretching)	conjugated acid
	3400-3300	3338	O-H (Stretching)	Alcohols, Phenols

Table 6 expresses the FTIR spectroscopy results of the aqueous extracts of *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L. showed the presence of the reducing agent. *Chromolaena odorata* L. extracts showed the vibration mode of -CH_2-OH groups of carbohydrate (1012 cm^{-1}) [57], C-O-H Bending (1395 cm^{-1}), C=O Stretching (1635 cm^{-1}), and O-H Stretching (3385 cm^{-1}) of alcohols or phenols [58]. The extracts of *Amaranthus virdis* L. leaves demonstrated the assignments of C=C Bending **(**660 cm^{-1}**),** -CH_2-OH groups of carbohydrate **(**1018 cm^{-1}**)** [57]**,** C-O-H Bending **(**1378 cm^{-1}**),** C=O Stretching **(**1598 cm^{-1}**)** and O-H Stretching (3345 cm^{-1}). The interaction and assignment of *Cyperus Rotudus* L. presented -CH_2-OH groups **(**1020 cm^{-1}**)** [57]**,** C-O-H Bending **(**1369 cm^{-1}**),** C=O Stretching **(**1645 cm^{-1}**)** and O-H Stretching (3338 cm^{-1}) [58].

The FTIR results correlated with the following literature: Man and Amorn [47] discussed the standard range of the interaction and assignment, which indicated the molecules uniqueness of the extract constituent compounds. Mythily and Devika [48] performed the extraction and pure isolation of *Fragaria xananassa* fruit, whereby the phytochemical, pure constituents, and antioxidant activity were evaluated. *Fragaria xananassa* fruit extracts contain aliphatic amines, indicating C-N s stretching vibration (1114.1919 cm^{-1}). Prince *et al*. [49] reported amines (1161 cm^{-1}, 1231 cm^{-1}) and alkene (721 cm^{-1}) from polyherbal formulations. The phenol, alcohol (1377 cm^{-1}), and conjugated acid (1686 cm^{-1}) were measured.

The phytochemical constituents and FTIR analysis of *Clitoria ternatea* leaf extracts were evaluated. The active compounds and bioactive constituents were Phenols (3389.57 cm^{-1}), Primary amines (1632.33 cm^{-1}), and Carboxylic acids (1057.61 cm^{-1}) [51]. This analysis revealed the properties of the compounds extracted from the sample weeds and the reducing agent of the active constituents infused in the extracts for the synthesis of silver nanoparticles using a green chemistry approach.

3.4 Inhibition Activity Percentage

Antioxidant agents suppress or reduce oxidative reactions, affecting the formation of free radicals that cause disease [52]. The antioxidant activity percentage (AA%) was evaluated by comparing the extracts of weed samples to ascorbic acid, which was inhibition of DPPH radical (Table 7). The weed sample extracts expressed AA% of 71.33% for *Chromolaena odorata* L., 18.36% for *Amaranthus virdis* L., and 21.74% for *Cyperus Rotudus* L. which, when compared to ascorbic acid (95.38%) had significant differences at $p<0.05$. The samples had a lower percentage of antioxidants than the standard. Si *et al*. [53] described the results of ethanolic extracts of *Cancrinia discoidea* to have anti-nociceptive, antiinflammatory, and antioxidant activities. Inhibition activity changes with the value of active constituents infused in extracts. The antioxidant potential of unripe and ripe *Citrus aurantifolia* was measured and compared to ascorbic acid. The juice concentration of *Citrus aurantifolia* increased, which affected the antioxidant activity percentage as 28.23% for unripe and 23.56% for ripe [54].

Table 7. Antioxidant activity percentage (AA%) of aqueous extracts of *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L.

Sample	% inhibition $_{Avg}$	S.D.	% RSD
Ascorbic acid	95.38	0.0002	0.0680
Chromolaena odorata L.	71.33	0.0007	0.2223
Amaranthus virdis L.	18.65	0.0005	0.1678
Cyperus Rotudus L.	21.74	0.0001	0.1508
F-test	1057553.494		
P-value	0.000[†]		

[†]Between groups within a column: One-Way ANOVA, $p < 0.05$

3.5 Silver nanoparticle Identification (XRD-Analysis)

Silver nanoparticles have the properties of anti-pathogenic or drug delivery [50] as synthesized by the green approach [13]. The silver nanoparticles identity was achieved through X-ray diffraction technique (XRD). Silver particles have the pattern of 2θ (planes for silver) at 38°(1 1 1), 45°(2 0 0), and 65°(2 2 0) [55]. Black sedimentary powders were achieved from the synthesis of silver nanoparticles by using sample weed extracts (*Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus* L.)

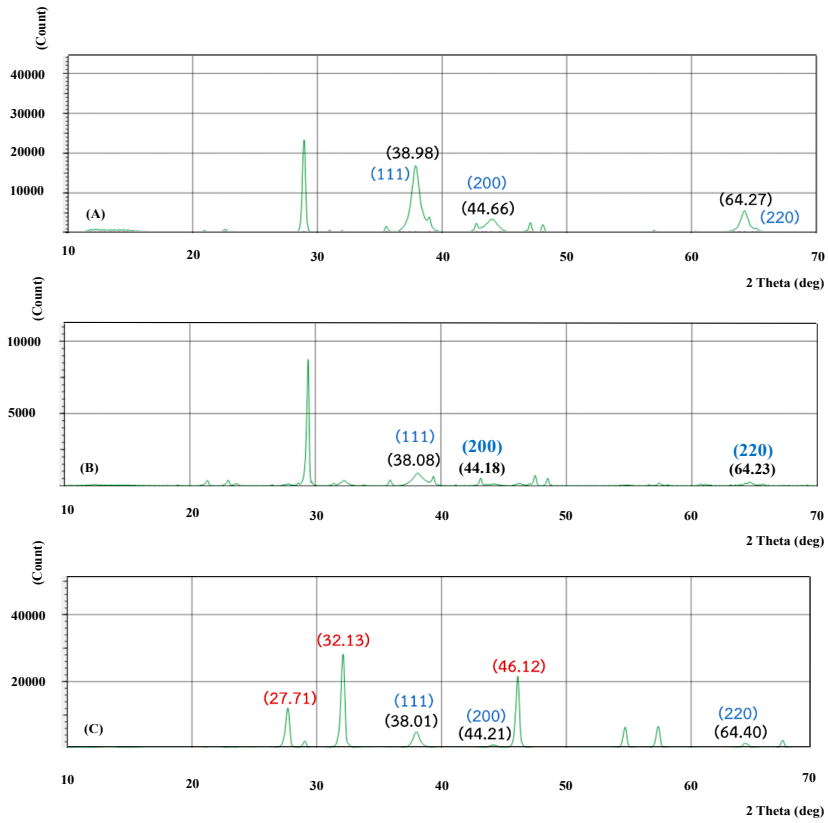

Figure 4. XRD patterns of AgNPs were obtained using (A) *Chromolaena odorata* L., (B) *Amaranthus virdis* L., and (C) *Cyperus Rotudus* L., reducing agents.

The product identity results are shown in Figure 4. The XRD patterns of silver nanoparticles synthesized from *Amaranthus virdis* L. extracts at 38.08°, 44.18°, and 64.23° were presented in planes (111), (200), and (220), respectively. Silver particle products from *Cyperus Rotudus* L. extracts expressed at 38.01°, 44.21°, and 64.40°, which were the planes at (111), (200), and (220), respectively. This corresponded to the XRD patterns of *Chromolaena odorata* L. at 39.98°(111), 44.66°(200), and 64.27° (220). Geetha *et al*. [59] discussed the synthesis process of silver nanoparticles by using the leaf extracts of *Cissus quadrangularis* L., which revealed the XRD-patterns of 2θ (1 1 1), (2 0 0), (2 2 0), and (311), that found the particle size of 11 nm. The silver nanoparticles synthesized from *Cyperus Rotudus* L. extracts demonstrated a high-intensity peak of 2θ at 27.71°, 32.13°, 46.12°. Picoli *et al*. [57] described the synthesized silver nanoparticles from *Fusarium oxysporum* extracts.

The XRD-patterns revealed the presence of three distinct peaks representing Bragg reflections of 27.9°, 32.3°, 46.3°, 55.0°, 57.6°, 67.6°, 74.6°, 76.9° and 85.7° relating to (1 1 1), (2 0 0), (2 2 0), (3 1 1), (2 2 2), (4 0 0), (3 3 1), (4 2 0) and (4 2 2) planes, respectively were AgCl characteristic at, which were aggregates of silver particles. The results of the experiment confirmed the ability to silver with plant extracts that contain water as solvents. The nanoparticles are dispersed only in water. [19]. Green synthesized silver nanoparticles using *Cyperus rotundus* L. extract were determined by dynamic light scattering (DLS) with a Zeta sizer Nano ZS (Malvern, UK) instrument at 25 °C. [60]. This is a suggested concept used to analyze the size and morphology of synthesized particles. To determine the size of the synthesized material from this research.

4. Conclusions

Phytochemical extraction from three local weeds was obtained in Sakon Nakhon Province: *Chromolaena odorata* L., *Amaranthus virdis* L., and *Cyperus Rotudus*. The preliminary testing of chemical constituents found secondary metabolites which had pharmacological activity. Phytochemical screening was correlated with the results of the GC-MS profile of the sample weed extracts, which expressed bioactive

compounds. The pharmacological activity can evaluate the sample weed's potential for the synthesis of silver nanoparticles. The antioxidant potential was performed by inhibition percentage based on DPPH. The sample weed extracts demonstrated a lower inhibition percentage than ascorbic acid, with significant differences at $p<0.05$. Reducing agent determination was carried out with conventional method and instrument analysis, revealing the reducing compounds as phenol and carboxylic acid. XRD patterns of black powder from the synthesized silver nanoparticles confirmed the Ag identity. The results showed the effectiveness of the weeds for medicinal application, which was the production of AgNPs. Literature has discussed using green synthesis for the nano-sized synthesis of silver nanoparticles. The results could guide how local weeds can be used for medicine, pharmacological activity, and synthesis of metal particles.

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Research article

Increasing Sweet Potato (Ipomoea batatas) Root Crop Yield Based Scientific Participatory Research

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Abstract: This study aims to improve sweet potato production in the Philippines by identifying the challenges facing sweet potato farmers and providing effective interventions to address these issues. Participatory Action Research (PAR) was used to foster community participation and action and to establish a baseline profile of farmers, assess participation rates, evaluate interventions using high-yield varieties and proper training on the use of the stem-cutting system and staggered planting, and identify challenges after the intervention. The mean age of the participants was 52.44 years old, indicating that they belong to the marginalized adult group, and their average monthly income from sweet potato production was relatively low. In addition to sweet potato farming, the key informants are also engaged in other crop production, such as rice (100%), banana (89%), cassava (67%), corn (67%), taro (67%) and potato (22%). Regarding fertilizer use in sweet potato farming, key informants use urea and complete 14-14-14 fertilizers. Using NSIC Sp-36 and UPL Sp-17 varieties combined with staggered planting and stem-cutting techniques effectively increases sweet potato yield per hectare. The total yield from the scientific interventions was 8,000 kilograms or 8 tons, twice the target yield of 4,000 kilograms or 4 tons. While the adaptation of the farming strategy effectively increased to 160% actual yield, there is a low market demand due to the lack of production takers, which constrained the success of this intervention. Moreover, some farmers could not attend the activity, which is essential to note. These interventions can potentially improve the productivity of sweet potato farming and increase food security and income for small-scale farmers. However, there is a need to intensify the marketing strategy.

Keywords: New variety; percent of yield loss; staggered planting; sweet potato

1. Introduction

Sweet potato (SP) is the seventh most important crop globally and the fifth most important food crop produced annually [1]. The world consumption of the said crop reached up to 130 million metric tons in 2017. China is the top global producer, with approximately 7.2 million metric tons, followed by Malawi at about 5.4 million metric tons. The same year, the Philippines ranked

22nd among the countries producing sweet potatoes with only 537,303 metric tons [2].

Based on the aforecited data, there is an excellent opportunity for sweet potato farmers/producers. In the 2017 International Potato Center (IPC) data, the Philippines has made few contributions to overcoming the global market's challenges. The center has reported that the Philippines' sweet potato production has been declining. It used to be 250,000 hectares in 1980, and in 2017 it is less than 80,000 hectares. There is a gap of 170,000 hectares over the three decades. The IPC has suggested that the government's best way to fill this gap is to link the farmers to the market through science and technology intervention.

In 2017, the volume of sweet potato production was contributed mainly by Bicol Region at 44,958 metric tons, followed by central Luzon at 31,459 metric tons. The third-largest contributor was Eastern Visayas at 25,363 metric tons. Sweet potato recorded a 1.7 percent gain from 133.94 thousand metric tons in 2016 to 136.25 thousand metric tons. This was explained by the increase in area planted in Caraga due to adequate rainfall during planting time and the bigger tubers harvested in Eastern Visayas because of sufficient soil moisture [3]. However, in June 2018, the production volume in Eastern Visayas decreased to 24,676 metric tons, with almost 960 metric tons of production loss in just a year. Samar, one of the provinces in Eastern Visayas, recorded a decrease of about 14% from 3,647 ha planted/harvested in 2012 to 3,135.10 ha grown/harvested in 2018 [3]. This problem is due to a lack of clean planting materials, the type of sweet potato varieties used by the farmers, and the traditional planting strategy.

In Samar, the challenges sweet potato farmers face in relation to variety selection and traditional planting strategies significantly impact sweet potato production. The limited variety selection, primarily yellow and purple, poses several limitations. These dominant varieties may not fully meet the dynamic market demands for sweet potatoes, potentially resulting in missed opportunities to maximize yields, improve resistance to pests and diseases, and enhance market competitiveness. The lack of diversity in varieties restricts the potential for farmers to adapt to varying market preferences and exploit the full range of benefits offered by different sweet potato cultivars [4]. Moreover, the reliance on traditional planting strategies in Samar poses an additional hurdle to sweet potato production. These conventional methods, often passed down through generations, may no longer be aligned with modern agricultural practices and advancements. Consequently, these outdated techniques fail to optimize productivity and efficiently utilize land and resources. Farmers adhering to traditional planting strategies may miss opportunities to increase yields, improve resource efficiency, and overcome challenges associated with climate variability and other environmental factors [5].

The limited variety selection and traditional planting strategies in Samar exacerbate the difficulties faced by sweet potato farmers, hindering their ability to achieve optimal productivity and compete effectively in the market. To address these challenges, interventions are needed to broaden the selection of sweet potato varieties and introduce modern farming techniques that align with current agricultural knowledge and best practices. By doing so, farmers in Samar can unlock the full potential of their land, increase their yields, enhance pest and disease resistance, and improve their overall market competitiveness. Farmers in Samar, Philippines, are small landholders with an average farm size of approximately one to two hectares. The sweet potato varieties grown in the said areas are the yellow and purple ones. Basey is one of the municipalities in Samar with high market demand for sweet potatoes. There are associations in the said place that strengthen the farming and production of sweet potato commodities, such as the Balante Agricultural Farmers' Association and Women's Association. However, during the conduct of the project Support Systems for Sweetpotato Value Chain Development and Establishment of SP Value Chains in Leyte and Samar implemented by the Visayas State University-Philippine Rootcrops Research and Training Center (VSU-PhilRootcrops), the municipality of Basey was not one of the beneficiaries [6].

The challenge now is to train Balante Agricultural farmers on the propagation techniques through the stem-cutting system and staggered planting so that they produce high-quality and disease-free planting materials to increase sweet potato production eventually. The stem-cutting system used UPL Sp-17 and NSIC SP-36 varieties. According to the National Seed Industry Council [7], aside from being a high-yielding crop, these are also more resistant to pests, particularly from scab and weevil, which attack sweet potatoes. Generally, these varieties grow in all regions in the Philippines. To address these problems, this study aims to

improve the livelihoods and well-being of sweet potato farmers by identifying their challenges related to sweet potato production and providing effective interventions to address these issues. It specifically aims to: (1) establish a baseline profile of the key informants involved in the research study, including their demographics, income, and crop planting practices, to gain an understanding of the context of the study; (2) provide information on the participation rate of sweet potato farmers in the activity or program being conducted, and assess the impact of the interventions; (3) provide evidence of their effectiveness in improving sweet potato production from 4 tons to 6 tons through the introduction of new varieties and scientific ways of farming, and (4) identify the challenges faced by sweet potato farmers in terms of selling their products and the lack of cooperation among members of the farmer association.

2. Materials and Methods

2.1 Research design

The study adopted a Participatory Action Research (PAR) approach, which is well-suited to the objectives of this research. PAR is an approach that emphasizes community participation and action. In line with the first objective, the study aimed to establish a baseline profile of the farmers involved, including their demographics, income, and crop planting practices [8]. This would provide an understanding of the context of the study and the needs and challenges facing the farmers in sweet potato production. In line with the second objective, the study aimed to provide information on the participation rate of sweet potato farmers in the activity or program being conducted. This would help measure the interventions' impact and assess their effectiveness. The PAR approach used in the study would facilitate the participation of the farmers in the research activities, allowing for more accurate data collection and analysis.

Third, the study aimed to assess the impact of the interventions and provide evidence of their effectiveness in improving sweet potato production. Using staggered planting and the stem-cutting system was expected to increase sweet potato production. The PAR approach used in the study would enable the farmers to evaluate the interventions actively, ensuring that their perspectives and experiences were considered. Finally, to achieve the last objective, the study sought to identify the challenges faced by sweet potato farmers regarding the sale of their products and the lack of cooperation among members of the farmer association. To address this issue, the PAR approach was used to foster collective inquiry and experimentation. The researcher-agriculturist trained the farmers on staggered planting and the stem-cutting system, which was participatory, allowing the farmers to share knowledge and learn together. This was intended to facilitate the development of collaborative efforts through community empowerment. Overall, the PAR approach was highly suitable for this study, as it allowed for community participation, collaboration, and action. Through this approach, the study aimed to improve sweetpotato production by addressing farmers' challenges in selling their products and promoting cooperation within the farmer association.

2.2 Data collection method

2.2.1 Social preparations for a participatory approach

The researchers conducted social preparations to achieve the first three objectives of the study. This was carried out in a two-day session that included baseline assessments and focused group discussions (FGD) for the challenges of engaging in sweet potato farming. *Survey questionnaire:* The baseline profile of key informants was collected using a survey questionnaire. The questionnaire included questions about key demographic information, such as age and average monthly family income. It also had questions about sweet potato production, such as average monthly family income from sweet potato farming, other crops planted, and fertilizer use. The survey questionnaire was administered using a face-to-face approach with a guided approach. The researchers presented the questions orally during the first day of social preparation activities for the participatory research. This approach ensured that key informants clearly understood the questionnaire and could provide accurate responses. *Attendance sheet:* The researchers used an attendance sheet or register to monitor the attendance of study participants in participatory research. This involved creating a physical document or spreadsheet that allowed researchers to record the names of study participants, the dates and times of scheduled activities or meetings, and whether or not each participant attended. For several reasons,

collecting attendance data during the research process among sweetpotato farming interventions is important. Firstly, it allows researchers to track participant engagement and ensure they are actively involved in the intervention activities. By monitoring attendance, researchers can identify participants struggling to attend or engage with the program and provide additional support or resources as needed. Secondly, attendance data can be used to assess the effectiveness of the intervention. If attendance rates are consistently high, it may suggest that participants find the program valuable and motivated to continue participating [9]. On the other hand, low attendance rates may indicate that the program needs to be adjusted, or additional support must be provided to help participants engage more effectively. Finally, attendance data can be used to evaluate the overall success of the intervention. By tracking attendance over time, researchers can assess whether the program meets its goals and objectives and make adjustments to ensure that the intervention effectively improves sweet potato farming practices and outcomes.

2.2.2 Focused Group Discussion (FGD):

After the scientific farming intervention, an FGD was conducted. This aimed to identify the various challenges faced by farmers in sweet potato farming. The FGD involved 32 farmer participants divided into four groups of eight members each. The groups discussed the challenges existing farmers' organizations encountered in sweet potato farming and provided reasons why training interventions were needed. This approach allowed for a deeper understanding of the challenges faced by farmers in sweet potato farming, as well as the specific needs of farmers' organizations in terms of training and support. By conducting the FGD during the first day of the participatory research training, participants could establish a strong foundation for future research and interventions based on a shared understanding of the challenges and needs of the community.

2.2.3 Experimental process

The researchers trained the 32 farmers and followed these Philippine Rootcrop [4] standards for sweet potato farming: *Use of clean planting materials:* The clean planting materials were acquired from Philippine Rootcrops, Baybay Leyte, and after 2 months, the demo farms became the source of SP clean planting materials through stem cutting. The demo farms are located adjacent to Samar State University-Basey Campus, a one-hectare agricultural land. In the first demo farm UPL Sp-17 was planted and NSIC Sp-36 in the second demo farm. The planting materials per hectare were approximately 40,000 cuttings (1,800 kg). Since there are two farms or the equivalent of 2 hectares to serve as nurseries and sources of planting materials for other farmers, the needed planting materials were approximately 80,000 cuttings (3,600 kg).

The stem cutting system (SCS) was the stage of planted SP from five to six leaves, and stems were cut from the plantlets with a sterile surgical blade. The stem cutting consisted of at least one axial bud and two leaves. *Fertilizer, nutrient, and water management.* In terms of nutrient content, fertilizers were applied two weeks after planting. For vegetative growth, a complete fertilizer (14-14-14). Then one and half months after planting, another set of fertilizers using a combination of nitrogen and potassium for a bulking period. The amount of fertilizer for each sweet potato hill was approximately 0.5 grams. The depth typically varies for water management from two (2) to six (6) inches. After five to 10 days, root depth can range from four (4) to eight (8) inches depending on moisture uniformity and the presence of a hard pan. The varieties introduced to the farmers are considered 'sun-loving' and do not necessarily require to be watered daily. *Pest and disease management.* There was crop sanitation to prevent or eradicate sources and vectors of pests and diseases. Planting materials should be healthy and pest and disease-free. Old plant materials, weeds, or volunteer plants that could act as an infestation source for the new crop were removed. Crop rotation prevents the build-up of crop-specific pests and diseases in one field area. Cultural control also included using pest and disease-free planting materials, growing the crop in ways that increase its resistance against pests and diseases by ensuring it is not suffering from soil nutrient deficiencies or water stress, and growing it in a climate it is well suited to. If any of the growth factors are sub-optimal, the crop will become stressed, and when plants are stressed, they have less resistance to pests and diseases. A healthy plant may be more resistant to attack by pests or diseases. *Harvesting period.* A specific schedule of activities prepared by the research team was given to the farmer-

cooperators to ensure the timely execution of technology interventions. Part of the research team is an agriculturist who handles training and discussion of root crop safety handling from planting to post-harvesting. The agriculturist was around during the demo farm applications.

3. Results and Discussion

3.1 Data analysis

For the survey questionnaire, demographic data such as age, average monthly family income, sweet potato production, other crops planted, and fertilizer were analyzed using descriptive statistics such as percentages and mean. The attendance data collected through the attendance sheet were analyzed using descriptive statistics to calculate the attendance rate for all the training conducted in social preparations. The total yield is calculated by adding the actual yields for each variety to evaluate the effectiveness of the scientific interventions. The target yield is then determined by dividing the actual yield by the percentage actual yield. This allows for a comparison between the target yield and the actual yield, providing insight into the success of scientific interventions in increasing yield production. For the FGD, a thematic analysis was conducted to identify common themes and patterns in the challenges faced by farmers in sweet potato farming. The study was performed manually or using the software NVivo. The identified themes were presented in a narrative presentation.

3.2 Baseline Profile of the Study Samples

The findings presented in Table 1 offer a valuable baseline profile of the 18 key informants involved in the study. The mean age of the participants is 52.44 years, indicating that they belong to the marginalized adult group, highlighting the importance of addressing their specific needs and challenges. The average monthly income of the participants, excluding their earnings from sweet potato farming, is Php 6,883.33. This figure sheds light on the economic circumstances of the informants, providing insights into their financial situation beyond their involvement in sweet potato production. Regarding income derived explicitly from sweet potato farming, the participants' average monthly earnings amounted to Php 1,116.67. This figure is relatively low compared to the estimated family income of Php 22,000.00 in 2015, as reported by the Philippines Statistics survey [10]. This suggests room for improvement and potential for increasing revenue through interventions to enhance sweet potato production and marketing strategies. The profile of the research participants also includes their educational background. The findings indicate that among the participants, 16.67% have had no schooling, 38.89% have reached elementary level education, 16.67% have graduated from elementary school, 11.11% have earned high school level education, 5.55% have graduated from high school, and 11.11% have pursued college-level education.

Furthermore, the key informants are involved in other crop production alongside sweet potato farming. They all cultivate rice, while 89% are also in banana production. Additionally, 67% of the participants grow cassava, corn, and taro, showcasing their diverse agricultural activities. A smaller percentage, 22%, cultivate potatoes as well. Regarding fertilizer use in sweet potato farming, the data reveals that 22% of the informants utilize urea only, while 27.78% apply a combination of urea and complete 14-14-14 fertilizers. These findings highlight the prevailing practices in fertilizer application among the participants and can inform future interventions and recommendations regarding appropriate fertilizer usage for optimal sweet potato production.

The baseline profile of the research participants provides valuable insights into their demographic characteristics, income levels, educational backgrounds, and engagement in other crop production. Understanding these aspects is crucial for designing interventions that address the specific needs and challenges sweet potato farmers face. The income, educational background, and crop diversification findings can guide efforts to enhance income generation, improve agricultural practices, and support sustainable livelihoods in the study area.

Table 1. Profile of the Research Participants as Baseline Data

Profile (n=18)	Result
Mean Age	52.44-year-old
Educational Background	No Schooling (3 or 16.67%) Elementary Level (7 or 38.89%) Elementary Graduate (3 or 16.67%) High School Level (2 or 11.11%) High School Graduate (1 or 5.55%) College Levels (2 or 11.11%)
Average Monthly Income (Other than engagement in sweet potato farming)	Php 6, 883.33
Average Monthly Income on Sweetpotato Production	Php 1, 116.67
Planted crops other than Sweet potato	Rice (18 or 100%) Banana(16 or 89%) Cassava (12 or 67%) Corn(12or67%) Taro(12or67%) Potato (4 or 22%)
Fertilizer Used	Urea only (4 or 22%) Urea and Complete 14-14-14 Combination (5 or 27.78%)

3.3 Participation Rate of Sweetpotato Farmers

The high participation rate of sweet potato farmers (91%), as shown in Table 3, indicates that they are willing to attend activities related to their farming practices. This result is significant as it implies that the farmers are interested in improving their sweet potato production and are willing to invest their time and effort to attend related activities.

However, despite the high participation rate, it is essential to note that there are still farmers who could not attend the activity, as shown in the average number of absences (3) and percentage of absences (9%). The in-depth interviews conducted by the researchers revealed that the lack of participation of other informants is a common challenge the farmers face. Identifying the reasons behind their absence and addressing them accordingly is essential to improve their participation rate.

Table 2. Participation Rate of Sweetpotato Farmers

Number of participants	Average number of attendees	% of participation	Average number of absences	% of absences
32	29	91%	3	9%

3.4 Yield per Hectare through Scientific Interventions

The table presents data on sweet potato yield per hectare through scientific interventions for two different varieties of sweet potato: NSIC Sp-36 and UPL Sp-17. The data shows the number of cuttings used, the cutting losses (20%), the cutting survival (80%), the theoretical yield (in kilograms) [11], and the actual yield (in kilograms) for each variety.

Table 3. Sweet potato Yield per Hectare through Scientific Interventions

Variety	Number of Cuttings	Cuttings losses (20%)	Cuttings Survival (80%)	Target (Theoretical) Yield (kilograms)	Actual Yield (kilograms)
NSIC Sp-36	33,000	2760	18,400	2,300	4,600
UPL Sp-17	7,000	2040	13,600	1,700	3,400
TOTAL	40,000	4,800	32,000	4,000	8,000
% actual yield= actual yield/theoretical yield*100					200%

For NSIC Sp-36, 33,000 cuttings were used, 20% of which were lost, leaving 26,400 surviving cuttings. The target theoretical yield was 2,300 kilograms per hectare, but the actual yield was 4,600 kilograms per hectare. For UPL Sp-17, 7,000 cuttings were used, 20% of which were lost, leaving 5,600 surviving cuttings. The target theoretical yield was 1,700 kilograms per hectare, but the actual yield was 3,400 kilograms per hectare. The total number of cuttings used for both varieties was 40,000, with 4,800 cuttings lost and 32,000 surviving. The theoretical yield for both varieties combined was 4,000 kilograms per hectare, but the actual yield was 8,000 kilograms per hectare. The percentage of actual yield achieved compared to the theoretical yield is 200%, indicating that the real yield exceeded the target yield by two times. The data suggests that the scientific interventions used in sweet potato cultivation using staggered planting successfully increased the yield for both sweet potato varieties. The high yield obtained from the scientific interventions indicates that these interventions can improve the productivity of sweet potato farming. Using NSIC Sp-36 and UPL Sp-17 varieties combined with staggered planting and stem-cutting techniques effectively increased the yield per hectare. However, it is essential to note that there were yield losses of 20%, which is not negligible. This could be due to several factors, such as pest infestation, disease, or other environmental factors. Future studies could further investigate ways to reduce these yield losses and improve overall yield per hectare.

Adopting new sweet potato varieties and improved farming techniques has significantly increased yield per hectare. Research studies support the use of NSIC Sp-36 and UPL Sp-17 varieties. A study by Aikpokpodion and Osaigbovo [12] found that the NSIC Sp-36 variety had a high yield potential and was suitable for cultivation in other countries like Nigeria. Another study by Khan et al. [13] reported that UPL Sp-17 exhibited good resistance to pests and diseases and had a high yield potential in India. Moreover, staggered planting and stem-cutting techniques have improved sweet potato yields. A study by Adal and Abraha [14] found that staggered planting increased the number of vines per hectare and yielded a higher product than traditional planting methods. Additionally, stem-cutting techniques have been reported to increase the number of plants per hectare, resulting in higher yields [15]. The statement further indicates that the total yield from the scientific interventions was 8,000 kilograms (8 tons), twice the target yield of 4,000 kilograms (4 tons). This demonstrates the potential of these interventions to improve the productivity of sweet potato farming significantly. Using NSIC Sp-36 and UPL Sp-17 varieties combined with staggered planting and stem-cutting techniques effectively increases sweet potato yield per hectare. These interventions can potentially improve the productivity of sweet potato farming and increase food security and income for small-scale farmers.

3.4 Challenges

3.4.1 Lack of Production Takers

According to the participants, some of them take it negatively because of low market demand due to a lack of production takers, as presented in the following utterances. *"waray namon nababaligyaan kay adi manla kami nagbabaligya* (we have no place to sell the commodity because we are selling it here in the locale)"- Key Informant 3. "*waray namon ginbabaligyaan, han hani ginsusudoy nam dinhi* (we have no place to sell the commodity, we just sell it by roaming around the community)"- Key Informant 4. "*it am baligya waray nam permanente nga ginbabaligyaan tapos nagbabaligya ngani kami diri nauubos* (We have no permanent location to sell

the commodity, and even if we tried selling it, it remained unsold)"- Key Informant 8. "*it problema an pagbaligya namun kay waray man nam naabaligyaan kay nasusumo na ginbabaligyaan nasusumo nala kay adlaw-adlaw* (the problem is we have no other targets to whom we can sell the commodity, buyers tend to refuse since it is regularly done)"- Key Informant 9. *"Diri gud naiimod, magbaligya man kami ha bungto kulang napalit* (we cannot sell them all, even we sell it in thetown proper of Basey there are few buyers)"- Key Informant 11.

The study's results showed that using the two new sweet potato varieties introduced to the key informants increased yield per harvest. However, the success of this intervention is constrained by the low market demand due to the lack of production takers, as mentioned by some of the key informants in the study. The inability to sell their sweet potato crops in a permanent location or to find enough buyers affects their motivation to continue planting and selling sweet potatoes. The key informants reported that they only sell their crops within the community or by roaming around the locality (Key Informants 3 & 4). Some also expressed that they have no permanent location to sell their products, and even if they tried selling them, they remained unsold (Key Informant 8). Moreover, Key Informant 9 stated that they have no other targets to sell their sweet potato, and buyers tend to refuse since it is regularly done. This means buyers are already accustomed to periodically buying from the same group of farmers and may not have enough demand for more sweet potatoes from other sources. Key Informant 11 added that even selling the sweet potatoes in the town proper of Basey resulted in few buyers, making it challenging for them to sell their products.

3.4.2 Presence of Conflict Among Farmer Association Members

Another identified problem contributing to the percentage of absences is the lack of cooperation among association members, as provided in the following responses of Key Informants. They are looking forward to the organization's unity such as *"Magka-urusa diri mag inaragway* (united and with no conflict)"- Key Informant 1, *"Magburubligay, mag-iristorya hin maupay para waray samok, mag-urusa* (Helping each other, discuss things in good ways to avoid conflict, unity)" - Key Informant 5, *"magburublig kami hit am grupo, para maupay it amon pagkaurusa, magburubligay* (help each other in the association so we can work together)"-Key Informant 6, "*An akon la han amon asosayon han amon bug-os nga magburublig kami* (For me, the members of association must work together)" - Key Informant 10, *"Kada miyembro magburublig ngan mayda nakatoka nga trabahuon* (each member must help and must have assignment of work)" - Key informant 12, "*Angay nga magburubligay ada pagtrabaho para diri makuri* (everyone must help so work will be easier)" -Key Informant 15, and *"bublig nga umunlad ito baga asosasyon, ngan magkamayda miyembro nga upayon gud, urusa kay maginaragway kit ano man it mahihimo? (everyone must help in the association, a member who is willing for the improvement of the association, as one, if we are more into conflict will it contribute to the association*)" - Key Informant 16.

The results indicate that lack of cooperation among association members is a significant factor contributing to absenteeism, and the key informants recognize this as a problem. They emphasize the importance of unity, teamwork, and communication in overcoming this problem. Key Informants 1, 5, and 16 all mention conflict as a hindrance to the association's progress and stress the need for resolving it. Key Informants 5, 6, 10, 12, 15, and 16 all emphasize the importance of working together, helping one another, and having assigned tasks to ensure the smooth functioning of the association. It is evident from the responses that the key informants understand the value of teamwork and its effect on the association's progress. They recognize that the lack of cooperation and conflicts can affect the association's functioning and, in turn, result in absenteeism. The key informants' responses suggest that the association needs to foster a sense of unity, trust, and cooperation among its members to address the absenteeism problem effectively. This can be achieved by promoting open communication channels, developing a culture of teamwork and collaboration, and establishing a clear hierarchy of tasks and responsibilities.

The participatory approach was central to this study, ensuring the active involvement of the key informants, sweet potato farmers, in every research stage. The study recognized the farmers as experts in their own right, valuing their knowledge, experiences, and perspectives. The participatory approach empowered the farmers through collaborative decision-making and problem-solving and allowed their voices to shape the study's design and implementation. This inclusive approach promoted a sense of ownership among the

farmers, fostering their commitment and motivation to improve sweet potato production. By actively engaging the key informants, the study aimed to enhance the interventions' relevance, effectiveness, and sustainability, ultimately benefiting the farmers and their communities.

The findings, including the participants' age, income, educational background, and farming practices, are a testament to the participatory nature of the study. The insights gained through this approach will help guide future interventions and recommendations, ensuring they are tailored to the specific needs and aspirations of the farmers and ultimately contributing to the overall success and impact of the study. The mean age of the participants is 52.44 years old, indicating that they belong to the marginalized adult group. This finding is consistent with previous research on rural communities in the Philippines, which shows that older adults often face challenges related to poverty and limited access to resources [16]. The average monthly income of the participants, other than from engagement in sweet potato farming, is Php 6,883.33, which is lower than the estimated family income in 2015 of Php 22,000.00 based on the Philippines Statistics survey results [3]. This suggests that the key informants may face economic challenges that could impact their ability to invest in their farming practices and improve their yields. Furthermore, the key informants are engaged in various crop production, including rice, banana, cassava, corn, taro, and potato. This finding is consistent with previous research on small-scale farming communities in the Philippines, which shows that farmers often engage in multiple crop production to diversify their income sources and reduce risks [17]. Regarding fertilizer use in sweet potato farming, most key informants (73%) do not use complete 14-14-14 fertilizers. Instead, only 4 out of 18 key informants (22%) use urea only, while 5 (28%) apply a combination of urea and complete 14-14-14 fertilizers. This suggests that there may be room for intervention in providing education and resources to key informants to improve their fertilizer use practices and ultimately increase their yields.

The high participation rate of sweet potato farmers in the study (91%) is a positive finding attributed to their interest in improving their production. However, the study also revealed that some farmers could not attend the activity, as indicated by the average number of absences (3) and percentage of absences (9%). To address this challenge, it is important to identify the reasons behind the farmers' lack and address them accordingly. For example, providing incentives such as transportation allowances, refreshments, or training certificates may motivate farmers to attend the activity [18]. Additionally, organizing the action at a convenient time and location may increase participation rates [19]. Adopting new sweet potato varieties and improved farming techniques has increased yield per hectare dramatically. Research studies support the use of NSIC Sp-36 and UPL Sp-17 varieties. A study by Aikpokpodion and Osaigbovo [12] found that the NSIC Sp-36 variety had a high yield potential and was suitable for cultivation in other countries like Nigeria. Another study by Khan et al. [18] reported that UPL Sp-17 exhibited good resistance to pests and diseases and had a high yield potential in India. Moreover, staggered planting and stem-cutting techniques have improved sweet potato yields. A study by Adal and Abraha[1] found that staggered planting increased the number of vines per hectare and yielded a higher yield than traditional planting methods.

Additionally, stem-cutting techniques have been reported to increase the number of plants per hectare, resulting in higher yields [19]. The statement further indicates that the total yield from the scientific interventions was 8,000 tons, twice the target yield of 4,000 tons. This demonstrates the potential of these interventions to improve the productivity of sweet potato farming significantly. Using NSIC Sp-36 and UPL Sp-17 varieties combined with staggered planting and stem-cutting techniques effectively increases sweet potato yield per hectare. These interventions can potentially improve the productivity of sweet potato farming and increase food security and income for small-scale farmers. However, while improved sweet potato varieties can increase yield, the lack of a stable market for smallholder farmers remains a significant challenge. Developing market linkages and promoting value addition can help smallholder farmers improve their income and livelihoods. This finding is consistent with previous studies that have identified the absence of a stable market as a significant barrier to increasing the revenue and productivity of smallholder farmers [20,21]. Further, the lack of cooperation and conflict among members of farmer associations has been identified as a common problem in many smallholder farming communities. This issue can significantly impact the success of collective action for community development and agricultural productivity [22]. Therefore, addressing the

lack of cooperation and conflict among the association members is essential to improve their productivity and livelihoods. One possible solution is to facilitate regular meetings and forums for the members to discuss their concerns and develop a shared vision for their association [23]. Additionally, capacity-building programs on leadership, communication, and conflict resolution can be conducted to promote effective collaboration among the members [22].

4. Conclusions

In conclusion, this study sheds light on various aspects of sweet potato farming practices among small-scale farmers in rural communities in the Philippines. The findings underscore these farmers' marginalized status, economic struggles, and engagement in diverse crop productions. The study emphasizes the need for targeted interventions to enhance fertilizer use practices and address absenteeism issues during farming activities.

Furthermore, the study highlights the potential of adopting new sweet potato varieties and improved farming techniques to increase yield per hectare significantly. This increase in productivity can positively impact food security and income generation for small-scale farmers. However, challenges such as the absence of a stable market and cooperation conflicts among farmer associations must be effectively tackled. To address these challenges and improve the livelihoods of small-scale farmers, it is crucial to develop market linkages, promote value addition, and conduct capacity-building programs focused on leadership, communication, and conflict resolution. These interventions can be pivotal in facilitating the sustainable development of small-scale farming communities in rural areas. In summary, this study underscores the importance of ongoing research and targeted interventions to support the sustainable growth of small-scale farming communities. By addressing the identified challenges and leveraging the opportunities highlighted in this study, it is possible to empower small-scale farmers, enhance their resilience, and contribute to the overall development of rural areas.

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Research article

Inexpensive Production of Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) from *Bacillus megaterium* PP-10 Using Pineapple Peel Waste

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Abstract: Pineapple peel waste has recently been interested in being utilized as a low-cost carbon source in PHA biosynthesis to reduce the production cost of PHA. The production of copolymer Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [PHBV] by the new *Bacillus megaterium* PP-10 was investigated. The bacteria were grown in a mixture of pineapple peel hydrolysate (PPH) and 3-hydroxyvalerate (3HV) precursor such as sodium propionate or sodium valerate at ratios of 1:1. Remarkably, the microbial growth and PHBV production in a mixture of PPH and sodium valerate exhibited higher biomass and higher PHA amount than that of sodium propionate, accounted about 2.40 ± 0.07 g/L of DCW and 0.71 ± 0.03 g/L of PHA concentration (PHA content of 29.6%DCW). Moreover, to control the 3HV molar fraction in PHBV, various sodium valerate concentration from 2 to 18 g/L was supplemented with PPH, and the result showed that the 3HV fraction increased linear trend with an increase in valerate concentration and was in the range between 6-35 mol%HV. In contrast, a maximum PHA concentration of 1.65 ± 0.04 g/L content (about 49%DCW) was obtained when *B. megaterium* PP-10 was cultivated in 18 g/L of total reducing sugar in PPH with 2 g/L of sodium valerate at 12 h of cultivation. Finally, the produced PHBV containing 20 mol%HV was further determined by some thermal properties and found that it possessed the melting and glass transition temperatures of 148°C and -10°C, respectively. Therefore, PHBV synthesized by *B. megaterium* PP-10 with various 3HV fractions was an excellent choice for biopolymer production.

Keywords: Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [PHBV], Pineapple Peel Waste, Pineapple Peel Hydrolysate (PPH), Sodium valerate, *Bacillus megaterium*

1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters synthesized and accumulated in bacteria as intracellular carbon and energy storage compounds under unbalanced growth conditions such as nitrogen limitation. [1-3]. Due to its physicochemical properties similar to synthetic plastics, i.e., polyethylene and polypropylene, PHAs are also completely biodegradable and show

biocompatibility properties. It has been extensively studied [3-5].Poly-3-hydroxybutyrate (PHB) is the first discovered and most commonly found in bacteria. However, PHB shows brittleness and high crystallinity [1,2,6]. An introduction of 3-hydroxyvalerate (3HV) to produce a copolymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) or PHBV exhibited superior properties by enhancing the biodegradability and reducing the crystallinity and melting point of the polymer resulted in better plasticity and more toughness [1-3]. The properties of the copolymers PHBV change depending on their HV molar fraction [1, 4-6]. PHBV is generally synthesized when the acid's organic acids or sodium salt form, i.e., sodium propionate or sodium valerate, is added into the culture medium [4, 7]. Notably, both 3HV substrate acts as direct precursor that triggers the formation of 3HV through the condensation of acetyl-coenzyme A and propionyl-coenzyme A molecules. In contrast, PHB was synthesized by condensing two molecules of acetyl-coenzyme A from a 3-hydroxybutyrate (3HB) precursor such as glucose [7-10].

Genus *Bacillus* has been reported previously. For example, *Bacillus cereus*, *Bacillus flexus*, *Bacillus aryabhattai* PHB10, and *Bacillus megaterium* were able to produce PHBV when fed with sugars or industrial wastes in the range of PHBV concentration of about 3.9-9.7 g/L with 2-84 mol% HV fraction [1]. However, the high production cost of PHA, which is more than 50% of the total cost is attributed to a carbon source, is a major obstacle for commercialized PHA; thus, the use of low-cost feedstock such as lignocellulosic waste is considered a good approach to reduce PHA production cost and at the same time can minimize waste disposal problem [7, 11]. Among these lignocellulosic waste, pineapple peel accounts for a significant part of waste accumulated in landfill which will further contribute to the release of greenhouse gases. However, with the rising pineapple demands worldwide such as Thailand that have been the largest exporter of pineapple products in the world with an estimation of the by-products from the canned pineapple industries based on the annual production year of 2020 account for more than 596,000 tons mainly, waste is pineapple peel waste (about 36%) followed by cores, stems, and crowns thus the abundance of pineapple peel waste and its disposal are a major concern currently[12, 13]. Other than the presence of protease in pineapple waste used for bromelain production and its high cellulose content, it is also a suitable substrate for the production of wine, vinegar, and organic acids, including use as a carbon source for supporting microbial growth due to its high sugar content, especially from the peel wastes [13, 14]. The highest reducing sugar concentration was obtained in pineapple peel waste compared with other feedstock, such as durian peel and sugarcane leaves, after being pretreated with 2.0%(w/v) sulfuric acid and autoclave [15]. The major components of pineapple peel consist of holocellulose, α-cellulose, hemicellulose, and low lignin content. For sugar compositions in pineapple peel hydrolysate (PPH), there are various fermentable sugars, i.e., glucose, fructose, galactose, arabinose, and xylose, as previously reported by Sukruansuwan and Napathorn [14]. These sugars can be readily available carbon sources for microbial growth and PHBV production. Besides, PPH contains various nutrients such as vitamins, minerals, and trace elements. These nutrients can provide essential elements for microbial growth and metabolic processes, potentially enhancing PHBV production [13, 14, 16]. Therefore, the objective of this study is to investigate the biosynthesis of PHBV from newly isolated *Bacillus megaterium* PP-10 by using pineapple peel waste as an inexpensive carbon source and supplemented with 3HV-precursor to achieve high PHBV production with various 3HV molar fraction for broader applications.

2. Materials and Methods

2.1 Preparation of Pineapple Peel hydrolysate (PPH)

The pineapple peel waste (PPW) samples were collected from the pineapple plantation area, Pa Bon District, Phatthalung, Thailand [7°16′12″N 100°10′12″E] and were then dried in a hot air oven at 65°C followed by grinding and sieved to the particle sizes between 0.841-0.420 mm. Then, 10 g of fined PPW samples were acid-pretreated by using 1% (v/v) H_2SO_4 in an autoclave heating under 121°C at 15 psi for 30 min (method modified from Sukruansuwan and Napathorn 2018) for fermentable sugars production [14]. The obtained pineapple peel hydrolysate (PPH) was filtered through Whatman filter paper No. 1 and finally adjusted pH to 7.0. In addition, the PPH has further analyzed the total reducing sugar (TRS) amount by DNS [17] before use as a 3HB precursor.

2.2 Bacterial strain and culture conditions

The bacterial strain *Bacillus megaterium* PP-10 (accession no. OQ859945) used in this study was newly isolated from environmental soil and identified in our laboratory (16S rDNA sequence similarity, morphological and biochemical characteristics). For seed preparation, *B. megaterium* PP-10 was cultured in a basal culture medium (BCM) [18]. Then, 5%(v/v) seed was transferred to nitrogen-limiting mineral salt medium (MSM) consisting in g/L: $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 2.0; Na_2HPO_4, 0.6; $MgSO_4 \cdot 7H_2O$, 1.0; 1 mL trace element [14, 18] supplemented with 1.0% (v/v) or 10 g/L of TRS in PPH and 10 g/L of 3HV precursor, i.e., sodium propionate (SP) or sodium valerate (SV). The pH of the medium was then adjusted to 7.0. The culture was incubated in a rotary shaker at 35°C and 200 rpm and the fermentation studies were conducted in 250 mL flasks with 50 mL culture medium for 48 h.

2.3 Control of 3HV monomer composition

Two different precursors of 3HV, such as sodium propionate (SP) or sodium valerate (SV), were compared in this study to achieve the high PHBV production with desired 3HV compositions by varying concentration of 3HB precursor, e.g., PPH and 3HV precursor from 2 to 18 g/L to reach a final concentration of carbon source at 20 g/L.

2.4 Analytical procedures

2.4.1 Dry cell weight

The cells were harvested by centrifugation (4,000x*g* for 10 min) followed by washing once with sterile distilled water and then lyophilized until constant cell weights were obtained. The dry cell weight (DCW) was calculated in g/L [5]

2.4.2 PHA quantification

Approximately 20 mg lyophilized cells were then subjected to methanolysis in the presence of 15% (v/v) sulphuric acid and 85%(v/v)methanol. The resulting methyl esters were then analyzed by gas chromatography (GC) analysis using poly(3-hydroxybutyric-*co*-3-hydroxyvaleric acid)(PHBV)(12% valerate, Sigma Aldrich) and benzoic acid as the external and internal standard, respectively to determine the PHA content and composition. The GC condition was performed according to the method described by Comeau et al. [18, 19]. The molar fraction of 3HV was calculated as this following equation; mol %3HV = [moles of 3HV/ total moles in PHBV] x 100 [1,5,10,19]

2.4.3 Detection of PHA granule

PHA accumulation was confirmed by transmission electron microscope (TEM) analysis. Briefly, the cell pellets were washed with a saline buffer (pH 7.2), and were then re-suspended in a 2.5% (v/v) glutaraldehyde solution overnight at 4°C before being fixed with 1.0% osmium tetroxide. The ultrathin sections were stained with uranyl acetate followed by lead citrate before viewing with JEOL JEM 2010F TEM (JEOL, Tokyo, Japan), with an accelerating voltage of 150-200 kV [20].

2.4.4 Total reducing sugar

DNS measured the total reducing sugar (TRS) concentration. Briefly, 500 µL of cell-free supernatant was added to 500 µL of the color reagent. These solutions were heated in boiling water for 10 min and immediately transferred to ice, and the absorbance was measured at 540 nm when the calibration curve was glucose [17].

2.5 Thermal properties of PHA

The thermal properties of the PHA sample were characterized by a differential scanning calorimeter (DSC) thermal analysis system (Perkin Elmer Pyris 1) in the range of -50 to 250°C at a heating rate of 20 °C/min. The glass transition temperature (T_g) and melting point temperature (T_m) were determined from the second scan of the DSC thermogram [21].

2.6 Statistical analysis

All the data represented the results of three independent experiments and were expressed as the mean values ± standard deviations (SD). The values were subjected to an independent t-test and values $p<0.05$ were taken as statistically significant [22]

3. Results and Discussion

3.1 Sugar analysis of Pineapple Peel hydrolysate (PPH)

The sugar compositions and concentrations in PPH were analyzed and it found that glucose was a significant component which reached about 1.51±0.02%(w/v) followed by fructose (1.30±0.01%w/v) and minor of sucrose and xylose (unpublished data). Moreover, the concentration of total reducing sugar (TRS) in PPH was determined and the result showed that the concentration of TRS in PPH was about 26.40±0.02 g/L. Similar to the previous study, the TRS concentration in PPH obtained in the range of 20 to 35 g/L after the pineapple peel residue was pretreated and showed that a major fermentable sugar in PPH was glucose followed by xylose, fructose, galactose, and arabinose, respectively [14]. However, the sugar compositions in PPH vary depending on the plant's age, growth conditions, soil conditions, geographic location, climate, and other environmental factors, such as temperature, stress, and humidity [13, 15].

3.2 The biosynthesis of PHBV from *Bacillus megaterium* PP-10

Incorporating 3HV monomer units into the PHBV polymer typically requires the addition of 3HV precursors, such as propionate or valerate, during the fermentation process. The only reported wild-type bacteria which can naturally synthesize PHBV from unrelated carbon sources like glucose are various species belonging to the Gram-positive genera *Nocardia* or *Rhodococcus* [4]. Thus in this study, sodium propionate or sodium valerate has to be supplemented in the production medium to enhance the availability of 3HV building blocks [1, 3-5]. The bacterial strain PP-10 investigated the PHBV copolymer production in the MSM culture medium containing 1.0% (v/v) of TRS in PPH and sodium propionate or sodium valerate at 10 g/L as mixed carbon sources. *B. megaterium* PP-10 produced the highest DCW and PHBV concentration of 2.40 ± 0.07 g/L (**Figure 1)** and 0.71 ± 0.03 g/L (about 29.6%DCW) after 12 h of cultivation (**Figure 2).** After 12 h, PHA content was likely to decrease along the cultivation time to maintain microbial growth.

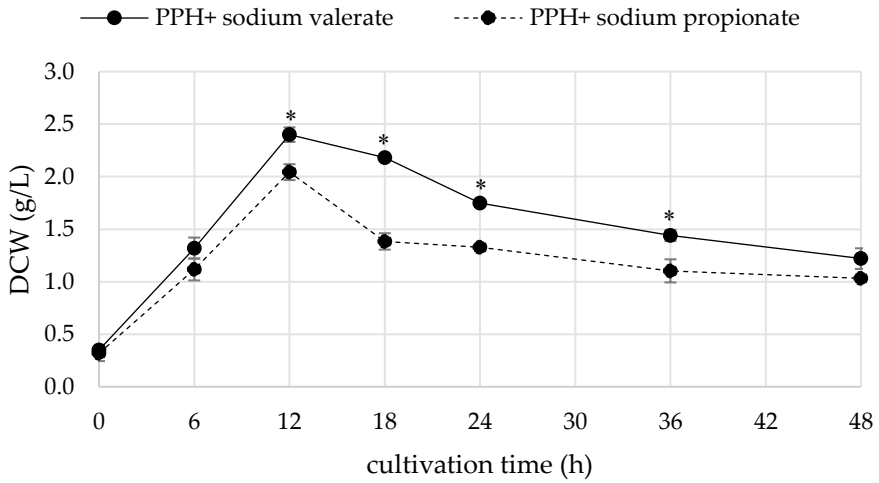

Figure 1. Cell growth (dry cell weight-DCW) produced by *B. megaterium* PP-10 when grown in the cells in PPH supplemented with sodium propionate or sodium valerate as 3HV-precursor for 48 h (* represents two independent groups were statistically significant, $p < 0.05$)

Interestingly, when growing, the cells in SV showed higher biomass than in SP. These results can be explained that the effect of propionic acid is more potent than valeric acid since carboxylic acids with shorter n-alkyl chains exhibit higher toxicity. In response to proton accumulation, free energy is released to expel protons out of cells to maintain the proton gradient [5, 10]. This excessive energy demand resulted in the decline of microbial activity, thus lowering the PHA yield. Therefore, valerate salt was identified as the better carbon substrate for synthesizing PHA compared to sodium propionate.

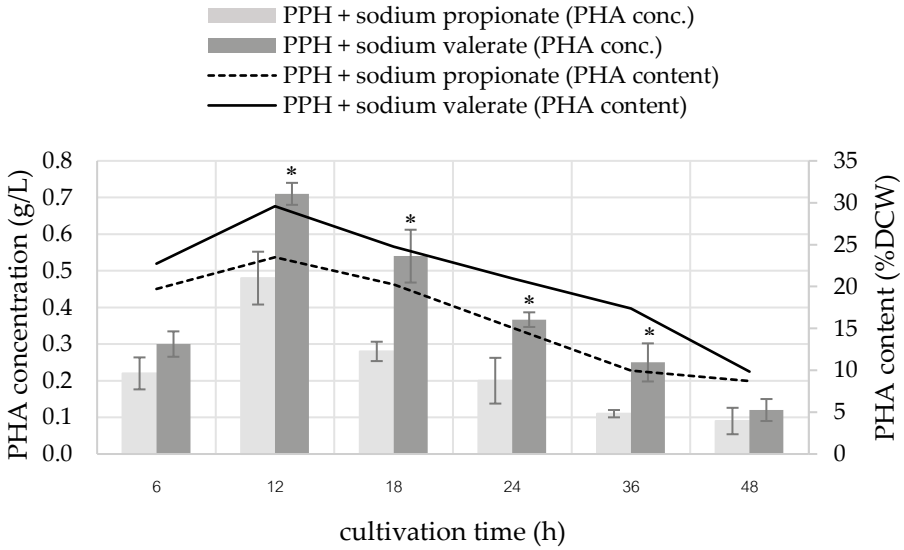

Figure 2. PHBV concentration (g/L) and the PHBV content (% DCW) produced by *B. megaterium* PP-10 when grown the cells in PPH supplemented with sodium propionate or sodium valerate as 3HV-precursor for 48 h (*represents two independent groups were statistically significant, $p < 0.05$)

However, incorporating 3HV precursors into the culture medium for PHBV biosynthesis is observed to reduce cell growth and PHBV content due to several factors, such as the toxicity and the induction of stress responses of 3HV precursors on microbial cells. In addition, 3HV precursors can disrupt the metabolic balance of the microbial culture through absent or insufficient enzymes responsible for the incorporation of 3HV monomers, resulting in inefficient utilization of the precursor [1, 5, 8]. This can divert cellular resources and energy toward stress mitigation. Moreover, adding 3HV precursors can introduce competition for carbon sources within the culture. For example, the 3HV precursor is metabolized preferentially over the primary carbon source, i.e., glucose, which may reduce cell growth and polymer content [4, 5, 10].

3.3 Control of 3HV monomer composition

The PHBV biosynthesis with different molar fractions of 3HV was produced when a mixture of 3HB-precursor (PPH) and 3HV-precursor (sodium valerate) was fed in MSM culture medium [1, 4, 5, 10]. To obtain the various 3HV molar fraction, the ratios between PPH and sodium valerate were varied from 0-20 g/L. **Table 1** demonstrated the biomass and PHBV production with different %mol of 3HV. A maximum DCW and PHA content of 3.38±0.01 g/L and 48.82%DCW were obtained when 18 g/L of PPH and 2 g/L of SV were used as mixed carbon sources. In contrast, the highest 3HV molar fraction of 35 mol% was produced when the cells were grown in 2 g/L of PPH and 18 g/L of SV, with the lowest cell growth and PHBV accumulation detected. This finding can indicate that *B. megaterium* PP-10 efficiently uptake and conversion of valerate into PHA [7, 8]. Therefore, a mixture of sodium valerate and PPH-containing glucose is a practical regulation of the 3HV molar fraction for tailor-made PHBV composition and properties. In many previous works, have been reported that genus *Bacillus*, for example, *Bacillus cereus*, *Bacillus flexus*, *Bacillus aryabhattai* PHB10, and *Bacillus megaterium*, were able to produce PHBV when fed with sugars or industrial wastes in the range of PHBV

concentration about 3.9-9.7 g/L with 2-84 mol% 3HV [1] as showed in **Table 2**. The PHBV amount in this study was the average of the PHBV content among the previous studies [7, 8, 23, 24].

Table 1. Biosynthesis of PHBV containing various 3HV compositions from *B. megaterium* PP-10 using a mixture of PPH and sodium valerate

Precursor (g/L)		DCW (g/L)	PHA concentration (g/L)	PHA content (%DCW)	Molar fraction	
PPH 3HB-precursor)	SV (3HV-precursor)				3HB	3HV
2	18	0.24 ± 0.01	0.03 ± 0.01	12.50	65	35
4	16	0.31 ± 0.02	0.05 ± 0.01	17.74	70	30
6	14	0.88 ± 0.02	0.18 ± 0.02	20.45	72	28
8	12	1.75 ± 0.02	0.37 ± 0.04	21.14	75	25
10	10	2.40 ± 0.07	0.71 ± 0.03	29.58	80	20
12	8	2.88 ± 0.05	0.88 ± 0.03	30.56	82	18
14	6	3.05 ± 0.02	1.12 ± 0.02	36.72	68	12
16	4	3.22 ± 0.01	1.44 ± 0.01	44.72	90	10
18	2	3.38 ± 0.01	1.65 ± 0.04	48.82	94	6

[a] Incubated in MSM for 12 h at 35°C, pH 7.0, 200 rpm

Table 2. PHBV production in various *Bacillus* sp.

Bacterial strain	PHBV conc. (g/L)	PHBV content (%DCW)	3HV (mol%)	References
Bacillus megaterium	3.64	86.6	16.6	[7]
Bacillus aryabhattai PHB10	2.8	71.15	-	[8]
Bacillus cereus FA11	3.9	48.43	15	[23]
Bacillus flexus	4-9.7	32	2	[24]
Bacillus megaterium PP-10	0.03-1.65	12.50-48.82	6-35	This study

3.4 Detection of PHA granule and Monomer characterization

To confirm the accumulation of PHA in *B. megaterium* PP-10, transmission electron microscopy was then examined for PHA granules in the bacterial cell, and the TEM micrograph was presented in **Figure 3**. Moreover, the monomer composition of the PHBV from *B. megaterium* PP-10 was identified by GC analysis. The GC-chromatogram of the tested PHBV consisted of 20 mol%HV, and 80mol%HB showed three significant peaks with retention times of 3.78, 4.58, and 5.61 min that referred to 3HB, 3HV, and benzoic acid (internal standard), respectively. This result corresponded with the PHBV standard presented in **Figure 4**. The PHA granule demonstrated about 30%DCW of its polymer content.

3.5 Thermal properties of PHBV

After the produced PHBV with 20 mol%HV was determined with DSC analysis, the result revealed that the melting temperature (T_m) and glass transition temperature (T_g)were 148°C and -10°C, respectively. This result was approximately equal to the PHBV reported by Policastro et al. (2021) [1] (**Table 3)**. The presence of T_m and T_g proves that the PHBV has both amorphous and crystalline regions [1, 2, 6, 7, 10]. Sudesh et al. [6] suggested that incorporating HV units in a homopolymer (PHB) reduced melting point, thereby increasing polymer flexibility and decreasing its brittleness. This property is advantageous for diverse applications such as cohesive and packaging [2, 3, 9]. PHBV with valerate fractions ranging from 8 mol% to 10 mol% is produced industrially using the bacterium *Ralstonia eutropha* [4].

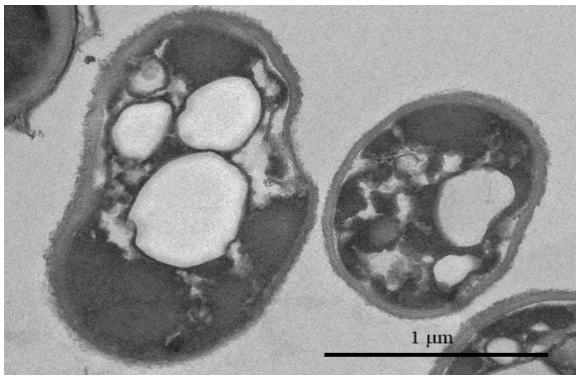

Figure 3. TEM images of *B. megaterium* PP-10 when grown in a mixture of PPH and sodium valerate for 12 h.

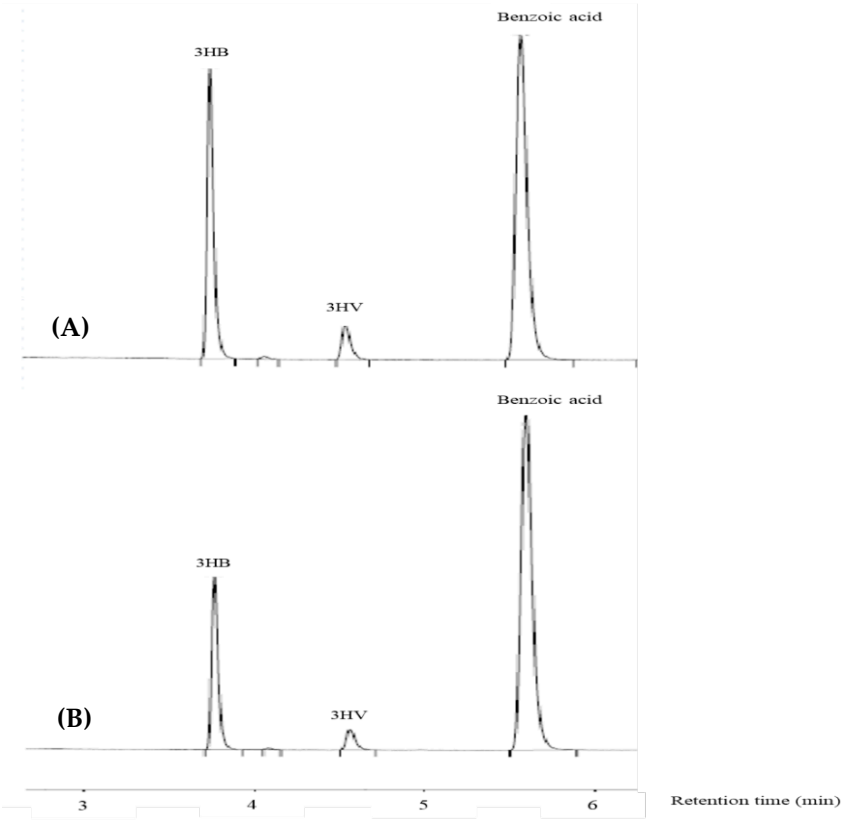

Figure 4. GC chromatogram of the poly (3-hydroxybutyrate-*co*-12 mol% 3-hydroxyvalerate) (A) PHBV with 20 mol%HV produced from *B. megaterium* PP-10 when cultivated the cells in PPH supplemented with sodium valerate at ratios of 1:1 for 12 h (B)

Table 3. Thermal properties of polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxy valerate) (PHBV) and low-density polypropylene (LDPE)

Polymer	melting temperature (°C)	glass transition temperature (°C)	reference
PHB	180	4	[1, 6, 9]
P(3HB-*co*-20mol%3HV)	145	-1	[1, 3]
P(3HB-*co*-20mol%3HV)	148	-10	This study
Low density polypropylene(LDPE)	130	-30	[9]

In this regard, *Bacillus megaterium* PP-10 is another attractive choice for the biosynthesis of copolymer PHBV from the low-cost feedstock. In addition, the tailored-made PHBV with various 3HV fractions was obtained in this study by varying the ratio of carbon substrate resulting in desired variations in the PHBV composition and properties. Moreover, the mechanical properties of the produced PHBV will be carried out.

4. Conclusions

In conclusion, the findings of this study demonstrate the successful accumulation of a copolymer of PHBV by *Bacillus megaterium* PP-10, reaching a significant concentration of 1.65 g/L. This copolymer accounted for 48.82% of the dry cell weight (DCW) and exhibited a 3HV molar fraction ranging from 6 to 35 mol%. The manipulation of 3HV-precursors enabled the regulation of different 3HV molar fractions, providing versatility in tailoring the polymer properties. Furthermore, the thermal properties of the produced copolymer, P3HB-*co*-20mol%3HV, were investigated. Notably, this copolymer exhibited a relatively low melting temperature (Tm) of 148°C and a glass transition temperature (Tg) of -10°C. These lower Tm and Tg values enhance polymer elasticity, making it more desirable for applications than PHB. Although the microbial growth and overall PHBV content achieved in this study are still relatively low, it is crucial to recognize the potential for further optimization of fermentation conditions. By fine-tuning various parameters such as the concentration of 3HV precursors, carbon source availability, carbon-to-nitrogen ratios, and the expression of relevant enzymes, as well as considering culture conditions and employing fermentation strategies like fed-batch cultivation, it is possible to overcome the current challenges and achieve higher cell growth and PHBV content. The pursuit of large-scale production of PHBV presents exciting opportunities for future research. By tackling the challenges mentioned above and exploring genetic engineering approaches, the potential for scaling up the production process becomes even more promising. These advancements would pave the way for the broader utilization of PHBV and its copolymers in various industrial applications, contributing to a more sustainable and eco-friendly future.

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Research article

Microwave-Assisted Extraction of Phenolic Compounds from *Smilax ovalifolia* Roxb. and Chemical Compositions

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Abstract: *Smilax ovalifolia* Roxb. is a medicinal herb in southern Thailand. However, there are not many studies on active compounds from this plant. This study aimed to optimize extraction conditions for total phenolic content (TPC) from *S. ovalifolia* root using microwave-assisted extraction (MAE) and to determine the phytochemical compositions and antioxidant activity of the extract. The result showed that the optimal conditions of phenolic compounds extraction include ethanol concentration of 85% v/v, a solid-to-solvent ratio of 1:30 (g/mL), microwave power of 450 W, and extraction time of 150 s. The phenolic-rich extract exhibited strong antioxidant activity with IC_{50} of 6.31 ± 0.05 µg/mL. LC-MS was used to fingerprint analysis of the extract. The result revealed the presence of 18 bioactive compounds. The main components of *S. ovalifolia* root extract were some flavonoids, including catechin, epicatechin, procyanidin B2, and quercetin.

Keywords: *Smilax ovalifolia* Roxb., microwave-assisted extraction, chemical compositions

1. Introduction

Microwave-assisted extraction (MAE) is a green extraction technology. Microwaves can convert some of the absorbed electromagnetic energy to heat energy, which are waves of frequency between 300 MHz to 300 GHz. The heating mechanism under microwave depends on the conduction of ions and dipole rotation, which enhances the solvent penetration into the sample matrix [1]. Generally, MAE is used with polar solvents for extracting organic components from dried plant samples. It has been widely used for extracting bioactive compounds from many plants due to the use of nontoxic solvents. The advantages of MAE are time reduction, reduced solvent usage, and provided high extraction yield [2]. Many reports presented the optimal MAE conditions for phenolic compounds extraction from plants were the mixture of ethanol and water (42-95%), 1:10 to 1:32 (g/mL) solid to liquid ratio, 62 s to 5 min extraction time, and 100 to 500 W microwave power [2-5]. These conditions gave high polyphenol content extracted from various plants.

Smilax ovalifolia Roxb. belongs to the Smilacaceae family, locally known as Hau-Ai Lek (Figure 1). It is grown in the southern regions of Thailand, especially in Phatthalung and Satun provinces. Hau-Ai Lek roots have been used in Thai folklore medicine for treating and preventing diseases in Kongra District, Phatthalung Province, Thailand. The rhizome of *S. ovalifolia* is used as traditional medicine in northern Thailand for treating aphthous ulcers, scars, body pain,

cancer, hypertension, and diabetes [6]. Moreover, Shah's study also showed that *S. ovalifolia* roots could be used in various diseases such as sexual diseases, uterine diseases, rheumatism, skin diseases, and wound healing. The chemical analysis of root extract showed that carbohydrates, steroids, flavonoids, tannins, and phenolic compounds were composed of the extract [7]. However, there have been few reports on the bioactivity and chemical composition of this plant. There was a report of antioxidant activity in a methanol root extract from *S. zeylanica* [8]. Moreover, steroidal saponins and polyphenols were isolated from the root of *S. china* and showed anti-inflammatory activity and anti-tumor activities [9,10]. Our preliminary study found that an ethanol extract from the roots of *S. ovalifolia* exhibited strong antioxidant activity. There have been no previous reports on LC-MS study of the root of this plant. Therefore, this study investigated the optimization of MAE parameters (Microwave power, extraction time, ethanol concentration, and ratio of dried plant material to solvent) for extracting phenolic compounds from *S. ovalifolia* root and determining the antioxidant activity of the phenolic-rich extract. In addition, this study also intended to determine the chemical compositions of the root extract.

Figure 1. The fresh *Smilax ovalifolia* Roxb. roots

2. Materials and Methods

2.1 Chemicals

Sodium carbonate (Na_2CO_3) and gallic acid were purchased from Sisco Research Laboratories (India). Folin-Ciocalteu' s phenol reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), and quercetin were purchased from Loba Chemie (India). Absolute ethanol (99.9%) was purchased from RCI Labscan (Thailand).

2.2 Plant material

The roots of *S. ovalifolia* Roxb. were purchased from Kong Ra District, Phatthalung Province, Thailand. The plant was identified according to Smitinand and Larsen [11]. Fresh roots were cut and dried at 60 °C for 48 h in a hot air oven and then ground using a grinder. The dried powder was stored in a desiccator at room temperature.

2.3 Microwave-assisted extraction

Dried root powders were extracted using a domestic microwave oven (Samsung model, MS23F300EEK/ST). The extraction conditions were: ethanol concentration (65, 75, 85, and 95% v/v), a ratio of dried plant material (g) to solvent (mL) (1:10, 1:20, and 1:30), microwave power (100, 300 and 450 W), and extraction time (90, 120 and 150 s). After extraction, the root extracts were filtered through a Whatman No. 4 filter paper and the solvent was evaporated under reduced pressure at 60 °C using a rotary evaporator. The MAE extracts obtained from each condition were analyzed for the total phenolic content (TPC).

2.4 Determination of total phenolic content (TPC)

The total phenolic content of root extracts was determined using the Folin-Ciocalteu method previously reported by Lovric et al. with a slight modification [2]. A volume of 500 µL of the extract was mixed with 500 µL of 10% (v/v) Folin-Ciocalteu reagent and 9.5 mL of distilled water, and after 5 min, 2 mL of 10% (w/v) Na_2CO_3 was added. This mixture was incubated at room temperature for 30 min. The absorbance was measured at 760 nm using a spectrophotometer (model GENESYS 180, Thermo Scientific, USA). The TPC was calculated according to the gallic acid calibration curve and expressed in mg of gallic acid equivalents (GAE) per g of extract. Each sample was analyzed in triplicate.

2.5 Determination of antioxidant activity

The phenolic-rich extract obtained from optimal MAE conditions (microwave power of 450 W, extraction time of 150 s, a solid-to-solvent ratio of 1:30, and ethanol concentration of 85%) was performed with an antioxidant activity using the DPPH method [12]. Briefly, a volume of 1 mL of the sample was mixed with 1 mL of 0.2 mM DPPH. This mixture was incubated at room temperature (25 °C) for 30 min. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Quercetin was used as a positive control. The DPPH radical scavenging activity of the extract was calculated as follows:

$$\% \text{ scavenged} = (1\text{-}As/Ac) \times 100$$

Where: As = the absorbance of the sample

Ac = the absorbance of the control

The half-maximal inhibitory concentration (IC_{50}) value of each sample was determined using a calibration curve.

2.6 LC-MS analysis

The chemical composition of *S. ovalifolia* root extract was evaluated by liquid chromatography-mass spectrometry (LC-MS/QTOF) method (model X500R QTOF, SCIEX). The analysis was performed on a Kinetex® C-18 column (150 mm × 3 mm × 2.6 µm) with 40 °C. The gradient mobile phase was a mixture of 0.5% acetic acid and methanol at a 0.5 mL/min flow rate. Mass spectra were recorded with electron ionization (EI) mode. The ion source temperature was 350 °C. The constituents were identified by comparing their retention time and mass spectral data with the National Institute of Standards and Technology (NIST) library.

2.7 Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The statistical comparisons were evaluated with the Kruskal-Wallis test.

3. Results and Discussion

3.1 Optimization of MAE

This study established the effect of MAE processing parameters including microwave power, extraction time, ethanol concentration and solid/solvent ratio, to extract polyphenols from the root of *S. ovalifolia*. The results are shown in Figure 2. The results indicated that the optimal microwave power for phenolic compounds extraction from *S. ovalifolia* root was 450 W, the most effective extraction time was 150 s with ethanol 85% (v/v), and the ratio of dried plant material (g) to solvent (mL) was 1:30. These conditions obtained higher TPC than other extractions. These results are related to that reported by Zaki et al., in which the authors studied the effect of MAE on TPC and antioxidant activity of the leaf of *Pandanus amaryllifolius* extract. Their results showed that the highest TPC was 1.557 mg/g GAE and DPPH scavenging activity was 77.7%, respectively, with an ethanol concentration of 75% v/v, microwave power of 450 W, extraction time of 10 min, and the solid to-solvent ratio of 3:100 (g/mL) [13]. In the extraction of *Centella asiatica* leaves with MAE, the optimal conditions were 75% ethanol concentration, 450 W microwave power, and 10 min irradiation time [14]. Moreover, Singh et al.'s study also showed that the highest values for the total phenolic content (83.53 mg GAE/g) and flavonoid content (18.98 mg QAE/g) were reached when MAE at 320 W during 3 min

extraction time and ethanol concentration of 50% (v/v) [15]. These data suggest that applying the optimal microwave power and extraction time may effectively extract phenolic compounds under MAE. If the extraction time is too long, the microwave energy will degrade unstable compounds, but if the irradiation is too short, microwave energy may be insufficient [16]. In the same way as microwave power, when the power of the microwave is too high, the extraction yield decreases, and the degradation of phenolic compounds at high temperatures may cause a decrease. There is evidence supporting this hypothesis. The TPC is highest at an extraction temperature of 60-80 °C [17]. This study found that when the intermittent microwave-assisted extraction time was 150 s and microwave power of 450 W, the polyphenol content was highest (221.65 ± 1.30 mg GAE/g extract). Moreover, 85% (v/v) ethanol concentration in water and the ratio of solvent to plant material 30 mL/g were considered to be optimal for the extraction of the phenolic from *S. ovalifolia* root because ethanol-water mixtures have different polarity, as a result, obtained the desired properties of the solvent and the extraction capability of the mixture was higher than a single solvent [4]. In addition, Rostagno and Prado's study found that if the solvent used for extraction is too small, the plant material does not absorb enough solvent during extraction. On the other hand, the higher the solvent content, the more significant the time needed to achieve the required temperature for extraction [18]. Our study showed that the highest TPC (195.64 ± 1.48 mg GAE/g extract) with an ethanol concentration of 85% and the solid-to-solvent ratio of 1:30. When the ethanol concentration was 95%, the content of phenolics was decreased may be caused by some polar compounds are insoluble in this mixture.

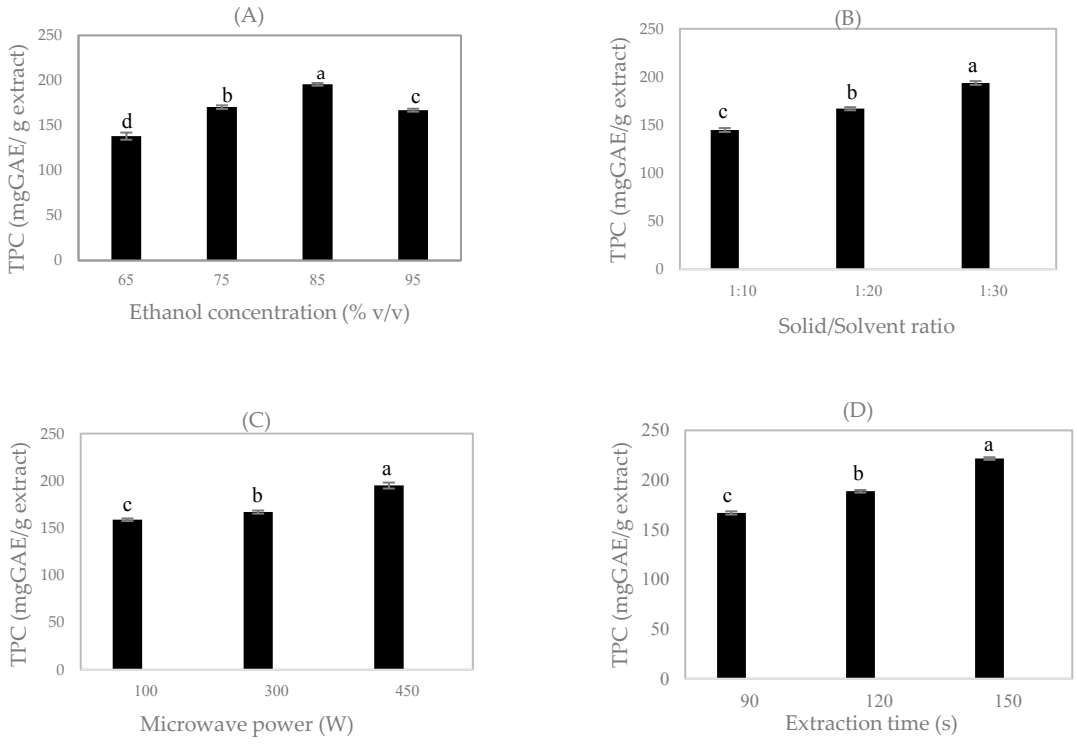

Figure 2. Influence of extraction parameters on the total phenolic content (TPC); (A): ethanol concentration, (B): solid/solvent ratio, (C): microwave power, (D): extraction time. The means presented by the various alphabets differ significantly at $p < 0.05$.

Table 1. The DPPH radical scavenging activity of phenolic-rich extract from *S. ovalifolia* root.

Extract	IC_{50} (μg/mL)
Phenolic-rich extract	6.31 ± 0.05
Quercetin (positive control)	6.79 ± 0.01

3.2 Chemical compositions and antioxidant activity of *S. ovalifolia* root extract

Chemical compositions of *S. ovalifolia* root extract were identified by liquid chromatography-mass spectrometry (LC-MS) analysis (Figure 3). The main component of *S. ovalifolia* root extract was flavonoids, including catechin, procyanidin B2, epicatechin, quercetin, quercetin glycosides (hyperoside, guaijaverin), diosmetin, morin, nepetin 7-O-glucoside, isorhamnetin 3-O-glucoside and orientin (Table 2). Because of these flavonoids (flavanols, flavonols, flavones, anthocyanidins), the phenolic-rich extract from *S. ovalifolia* root showed strong antioxidant activity with IC_{50} of 6.31 ± 0.05 μg/mL as same as quercetin (Table 1). Many reports showed that catechin, epicatechin, and quercetin have some biological activities, such as antioxidant, anticancer, antibacterial, antiviral, and anti-inflammatory activity [19-20]. Paneru and Rajbhandari showed the presence of carbohydrates, phenolics, and flavonoids in the methanolic *S. ovalifolia* leaf extract, which showed antimicrobial and antioxidant activities [21]. Moreover, Divya et al. reported that ethanol, as well as aqueous extracts of *S. ovalifolia* root, exhibited potent anti-inflammatory activity in Wistar albino rats because the root extract contained alkaloids, glycosides, flavonoids, and saponins [22].

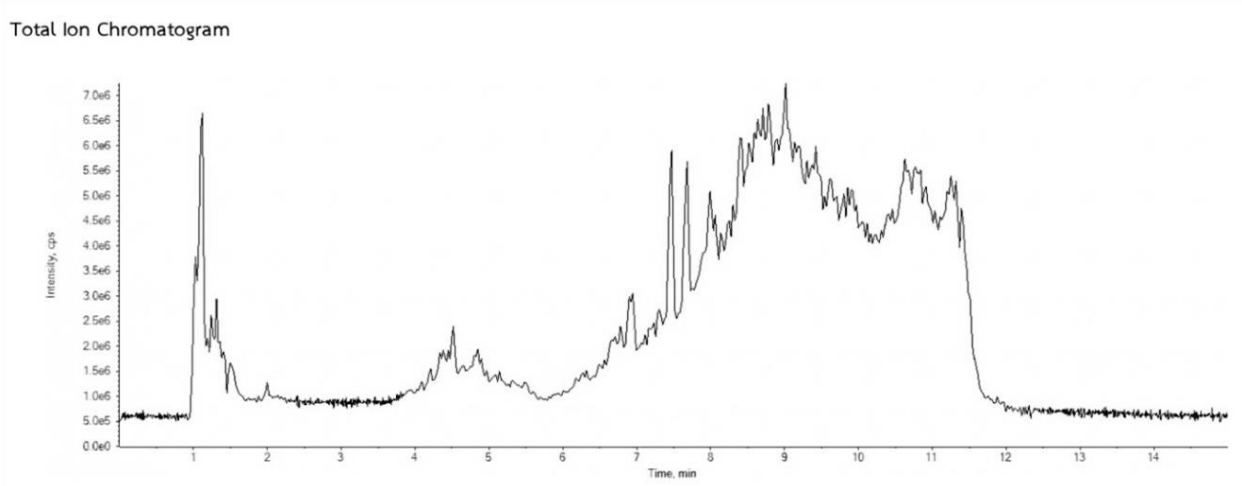

Figure 3. LC-MS chromatogram of *S. ovalifolia* root extract.

4. Conclusions

The results of this study indicated that the MAE was an efficient technique for phenolic compounds extraction from the root of *S. ovalifolia*. Optimal MAE conditions for extracting phenolic compounds were obtained with a microwave power of 450 W, an extraction time of 150 s, a solid-to-solvent ratio of 1:30 (g/mL), and an ethanol concentration of 85% (v/v). The LC-MS analysis revealed 18 bioactive components in *S. ovalifolia* root extract with catechin as the main compound. The major active compound from this plant was flavonoids. Results show that the root of *S. ovalifolia* has considerable potential as a source of natural bioactive components for healthcare products.

Table 2. Chemical compositions of *S. ovalifolia* root extract.

Compound Number	Retention time	Compound Name	Molecular formula	Molecular weight	Peak area (V)	Library Score
1	4.85	(-)-Catechin	$C_{15}H_{14}O_6$	291.08	3.194e+06	97.1
2	4.50	Procyanidin B2	$C_{30}H_{26}O_{12}$	579.13	2.112e+06	96.4
3	4.85	(-)-Epicatechin	$C_{15}H_{14}O_6$	291.08	1.506e+06	97.6
4	6.93	Quercetin	$C_{15}H_{10}O_7$	303.04	1.480e+06	97.5
5	6.93	Hyperoside	$C_{21}H_{20}O_{12}$	465.09	8.619e+05	97.6
7	1.31	Betaine	$C_5H_{11}NO_2$	118.08	2.596e+05	95.2
8	7.42	Diosmetin	$C_{16}H_{12}O_6$	301.06	2.582e+05	92.8
9	1.24	Stachydrine	$C_7H_{13}NO_2$	144.09	2.393e+05	96.7
10	7.83	Morin	$C_{15}H_{10}O_7$	303.04	2.163e+05	90.0
11	1.29	Salsolinol	$C_{10}H_{13}NO_2$	180.09	1.857e+05	93.1
12	6.65	Nepetin 7-glucoside	$C_{16}H_{12}O_7$	316.26	1.760e+05	95.2
13	7.14	Guaijaverin	$C_{20}H_{18}O_{11}$	435.08	1.522e+05	94.6
14	6.51	Emodin	$C_{15}H_{10}O_5$	271.05	1.203e+05	91.0
15	9.01	13-Keto-9z,11E-octadecadienoic acid	$C_{18}H_{30}O_3$	295.22	8.759e+04	93.7
16	1.54	Niacinamide	$C_6H_6N_2O$	123.05	2.904e+04	96.8
17	7.35	Isorhamnetin 3-O-glucoside	$C_{22}H_{22}O_{12}$	479.10	2.715e+04	97.5
18	6.40	Orientin	$C_{21}H_{20}O_{11}$	449.10	2.311e+04	96.4

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Research article

Enhancing Oil Pollution Remediation Sites with Effective Emulsifying Property of Biosurfactant–Producing *Bacillus oceanisediminis* PM 08

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Abstract: *Bacillus oceanisediminis* PM 08, isolated from soil and seawater in southern Thailand, was evaluated as a potential biosurfactant with effective emulsifying properties. This strain produced a biosurfactant with a maximum emulsifying activity of 65% and a surface tension of 22.67 mN/m after 60 hours of cultivation under cultivation conditions. The yield of biosurfactant obtained by extraction with chloroform: methanol (2: 1, vol/vol) was 1.55 g/L. The stability of the biosurfactant was effective over a wide range of pH, temperature, and salinity. The high thermostability of the biosurfactant was determined using a thermal simultaneous. In addition, a preliminary chemical composition using FT-IR revealed that it is a glycolipid biosurfactant. The properties of this biosurfactant make it a good product for many industrial applications under extreme conditions.

Keywords: Biosurfactant, emulsifying, oil pollution remediation, *Bacillus oceanisediminis* PM 08

1. Introduction

Biosurfactants are amphipathic surface-active compounds consisting of structurally distinct biological macromolecules and functional groups produced by eukaryotic and prokaryotic microorganisms. [1] They are extracellular secondary metabolites that play an important role in the survival of the organisms that have them, either by interfering with host-microbe interactions or acting as antimicrobial agents. [2] These compounds are amphiphilic molecules with hydrophobic and hydrophilic moieties that act between fluids of different polarities, providing access to hydrophobic substrates, causing a reduction in surface tension, and an increase in contact area for insoluble compounds [3]

The lipophilic moiety may be a protein or peptide with a high proportion of hydrophobic side chains or a hydrocarbon chain of a fatty acid with 10 to 18 carbon atoms, although fatty acids with a higher molecular weight have also been reported. The hydrophilic moiety can be an ester, hydroxyl, phosphate, carboxylate

group, or sugar. [4] Biological surfactants are generally classified into low molecular mass molecules, effective in reducing surface and interfacial tensions, and high molecular mass polymers, more effective as emulsion stabilizers. The major classes of typical molecular mass surfactants are glycolipids, lipopeptides, and phospholipids, while high molecular mass surfactants include polymeric and particulate surfactants. [5] Biosurfactants offer a number of advantages over chemical surfactants, such as biodegradability due to their simple chemical structure, environmental compatibility, low toxicity allowing their use in the cosmetic, pharmaceutical, and food industries, high selectivity due to the presence of specific functional groups allowing specific detoxification of certain pollutants, and activity under extreme temperature, pH and salinity conditions. These properties contribute to the applicability of biosurfactants in various industries. Large quantities of crude oil entering the marine environment, groundwater, and soil can cause significant harm to the organisms living there. [6] Petroleum is a hydrophobic hydrocarbon with adverse effects on cell membranes' structural and functional properties in living organisms, posing a significant contamination risk to marine and terrestrial ecosystems. [7] When oil and its by-products come into contact with water, they spread and form a thin layer on the surface that prevents gas exchange between air and water, blocks the passage of sunlight, and impedes respiration and photosynthesis. In this way, hydrocarbon debris affects phytoplankton communities and causes a fundamental breakdown of the food chain. [8] The potential threat to human health from hydrocarbons is related to these compounds' physical and chemical properties, which are absorbed through the skin and spread rapidly through the organism when ingested or inhaled. The most common role of biosurfactants is to improve the dispersion of pollutants in the aqueous phase and increase the bioavailability of the hydrophobic substrate for microorganisms to remove these pollutants through biodegradation. [9] Based on the above facts, many studies have been conducted using different microbes to investigate the production of biosurfactants. Therefore, the present research focused on isolating biosurfactant-producing bacteria from palm oil-contaminated sites in southern Thailand. The isolate with the most powerful emulsifying property and petroleum-degrading strains from hydrocarbon-contaminated environments were identified and characterized.

2. Materials and method

2.1 Isolation and screening of biosurfactant-producing strains

Contaminated soil and seawater were collected from Pak Meng Port, Hat Yaw Port, and Libong Port in Trang Province in southern Thailand. Bacteria were isolated using mineral salt medium (MSM) containing (g/L): K_2HPO_4—0.8, KH_2PO_4—0.2, $CaCl_2$—0.05, $MgCl_2$—0.5, $FeCl_2$—0.01, $(NH_4)_2SO_4$—1.0 and NaCl—5.0 [10]. The pH of the medium was adjusted to 7.0 before autoclaving. One gram/mL of sample was added aseptically to 5 mL of 0.85% NaCl, and 100 μL of the suspension was spread on MSM agar supplemented with palm oil (1%, vol/vol) as a carbon source followed by incubation at 30 ± 3°C for 4–5 days. Subsequently, bacterial colonies with different morphologies were picked, purified on MSM agar, and kept in nutrient broth containing 20% glycerol at –20°C. The bacterial isolates were screened for their ability to produce biosurfactants. Inocula of cultures of isolated bacteria were prepared in nutrient broth (NB, HiMedia, India), and OD^{660} was adjusted to 0.5. Then, it was inoculated in 20 mL MSM medium supplemented with 1% (vol/vol) palm oil or 1% (wt/vol) glucose as a carbon source, followed by being kept on an incubator shaker (200 rpm) at 30 ± 3°C for 48 h.

2.2 16S rRNA gene sequence analysis

Species identification of selected bacterial isolates was made based on 16S rRNA gene sequencing. The genomic DNA was extracted, and the 16S rRNA sequence was amplified by using universal primer 27F and 1492R (5'-AGAGTTTGATCATGGCTCAG-3'; 5'-GGTACCTTGTTACGACTT-3') [11]. The resulting sequence was compared with lines in the GenBank database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the nucleotide blast (BLASTn) network service.

2.3 Culture medium optimization and time course of biosurfactant production

MSM medium was used throughout this experiment. The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed under specific conditions.

Two factors were chosen to obtain a biosurfactant's higher productivity: carbon source (C) and nitrogen source (N). The carbon sources used were 1% (wt/vol) of glucose, commercial sugar, molasses, soybean oil, palm oil, and used palm oil, with 0.1% (wt/vol) $(NH_4)_2SO_4$ as a nitrogen source. A medium with no carbon source was used as the control. For evaluation of the most appropriate nitrogen sources for the production of a biosurfactant, peptone, yeast extract, urea, $(NH_4)_2SO_4$, NH_4Cl, and $NaNO_3$ was employed at a concentration of 0.1% (wt/vol) with the optimum carbon source. A medium with no nitrogen source was used as a control assay. Finally, the optimum amount of carbon and nitrogen sources was determined. To find optimum conditions for biosurfactant production, the selected strain was grown in a 250 mL flask containing 50 mL of MSM medium using 5% (vol/vol) of inocula containing the optimum amount of carbon and nitrogen sources with pH 7.0, incubated in an orbital shaker (150 rpm) at 30 ± 3°C for 72 h. Samples were taken at different intervals to measure microbial growth by dry cell weight and its ability to emulsify palm oil by measuring emulsion activity (%EA and %EI).

2.4 Recovery of biosurfactant

For recovery of crude biosurfactant, cells were separated from the culture broth by centrifugation at 6000 × g for 10 min at 4°C. The supernatant was tested for biosurfactant extraction. Five precipitation methods (acid, acetone, $(NH_4)_2SO_4$, methanol, and ethanol) and solvent extraction (chloroform-methanol (2: 1, vol/vol)) for recovery of biosurfactant were performed according to Saimmai et al. [12] with modifications. The method showing the highest emulsion activity was used to recover the crude biosurfactant from *B. oceanisediminis* PM 08 Biosurfactant production in the culture broth was detected by the method described by Cooper and Goldberg [13]

2.5 Characterization of biosurfactant

Stability of biosurfactant. The crude biosurfactant (1 mg/L) was dissolved in distilled water. To investigate the effects of pH, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), and temperature on emulsion activity, the biosurfactant solution was adjusted with 1 N HCl or 1 N NaOH to obtain a pHs from 2.0 to 12.0. NaCl was added to the sample to get the final concentrations of 0–12% (wt/vol), and $MgCl_2$ and $CaCl_2$ were used in the range of 0–0.1% (wt/vol). For the thermal stability study, biosurfactant solution was incubated at 25–100°C for 1 h and at 110 and 121°C for 15 min and cooled to 30°C. The remaining activity was then determined.

2.6 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is very useful for identifying types of chemical bonds (functional groups) and can therefore be used to analyze the components of an unknown mixture. The obtained biosurfactant (10 mg) was ground with 100 mg of potassium bromide and pressed with 7,500 kg for 30 s to get translucent pellets. Infrared absorption spectra were recorded on a Thermo Nicolet AVATAR 330 FT-IR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively. Each set of measurements consisted of 500 scans and a potassium bromide pellet was used as a background reference [14]

2.7 Analytical methods

Tests for determination of the emulsification activity (%EA) and emulsification index (%EI) were performed to evaluate the emulsifying ability of culture supernatant following the method described by Cooper and Goldberg. [13] The activity was determined by mixing 1 mL palm oil with 1 mL culture supernatant in a test tube, vortexing at high speed for 2 min, and the mixture was allowed to stand for 1 h (%EA) and 24 h (%EI). Upon standing, a creamy emulsion was formed when an emulsifier was present. Then the total height of the suspension and the height of the emulsified layer were determined by a measuring scale. Emulsion activity is defined as the height of the emulsion layer divided by the total height and expressed as a percentage. The surface tension of the culture supernatant was examined [15]. All experiments were carried out in triplicate to calculate the mean value. All chemicals used were of analytical grade. Statistical analysis was performed using SPSS 10.0 for Windows (SPSS, USA).

3. Results and Discussion

3.1 Isolation and screening of biosurfactant-producing strains

Isolation and screening of biosurfactant-producing strains. MSM with 1% (wt/) palm oil as the sole carbon source was used to isolate biosurfactant-producing bacteria from soil and seawater collected from many harbor sites in Trang province, Southern Thailand. 31 bacterial isolates were obtained, characterized by different colony morphologies. All samples were analyzed for biosurfactant production using 1% (wt/vol) palm oil as the sole carbon source. Among them, 31 bacterial isolates were identified as biosurfactant-producing bacteria using the emulsifying activity test (% EA). Most isolated bacteria (75%) were Gram-negative (Table 1). It has been previously reported that most bacterial isolates from sites that have been contaminated with oil in the past are Gram-negative, and this could be a characteristic that contributes to the survival of these populations in such harsh environments [16]. The potential biosurfactant should have an emulsifying activity of more than 50%. Only 13 isolates showed EA of more than 50%. All bacterial isolates had varying emulsification activity, which depended primarily on the microbial strain, carbon and nitrogen source, pH and temperature, and adding water-immiscible substrates to the media. The isolate with the highest percentage of EA was isolated PM08 (59.23%)—identification of the selected strain. Isolate PM08 was identified by combining the alignment results of 16S rRNA sequence analysis with biochemical and physiological characteristics. Based on the 16S rRNA gene sequence and using the tool GenBank BLAST, this isolate PM08 was closely related to *B. oceanisediminis* with a percentage similarity of 100%. It is a Gram-negative, rod-shaped (coccobacillus, straight/curved), the non-spore-forming bacterium that shows motility through a single, non-enveloped polar flagellum and produces large amounts of a non-dialysable biosurfactant. [17]

3.2 Effect of carbon and nitrogen sources on growth and biosurfactant production of B. oceanisediminis PM 08

This study began by examining the effect of carbon sources on biosurfactant production. *B. oceanisediminis* PM 08 was grown on all the tested carbon and nitrogen sources. Table 2 represents the cell growth and emulsion activity produced by *B. oceanisediminis* PM08 using different carbon sources. The results showed that *B. oceanisediminis* PM08 grew better in water-soluble carbon sources. Molasses was the most appropriate carbon source, with the maximum growth and emulsion activity. This result can be compared with that of Abbasi et al. [18] demonstrated that water-insoluble substrates were more effective in biosurfactant production in *Pseudomonas aeruginosa*. Biosurfactant production is the most effective in the presence of vegetable oils. Maximum biosurfactant yield was obtained when corn and soybean oils were used as carbon sources. Moreover, there is evidence that nitrogen plays an important role in microorganisms' production of biosurfactant compounds. *B. oceanisediminis* PM08 could utilize a wide range of nitrogen sources for growth. Among the nitrogen sources used, organic nitrogen sources better support the growth than inorganic nitrogen compounds. The highest growth occurred in the presence of peptone. However, $NaNO_3$ exhibited the most increased emulsion activity (Table 3). Similar observations have been reported by Saimmai et al. [12], which showed that $NaNO_3$ was the most efficient nitrogen source for *Oleomonas sagaranensis* AT18 to produce biosurfactants.

3.3 Time course of growth and biosurfactant production by B. oceanisediminis PM 08

The growth characteristics and biosurfactant production of *B. oceanisediminis* PM 08 were studied by using MSM medium containing 5% (vol/vol) of inocula, 1% molasses as a carbon source, 0.1% $NaNO_3$ as a nitrogen source (pH 7.0) during incubation at 30 ± 3°C and 150 rpm for 72 h. Bacterial growth (DCW), biosurfactant concentration (g/L), %EA, %EI and pH of culture broth were monitored during incubation time (Fig. 1). The biosurfactant production by this strain started during the exponential phase beginning after 6 h of growth and continued up to the stationary phase. The maximum growth and biosurfactant production occurred after 60 h of cultivation. The highest biomass yield was 4.48 g/L of DCW, and the highest yield of biosurfactant production, as determined by acid precipitation, was found to be 3.55 g/L after 60 h of cultivation. Moreover, the culture was tested for %EA and surface tension. It can be seen that a cultivation time of 60 h gave the highest activity of 65% EA and 22.67 mN/m. It evidenced the growth-associated pattern of biosurfactant production. The production profile of biomass and biosurfactant as a function of time revealed that it was the primary metabolite. However, in other reports, a partial growth-associated biosurfactant production profile was observed, in which the biosurfactant production continued during the stationary growth phase.

Table 1. Gram's staining and emulsification activity (%EA) of isolated strains

Isolate	Gram's stain	EA, %
PM01	Negative	45.06 ± 5.17^{b}
PM02	Negative	42.18 ± 4.11^{c}
PM03	Positive	44.17 ± 7.46^{b}
PM04	Negative	47.48 ± 5.48^{b}
PM05	Negative	45.21 ± 7.25^{b}
PM06	Negative	52.39 ± 8.29^{a}
PM07	Negative	50.58 ± 6.58^{a}
PM08	Positive	59.23 ± 5.26^{a}
PM09	Negative	54.28 ± 5.25^{a}
PM10	Negative	49.83 ± 7.49^{b}
HY01	Negative	52.64 ± 6.12^{a}
HY02	positive	55.26 ± 8.14^{a}
HY03	Negative	50.56 ± 9.75^{a}
HY04	Negative	49.46 ± 5.27^{b}
HY05	Negative	51.09 ± 9.23^{a}
HY06	Negative	48.37 ± 6.85^{b}
HY07	Positive	44.12 ± 6.12^{c}
HY08	Negative	55.36 ± 9.42^{a}
HY09	Negative	50.56 ± 5.87^{a}
HY10	Negative	49.25 ± 7.28^{b}
HY11	Negative	42.38 ± 6.57^{c}
HY12	Negative	40.59 ± 5.96^{c}
LB01	Positive	48.27 ± 5.25^{b}
LB02	Negative	43.49 ± 6.12^{c}
LB03	Negative	57.23 ± 9.52^{a}
LB04	Negative	48.27 ± 6.29^{b}
LB05	Positive	52.26 ± 9.63^{a}
LB06	Negative	44.28 ± 6.84^{c}
LB07	Negative	47.29 ± 5.28^{b}
LB08	Negative	44.58 ± 6.47^{c}
LB09	Negative	55.08 ± 9.48^{a}

* Different letters in the same column indicate significant different ($p < 0.05$)

Table 2. Effect of carbon source on biosurfactant production by *B. oceanisediminis* PM08 cultivated in MSM medium at 30°C and 150 rpm for 48 h

C-source (1%, wt/vol)	Dry cell weight, g/L	Final pH	EA, %	EI, %
Control**	0.07 ± 0.03^{e}	6.72 ± 0.02^{a}	2.86 ± 0.45^{e}*	0
Glucose	2.12 ± 0.05^{d}	5.28 ± 0.03^{c}	32.58 ± 0.75^{d}	17.56 ± 0.13^{c}
Commercial sugar	2.84 ± 0.07^{b}	5.47 ± 0.01^{c}	45.29 ± 0.52^{b}	30.14 ± 0.23^{b}
Molasses	3.10 ± 0.08^{a}	6.58 ± 0.05^{a}	59.87 ± 0.58^{a}	38.72 ± 0.15^{a}
Soybean oil	2.50 ± 0.05^{c}	6.75 ± 0.03^{a}	39.72 ± 0.72^{c}	15.56 ± 0.15^{d}
Palm oil	2.86 ± 0.07^{b}	6.49 ± 0.02^{b}	45.27 ± 0.64^{b}	19.84 ± 0.26^{c}
Used palm oil	3.12 ± 0.06^{a}	6.28 ± 0.04^{b}	39.47 ± 0.55^{c}	26.68 ± 0.27^{b}

* Different letters in the same column indicate significant different ($p < 0.05$)

** Control: no carbon source. Results represented mean ± standard deviation from 3 determinations.

Table 3. Effect of nitrogen source on biosurfactant production by *B. oceanisediminis* PM08 cultivated in MSM medium at 30 °C and 150 rpm for 48 h (carbon source: 2.5% molasses)

N-source (1%, wt/vol)	Dry cell weight, g/L	Final pH	EA, %	EI, %
Control**	0.05 ± 0.01^{d}	5.23 ± 0.04^{c}	25.21 ± 0.48^{c} *	9.87 ± 5.02^{d}
Peptone	3.72 ± 0.03^{a}	8.35 ± 0.07^{a}	52.35 ± 0.17^{a}	45.14 ± 4.15^{a}
Yeast extract	3.24 ± 0.02^{a}	4.25 ± 0.06^{d}	48.14 ± 0.38^{b}	38.75 ± 5.24^{b}
Urea	3.08 ± 0.07^{b}	8.01 ± 0.03^{a}	45.28 ± 1.72^{b}	30.15 ± 3.86^{c}
$(NH_4)_2SO_4$	2.95 ± 0.02^{b}	4.53 ± 0.08^{d}	48.35 ± 0.25^{b}	30.35 ± 0.25^{c}
NH_4Cl	2.84 ± 0.08^{c}	4.32 ± 0.04^{d}	51.50 ± 2.56^{a}	40.83 ± 3.21^{b}
$NaNO_3$	2.91 ± 0.05^{b}	7.82 ± 0.05^{b}	46.05 ± 3.87^{b}	32.09 ± 5.10^{c}

* Different letters in the same column indicate significant different ($p < 0.05$)
** Control: no nitrogen source. Results represented mean ± standard deviation from 3 determinations.

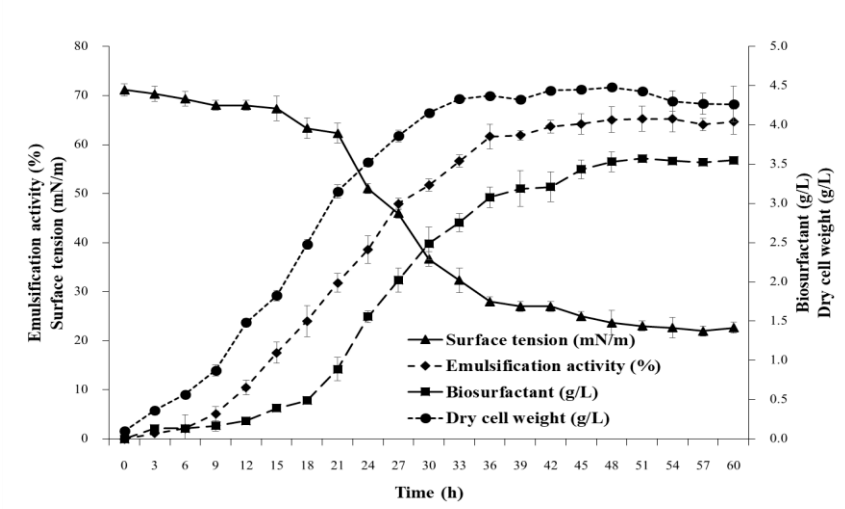

Figure 1. Time course of growth, biosurfactant production, and emulsion activity of *B. oceanisediminis* PM 08 under optimal medium conditions.

3.4 Recovery of biosurfactant

The biosurfactant was precipitated or extracted from the culture supernatant of B. oceanisediminis PM 08. Among six methods of precipitation and extraction, chloroform: methanol (2: 1) was the most efficient in biosurfactant recovery from the culture supernatant of this strain (Table 4). A recovery yield of 1.55 g/L was obtained from *B. oceanisediminis* PM 08. Panjiar et al. [19] reported the amount of emulsifier produced, as recovered by the acid precipitation method. It was found to be 3.07 ± 0.62 and 3.90 ± 0.3 g/L for *Lysinibacillus* sp. SP1025 and *Bacillus cereus* SP1035, respectively.

3.5 Characterization of biosurfactant. Stability of biosurfactant.

Crude biosurfactant was used to study the effect of pH, temperature, and salinity on emulsion stability. The impact of various pHs in the range of 2.0–12.0 on the emulsion activity (%EA and %EI) of biosurfactant are presented in Fig. 2a. Biosurfactant from *B. oceanisediminis* PM 08 showed a broad range of emulsion activity between pH from 4.0 to 10.0. The lowest emulsion activity was observed under acidic (pH < 4.0) and alkaline (pH > 11.0) conditions

Table 4. Effect of recovery method on yield, EA, and EI of the biosurfactant produced by *B. oceanisediminis* PM 08

Recovery method	Yield, g/L	EA, %	EI, %
Acid precipitation	5.87 ± 1.57[a]	40.65 ± 5.12[d]	35.17 ± 3.50[d]
Acetone precipitation	5.08 ± 1.42[b]	50.23 ± 4.11[c]	40.18 ± 3.49[c]
$(NH_4)_2SO_4$ precipitation	4.25 ± 1.34[b]	52.65 ± 5.52[c]	36.48 ± 2.58[d]
MeOH precipitation	2.88 ±1.35[c]	60.88 ± 5.35[b]	52.06 ± 4.28[a]
EtOH precipitation	1.98 ± 0.55[d]	65.32 ± 6.17[a]	49.82 ± 5.14[b]
CH_3Cl: MeOH extraction	1.55 ± 0.16[d]	69.81 ± 2.56[a]	55.26 ±5.86[a]

* Different letters in the same column indicate significant different ($p < 0.05$)

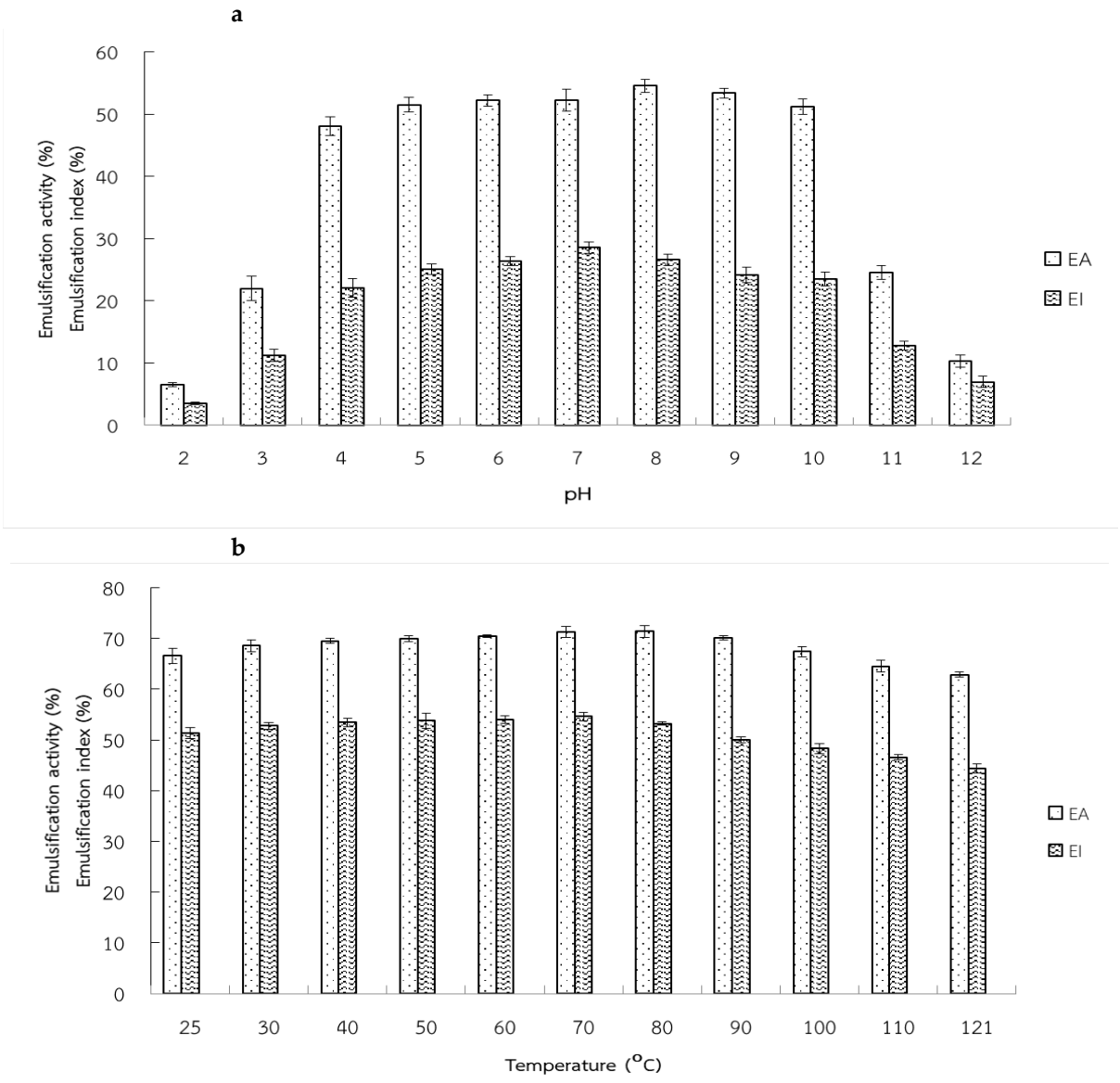

Figure 2. Effect of pH (a) and temperature (b) on emulsion activity of crude biosurfactant produced by *B. oceanisediminis* PM 08.

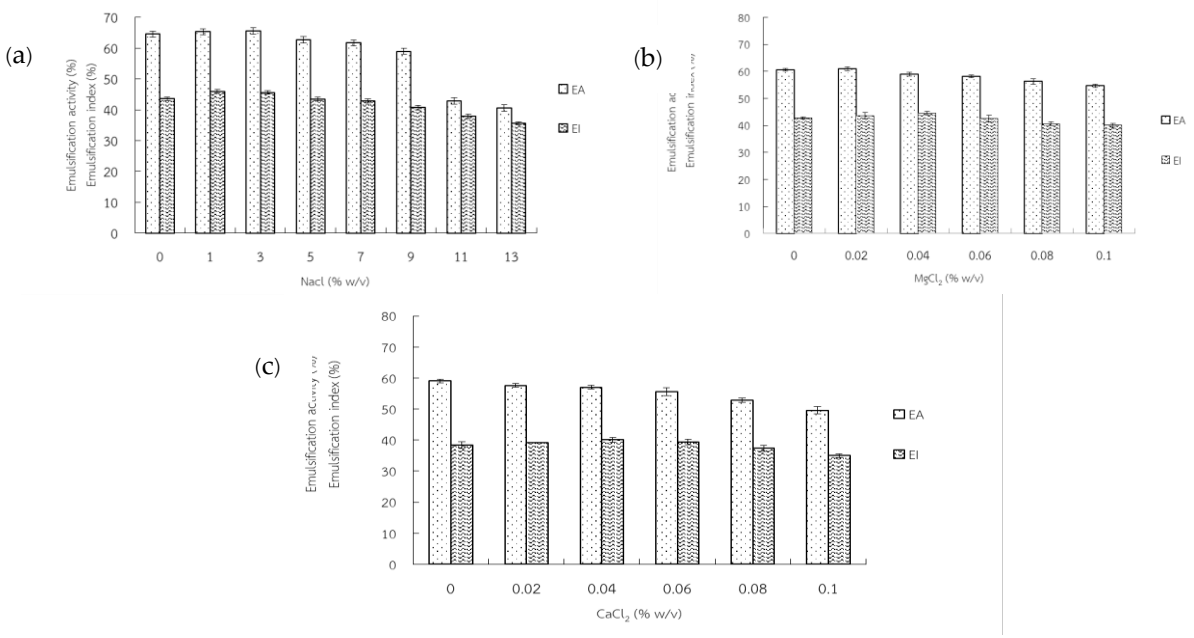

Figure 3. Effect of NaCl (a), $MgCl_2$ (b), and $CaCl_2$ (c) on emulsion activity of crude biosurfactant produced *B. oceanisediminis* PM 08.

Thermal stability analysis over a wide range of temperatures (25–121°C) showed that the biosurfactant had considerable stability under studied conditions (Fig. 2b). Furthermore, a study on the effect of salts and salt concentrations on emulsion activity was performed (Fig. 3). Emulsion stability retains up to 9% NaCl, 0.1% $MgCl_2$ and 0.06% $CaCl_2$. After that, a considerable reduction occurred at higher concentrations. Biosurfactants were stable at various temperatures, pH, and salinity. Gudina et al. [20] reported a novel bioemulsifier produced by a *Paenibacillus* strain isolated from crude oil. Its emulsifying ability was not affected by exposure to high salinities (up to 300 g/L), elevated temperatures (100–121°C), or a wide range of pH values (2.0–13.0). Similarly, the bioemulsifier from *Solibacillus silvestris* AM1 was to be thermostable and active in the pH from 5.0 to 9.0 and 0–5 M NaCl range [21]. Also, an *Ochrobactrum pseudintermedium* strain C1 that secrets an exopolysaccharide as a bioemulsifier showed its properties over a wide range of pH (2.0– 8.0), at moderate salinity (4–6% NaCl), and during exposure to elevated temperatures (100°C) [22] These exciting properties offer the opportunities for the biosurfactants to be investigated in the extreme environment for microbial enhanced oil recovery and in situ biodegradation of oil sludge. In addition, their use is possible in industrial processes for food and pharmaceutics, frequently involving exposure to extremes of temperature, pressure, pH, and ionic strength [23].

3.6 Biosurfactant Characteristics

It was submitted to FT-IR to identify the main functional groups present in the biosurfactant produced by B. oceanisediminis PM 08. Figure 4 shows the FT-IR spectrum of biosurfactants. The presence of a broad range band at 3082.20 cm–1 characteristic of the -OH group assigned to the carboxylic group of sugar moiety and a set of intense bands within the 1601–1451 cm–1 region (1601, 1583, 1541, 1493, and 1451 cm–1) were assigned to the vibration of the C–O and C–O–C glycosidic bands, demonstrating the occurrence of carbohydrates [24]. The 2923 and 2850 cm–1 spectra showed the presence of –CH aliphatic stretching and –CH2 methylene stretching [25]. In addition, the C–O stretching bands at 757 cm–1 and 700 cm–1 confirm the presence of the bands formed between a carbon atom and hydroxyl groups in the chemical structures of the glycoside part [26]. The FT-IR spectra showed the hydroxyl group of –OH stretching vibration, –CH aliphatic stretching, –CH2 methylene stretching, and an ester carbonyl group (C–O) revealed the lipid part in glycolipid biosurfactant such as rhamnolipid [27]. Overall, the FT-IR spectrum suggested that the biosurfactant produced

by B. oceanisediminis PM 08 has been classified as a glycolipid with a carbohydrate and lipid combination. The FT-IR spectra of this biosurfactant displayed a significant similarity in the adsorption of other glycolipids. [28]

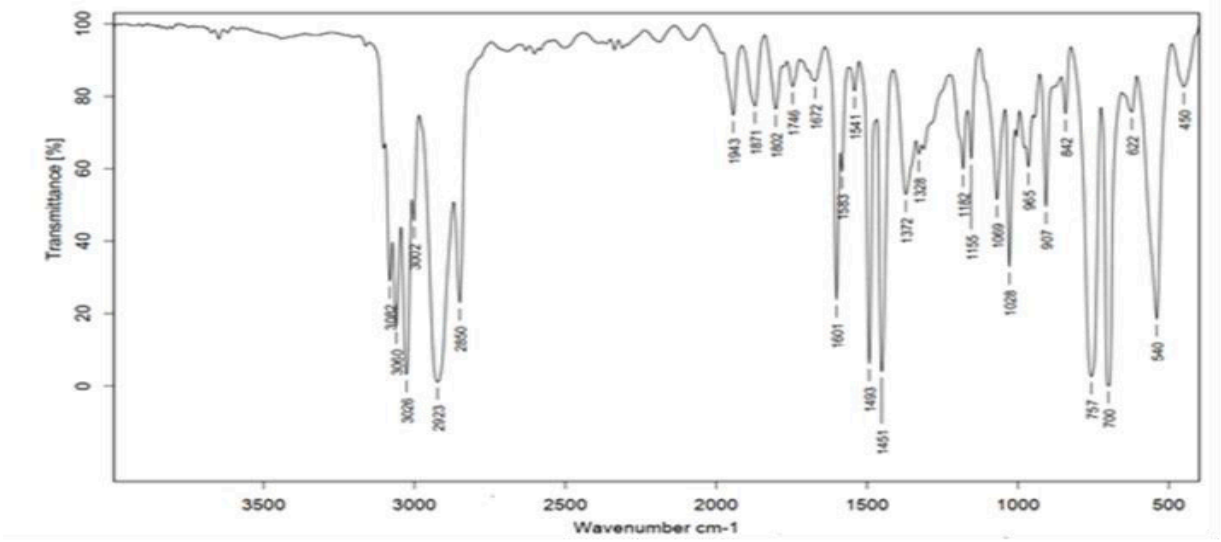

Figure 4. FT-IR spectrum of the biosurfactant produced by *B. oceanisediminis* PM 08

4. Conclusions

In this study, the biosurfactant produced by *B. oceanisediminis* PM 08 exhibited the highest emulsification activity after being grown under optimized growing conditions in MSM with molasses as a carbon source and $NaNO_3$ as a nitrogen source. The biosurfactant also proved stable under extreme pH, temperature, and salinity conditions. The biosurfactant from *B. oceanisediminis* PM 08 was extracted and characterized by FT-IR, indicating it is a glycolipid. The properties of this biosurfactant make it an interesting biotechnological product for many environmental and industrial applications.

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Research article

Prototype System for Production of Refuse Derived Fuel (RDF-5) from Municipal Solid Waste Using Natural Rubber as Binder

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Abstract: The development of RDF-5 fuel from community waste using natural rubber as a binder was investigated. The results showed that the calorific and heat values were increased proportionately to the amount of rubber added. The ratio with the highest calorific value was 50:50, followed by 75:25, 90:10, and 95:5. The calorific value was 40.67 MJ/kg (9720 kcal/kg), 40.24 MJ/kg (9,617 kcal/kg), 39.28 (9,389 kcal/kg) and 39.18 MJ/kg (9,365 kcal/kg), respectively. Compared with 100:0, have a calorific value of 38.84 MJ/kg (9,284 kcal/kg). The experimental addition of natural rubber as a binder makes the combustion process more complete, resulting in faster fuel ignition time or easier igniting, less ash content, and being environmentally friendly. The research team chose to develop the ratio of 95:5 because the economic analysis showed a fast payback period of only 5.72 years. So, the ratio of 95:5 is the optimal condition that can scale up to the industrial level. The production cost of RDF-5 is 1,935 baht/ton for 50 baht/kg of natural rubber. The amount of emission caused by the combustion process of RDF-5 (95:5) passed the standard. Therefore, the evaluation of this research found that RDF-5 fuels from community waste using natural rubber as a binder. It is one of the attractive alternatives to renewable fuel generation and solid waste management solutions and helps improve the environmental quality of communities.

Keywords: RDF-5 fuel, municipal solid waste, natural rubber binder, prototype system

1. Introduction

Satun is a small province with world-famous tourist attractions such as Koh Lipe, and on April 17, 2018, it was certified as Thailand's first world geopark (Satun Global Geopark). Satun has thus become a world-class new tourist attraction in terms of geology. It found that in 2018 there were 1.5 million visitors, of which 1.04 million were tourists, an increase of 8.43% from the previous year [1], resulting in the problem of overflowing waste. It found that the amount of waste generated in 2018 was 110,476 tons, the average municipal solid waste generation rate was 1.15 kg/person/day [2]. While the amount of waste continuously increases, the daily waste generation rate is

higher than the disposal rate. This results in a large amount of old waste left over, but the waste management on the floor is only a landfill method, and there is a limited amount of landfill space causing the responsible person to be unable to solve the problem of waste overflowing the city. In the experiment, the feasibility of RDF-5 production from the Integrated Waste Management Center, Kamphaeng Subdistrict, La-Ngu District, Satun Province. There are about 100,000 tons of residual and new waste, which the landfill plan designed to use as a landfill for 15 years. The average amount of waste entering the center is 50 tons per day, selected only 2 tons of organic waste, recyclable waste 8 tons, and landfill 40 tons. There are also 300 tons of waste from Koh Lipe per month, transported twice a month. Information reported by the Integrated Waste Management Center, Kamphaeng Subdistrict, La-Ngu District, Satun found the problem of overflowing waste in the area of La-ngu district and the finding urgent measures to deal with the problem, which, if left affected to the environment deteriorate in a short time, and this will affect the economy, income and the health of people in the community.

RDF-5 is a compression-densified fuel (density greater than 600 kg/m^3) and comes in various shapes, such as pellets, spheres, cuvettes, briquettes, or similar shapes [3]. The advantages of compression are increased bulk density, low moisture content, high calorific value, and ease of transportation [4, 5]. RDF can generally be manufactured using three leading technologies; piston press, screw press, pelletizing process, etc. [6]. Searching for research papers, no studies using natural rubber as a binder have been found for RDF-5. So not comparing RDF-5 production with past research reports, similar research papers have prepared RDF-5 from sawdust powder, cardboard, polyethylene bags, and dry cooked rice using glycerol as a binder [7]. It found that using glycerol as a binder increased overall thermal efficiency. Natural rubber has good cohesiveness. Therefore, natural rubber is expected to be a great binder in RDF-5 production. Additionally, using natural rubber as a binder can enhance the used quantity of natural rubber, which is economic promotion.

The research team is aware of such problems. Therefore, guidelines have been established to manage the waste overflow problem by producing RDF-5 solid fuel from municipal and combustible waste. It is the most likely choice because it has a heating value three times higher than biomass fuels. This research applies the concept of using natural rubber as a binder in the production of RDF-5 solid fuels. It has an explosive property, a higher heat value, and the standards required by the industry.

2. Materials and methods

2.1 Preparation of RDF-5

Our study aimed to study the feasibility of utilizing solid waste at the Integrated Waste Management Center, Kamphaeng Subdistrict, La-Ngu District, Satun Province, which could be utilized to produce RDF-5. In the experiment, municipal solid waste chose to use solid waste through the separation process before landfill which most solid waste of combustible components, such as non-recyclable plastics (not including PVC), paper cardboard, labels, and other corrugated materials, etc. After that, solid waste and natural rubber were ground pieces into flakes about 1-2 cm x 1-2 cm in size (Figure 1). Mixed the crushed municipal waste with natural rubber and varied the amount of dry rubber in the range of 5-50% of the weight (Table 1).

Figure 1. Materials used in the experiment; (A) Municipal solid waste, (B) Ground solid waste, and (C) Ground natural rubber

Table 1. The raw material ratio for the preparation of RDF-5

Ratio	Municipal solid waste (g)	Natural rubber (g)
100:0	50.0	0
95:5	47.5	2.5
75:25	37.5	12.5
50:50	25.0	25.0

Figure 2. A prototype system for the production of refuse-derived fuel (RDF-5) at a production capacity of 800 kg/day

2.2 Study on the physical and chemical properties of RDF-5 fuel

RDF-5 is produced in a prototype system shown in Figure 2. Raw materials from step 2.1 were sent to the extrusion system with a single-screw extruder with a temperature of 170 °C and cut the sample into pieces

of the required length to produce RDF-5 fuel. The rubber acts as a binder to the fuel ratio and cools rapidly to room temperature as the RDF-5 fuel leaves the extrusion. The produced RDF-5 waste fuels were analyzed for the performance of RDF-5 waste fuels and pollution according to ASTM standard methods as follows calorific value (suitable in the range of 13 - 18 MJ/kg), ASTM D 5865 volatile matter, ASTM D 3172 ash content (ash), ASTM D 3174, density, ignition time, and moisture content (moisture), which calorific value is a direct analysis. The sample of fuel waste is ground to a size of about 1 mm and analyzed by the Bomb Calorific Method. The heat value called Dry Solid Calorific Value (DSCV) is the heat value obtained from complete combustion. Finally, analyze emissions arising from the RDF-5 combustion process such as carbon dioxide (CO_2), oxide of nitrogen as NO_2, sulfur dioxide, total suspended particulate, etc.

3. Results and Discussion

3.1 Effect on physical and chemical properties of RDF-5 fuel

The addition of natural rubber results in a significant increase in heat. The heating value will increase in proportion (%) of the added natural rubber (Table 2). The heating value of the fuel is directly proportional to the amount of natural rubber. The highest heat was a ratio of 50:50, followed by 75:25 and 95:5 with a heat value of 40.67 MJ/kg (9,720 kcal/kg), 40.24 MJ/kg (9,617 kcal/kg) and 39.28 (9,389 kcal/kg) respectively, and when compared with solid waste alone (100:0), the calorific value was 38.84 MJ/kg (9,284 kcal/kg). The addition of rubber to RDF-5 fuel significantly promoted synergism, as shown in Figure 3. From Figure 4, it was found that every ratio of RDF-5 fuel had a higher heating value than the solid fuel production standard of 15 MJ/kg (ASTM standard). The thermal benefits of RDF-5 fuel production can be obtained. Therefore, results from research can bring each fuel ratio to produce RDF-5 fuel. It was found that all fuel ratios passed the production standard criteria. Solid fuels are fuels in each ratio that contain moisture content of fuel not more than 10% (Figure 5(A)). The ash content of each fuel ratio is in the range of 0.21-11.32%, with the ratio with the highest ash content being 100:0, followed by 95:5, 90:10, 75:25, and 50:50, respectively(Figure 5(B)).

Table 2. Resulting in the calorific value of RDF-5 fuel

Ratio	Municipal solid waste (g)	Natural rubber (g) (dry weight)	Results	
			Calorific value (kcal/kg)	Calorific value (MJ/kg)
100:0	50.0	0	9,284	38.84
95:5	47.5	2.5	9,365	39.18
90:10	45.0	5.0	9,389	39.28
75:25	37.5	12.5	9,617	40.24
50:50	25.0	25.0	9,720	40.67
Rubber (0:100) [8]	-	-	10,127	42.40
Municipal solid waste (100:0) [9]	-	-	8,927	37.50

Figure 6 showed that the density of each fuel was inversely proportional to the amount of natural rubber added, resulting in a decrease in the density value when the ratio of rubber added more rubber which is the opposite of the calorific value, possibly because natural rubber has high elasticity properties. It can quickly return to its original or similar size shape after deformation due to external force [10], resulting in the density of RDF-5 decreasing when natural rubber is added to the increasing ratio. Although adding rubber to the amount will help with the increased heating value and ash content decreased because it causes complete combustion. The addition of natural rubber must be in the appropriate amounts to promote synergism, and production costs must be considered when operating on an industrial scale. From the experiments, it was found that every ratio used in the experiment exceeded the ASTM standard of density at 600 kg/m^3. It could use as RDF-5 fuel (Figure 6). Ignition time is the amount of time the fuel temperature is heated. It can see from Figure 7 that adding natural rubber to RDF-5 fuel makes Rdf-5 fuel easier to ignite. Compared to 100:0, which is RDF-5 made from municipal waste alone, it takes 20.41 minutes when adding natural rubber, the ratio is 95:5,

90:100, 75:25, 50: 50, causing the ignition time to be reduced to 12.67, 12.40, 11.04 and 10.19 minutes, respectively. Adding a higher ratio of rubber nature resulted in a faster or easier ignition time of RDF-5 fuel significantly.

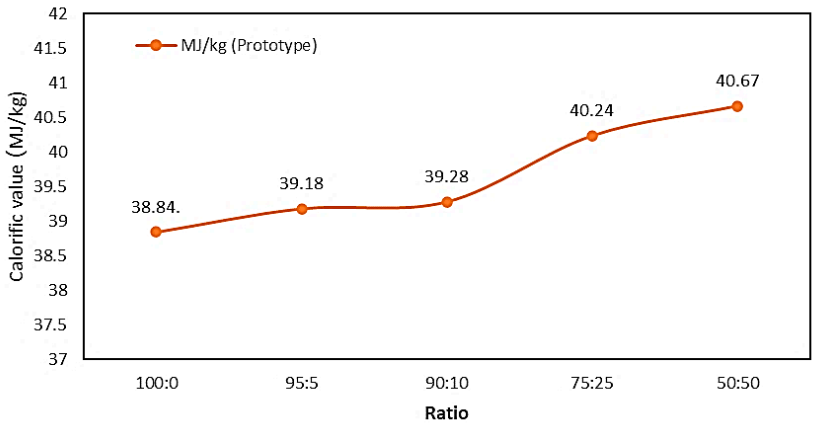

Figure 3. Effect of mixture ratio on the calorific value of RDF-5 fuel

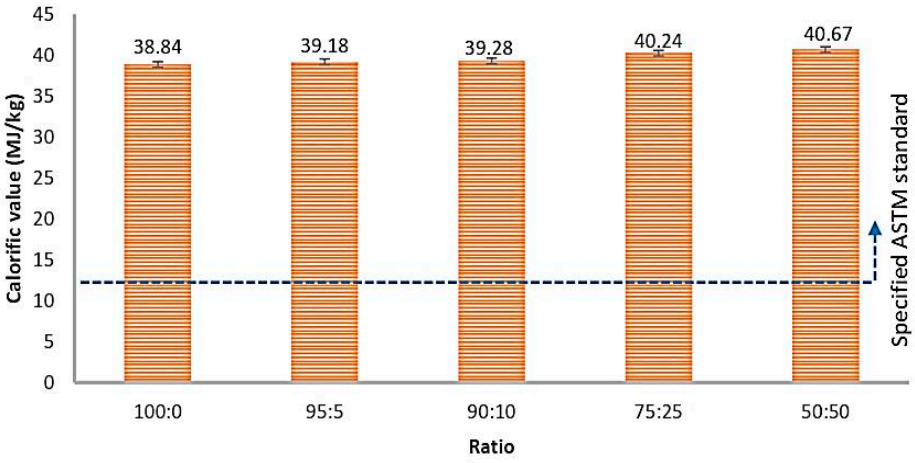

Figure 4. The calorific value of RDF-5 compared to ASTM standard calorific value

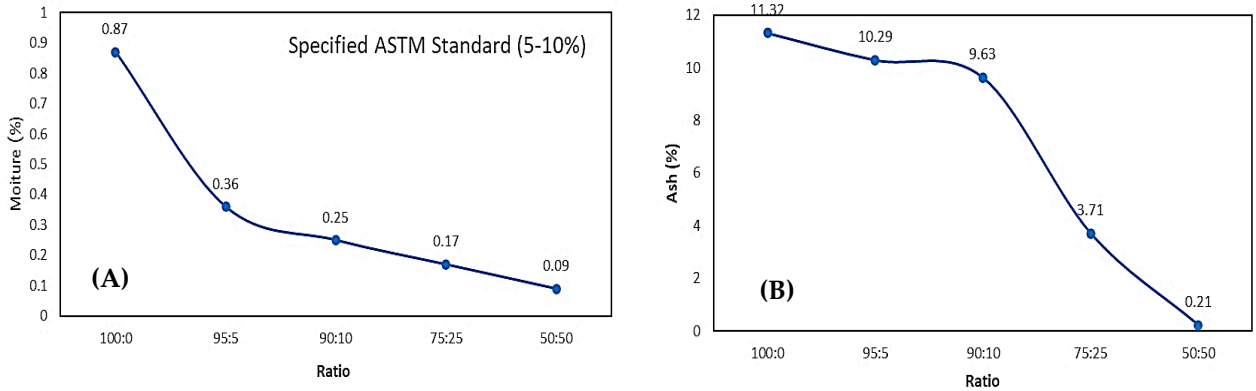

Figure 5. Effect of mixture ratio on (A) moisture and (B) ash of RDF-5 fuel

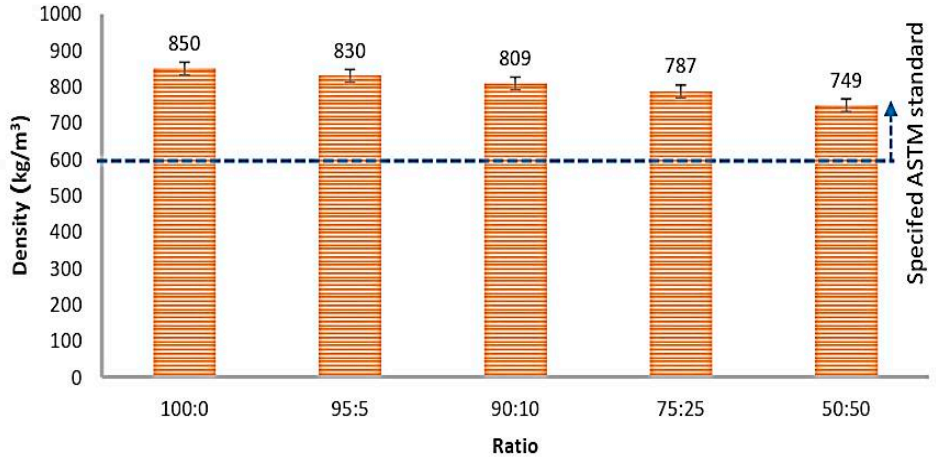

Figure 6. Effect of mixture ratio on the density of RDF-5 fuel

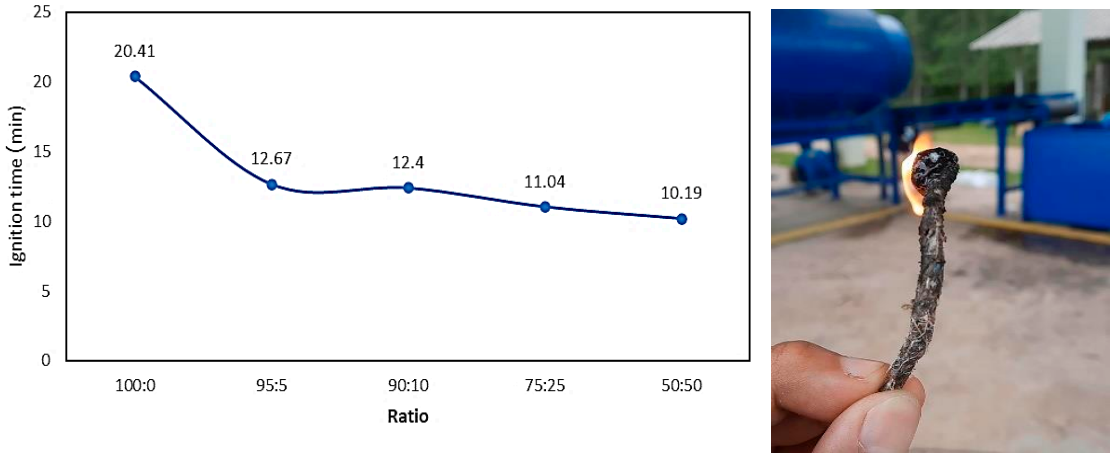

Figure 7. Effect of mixture ratio on ignition time of RDF-5 fuel

The experimental results in Table 3 show that all ratios can use as RDF-5 fuel. Making the combustion process more complete resulted in a faster or easier ignition time of the fuel and less ash content environmentally friendly, but adding the addition of natural rubber into the amount is too high and may affect the cost of production. As a result, the research team developed a 95:5 ratio as a commercially viable ratio because is a possibility of both production costs, combustion, and environmental performance. Prototype RDF-5 fuel production with rubber binder at 95:5 ratio, which could convert 800 kg./day of waste to produce 400 kg/day of fuel waste (RDF-5), dispose of 80 kg./day of organic waste, and lose water from the hot extrusion process by single screw extruder with temperature control of 170 ^{o}C, resulting in weight loss of 320 kg/day, with wastewater generated from the production process of 3.36 m^3/day. The material balance in the prototype system is shown in Figure 8.

Table 3. Summarize the fuel properties of RDF-5 from municipal waste with a rubber binder

properties	Ratio (%by weight)					Solid waste after the MBT process: crude oil sludge [5]
	100:0	**95:5**	**90:10**	**75:25**	**50:50**	**95:5**
Calorific value (MJ/kg)	38.84	39.18	39.28	40.24	40.67	38.13
Moiture (%)	0.87	0.36	0.25	0.17	0.09	1.00
Density (kg/m^3)	850	830	809	787	749	475
Ash (%)	11.32	10.29	9.63	3.71	0.21	6.26
Ignition (min)	20.41	12.67	12.40	11.04	10.19	-

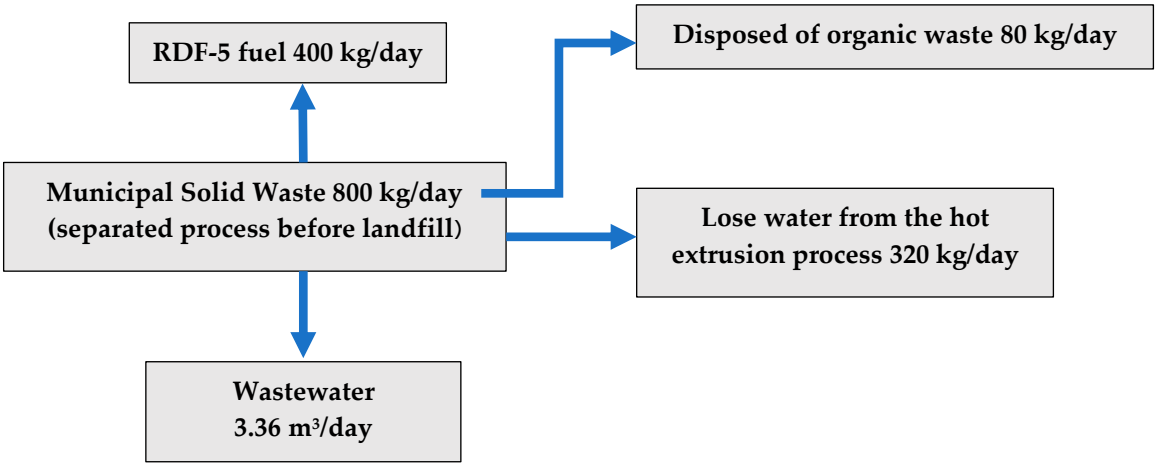

Figure 8. Material balance of prototype RDF-5 fuel production with rubber binder at 95:5 ratio

3.2 Emission effects from the RDF-5 combustion process

Emission analysis from the RDF-5 combustion process (95:5 is the optimal ratio) found that SO_2 was 1.05 ppm, CO_2 was 3.25 ppm, NO_x was 90.68 ppm, and particulate matter was 112.45 mg/m^3, all of which passed the standards of the Pollution Control Department and passed the emission standards of biomass power plants and is less than the emission from diesel combustion as shown in Table 4. In the combustion process of RDF-5, the amount of emission depends on the appropriate proportion of para rubber or the amount of para rubber used to mix. Especially carbon dioxide (CO_2) and sulfur dioxide (Sulfur Dioxide) should be little or none because the natural rubber used in the experiment does not use acids containing sulfur compounds in the production process. Additionally, the use of RDF-5 seems to reduce emission problems when comparing the amount of carbon generated with other fuels. Punin et al. [5] reported that RDF-5 has a beneficial effect on air emissions and residual ash compared to municipal waste incineration, which had SO_2 emission of about 0.16 ppm by the production of RDF-5 from solid waste after the MBT process: crude oil sludge of 95:5 ratio.

Table 4. Emission effects from the RDF-5 combustion process.

Emission	RDF-5 (95:5)	Diesel of diesel combustion [11]	Standard [12]	Standard of biomass power plants [13]
SO_2 (ppm)	1.05	-	< 30	No exceed 60
CO_2 (ppm)	3.25	4.28	-	-
NO_x (ppm)	90.68	104.25	< 250	No exceed 200
Particulate matter (mg/m^3)	112.45	-	< 400	No exceed 120

3.3 Production cost of RDF-5 fuel

Calculation of the RDF-5 production cost (95:5) compared with the price of natural rubber in the price range of 30 – 100 baht/kg found that the production cost of RDF-5 (95:5) from new waste (with waste cost) is 2,358 baht/ton (Rubber price 50 bath/kg for example), but without adding rubber, the charge is 2,133 baht/ton (Figure 9) and the production cost of RDF- 5 (95:5) from residual waste (no waste cost) at 1,935 baht/ton, without adding rubber, the charge is 1,685 baht/ton, as shown in Figure 10. The production cost of RDF-5 varies directly with the quantity of natural rubber used and rubber prices according to the economic conditions.

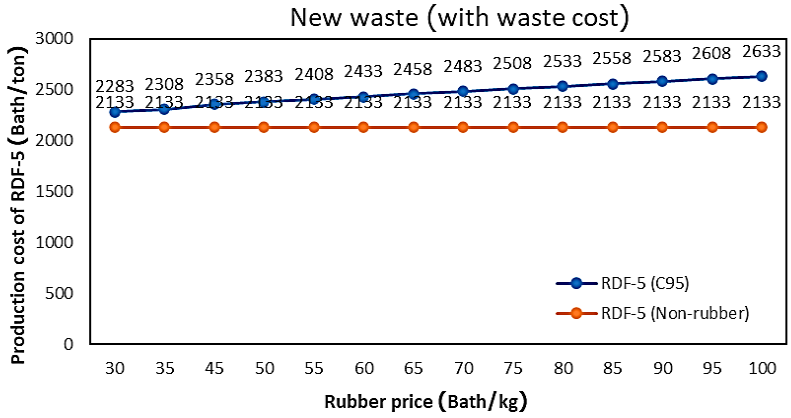

Figure 9. The production cost of RDF-5 at a 95:5 ratio from new waste (with waste cost)

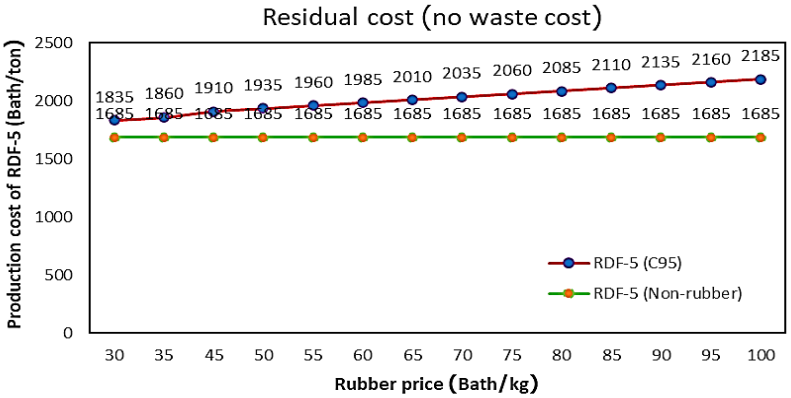

Figure 10. The production cost of RDF-5 at a 95:5 ratio from residual waste (no waste cost)

Table 5. Summary of the economic suitability analysis of RDF-5 fuel (95:5)

Issues of consideration	Units	RDF-5 (95:5)	Decision Criteria
1. Payback period (PBP)	years	5.72	PB ≤ 10 years
2. Net present value (NPV)	Bath	117,334.77	NPV ≥ 0
3. Internal rate of return (IRR)	%	12	> 10%

The results of the analysis of the economic suitability of RDF-5 fuel from municipal waste with natural rubber as a binder at the ratio of 95: 5 are shown in Table 5. The various assumptions used in economic calculations were time working (261 days/years), Total multiple solid wastes (50 tons/days), solid wastes used to produce RDF-5 (40 tons/days), amount of RDF-5 produced per day (20 tons/days). Comparative analysis results of the economic suitability of RDF-5 fuel found that the payback period is only 5.72 years with a positive net present value (NPV) and has an internal rate of return of 12%, which is 10% greater than the weighted average cost of capital required, as shown in Table 5. So, RDF-5 (95:5) is an alternative energy fuel in producing electricity or heat interesting, and it is economically worthwhile to invest.

4. Conclusions

Municipal solid wastes from the Integrated Waste Management Center, Kamphaeng Subdistrict, La-Ngu District, Satun Province, could be utilized to produce RDF-5 which an optimal ratio was 95:5 by using a natural rubber binder. The physicochemical properties of the optimal weight ratios (95:5 ratio) of RDF-5 found that the RDF-5 fuel passed the ASTM standards all property. The addition of natural rubber to RDF-5 fuel could significantly increase synergism. Emission analysis from the RDF-5 combustion process found that SO_2 was 1.05 ppm, CO_2 was 3.25 ppm, NO_x was 90.68 ppm, and particulate matter was 112.45 mg/m^3, all of which

passed the standards of the Pollution Control Department and passed the emission standards of biomass power plants and is less than the emission from diesel combustion. The economic suitability of RDF-5 fuel found that the payback period is only 5.72 years. Production of refuse-derived fuel (RDF-5) from municipal solid waste using natural rubber as a binder was the new product in the future due to fuel being environment-friendly and having high calorific value.

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Research article

Evaluation of Antibacterial, Antiinflammatory Activities and GC-MS Profiling of *Millingtonia hortensis* Linn. Leaf and Stem Bark Extracts

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Abstract: The present study aimed to evaluate the antibacterial and antiinflammatory activity of *Millingtonia hortensis* Linn. leaf and stem bark extracts and identify the bioactive compounds by GC-MS analysis. This study determined the antibacterial activities using the agar disc diffusion technique. The results revealed the ethanol extract of *M. hortensis* Linn. stem bark was the highest potential inhibitory against gram-positive bacteria *(Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus,* and *Bacillus cereus).* The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed. The highest inhibitory effect on *Enterococcus faecalis, Enterococcus faecium,* and *Bacillus cereus* with MIC and MBC values of 500 mg/ml. This investigation also indicated the *M. hortensis* Linn. leaf and stem bark extracts had no cytotoxicity against macrophage RAW264.7 cell line and strong antiinflammatory properties. The results of antiinflammatory activity showed that the ethanol extract of *M. hortensis* Linn. leaf and stem bark extracts were the most effective in inhibiting nitric oxide secretion with IC_{50} values of 0.25 and 0.41 mg/ml, respectively. GC-MS profiling analyzed the ethanol extract of *M. hortensis* Linn. stem bark, which had the highest antibacterial and antiinflammatory activity. There were three bioactive substances: 9,12-Octadecadienoic acid, ethyl ester, trans-13-Octadecenoic acid, and oleic acid. The natural functions were antiinflammatory, dermatitigenicity, antioxidants, and antifungal activities. Therefore, the results suggest that the ethanol extract of *M. hortensis* Linn. stem bark had the highest potential usefulness for treating gram-positive bacteria infection and inflammation-induced ailment. This research will be helpful toward the better acceptability of this extract in therapeutics.

Keywords: Antibacterial activity; antiinflammatory activity; GC-MS; bioactive substance

1. Introduction

The several properties of medicinal plants have been helpful in various categories of human ailments and conditions. Traditional herbal medicine is used worldwide for healthcare management [1]. The acceptance of herbal medicine is increasing because of its safety, no side effects, efficacy, and low

cost. Mainly, medicinal plants produce a diverse range of bioactive substances as a global source of therapeutic compounds to play a main role in several mechanisms of human health [2,3].

Millingtonia hortensis Linn. (Syn *Biognonia suberosa* Roxb.) is an important herb in South Asia, ranging from Thailand, India, Southern China, and Burma. It belongs to the family of Bignoniaceae [4]. It is a tall deciduous tree, a height of between 18 and 25 meters. It has corky stem bark, a straight trunk, and a few branches. In winter, the tree blooms with fragrant white flowers [5]. The leaf is large, dark green, has no odor and tastes slightly bitter. The stem bark is dark brown colored and has a characteristic odor. In traditional uses, flower buds are used in the treatment of asthma, sinusitis, and tonic. Its leaf is used for anticancer, antimicrobial, and anti-asthmatic activity. The stem bark also has great medicinal value as a treatment for throat ailments, coughs, and hangovers [6]. The phytochemical reports revealed some bioactive compounds from *M. hortensis* flowers, such as millingtonine, hispidulin, scutellarein, scutellarein-5-alactoside, and hortensin. The natural compounds from its leaf and stem bark were strong antimicrobial substances such as tannins, β-carotene, dinatin, and rutinosid [7]. In prior reports, the acetone extract of *M. hortensis* leaf had larvicidal activity against *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles stephensi*. The different of stem bark extracts showed anthelmintic activity against the adult earthworm *Pheretima posthuma* [8,9,10]. The methanol extract of *M. hortensis* leaf was more potent than fluconazole against *Candida krusei* and *Saccharomyces cerevisiae* [11]. The efficacy of acetone and methanol extract of *M. hortensis* stem bark were reported in their natural compounds as Dl-alpha-tocopherol, Vitamin E, Squalene, Bicyclo[4.1.0]heptane,7-pentyl-, Methyl 6,9-octadecadienoate could be the potential for the treatment of dapsone resistance of leprosy [12].

Inflammation is one of the physical responses of living tissues to toxins, damage, injuries with chemical stimuli, and contains infections. Inflammatory reactions result from the immune response against irritants and pathogens, and activated macrophages such as RAW 264.7 cells are involved in the process of inflammatory mechanism [13]. The inflammation is generally acute but can become chronic, leading to many diseases. The activated macrophages produce increased expression of inflammatory mediators such as nitric oxide (NO), prostaglandins 2 (PGE2), and tumor necrosis factor-alpha (TNF-α). Nitric oxide is a pro-inflammatory mediator that induces inflammation due to its overproduction in abnormal tissue responses. Therefore, NO inhibitors represent crucial therapeutic occurrences in managing inflammatory diseases because NO is involved in the pathogenesis of inflammatory disorders [13].

However, the herbal source comes into focus as natural components with effective antiinflammatory agents. In a prior report, the potency of the aqueous extract of *M. hortensis* stem bark revealed an antiinflammatory effect by carrageenan-induced paw edema on rats [14]. There is no information on the chemical components and antiinflammatory effects of *M. hortensis* leaf and stem bark extract. Therefore, the present investigation aimed to find scientific data for evaluating bioactive substances from *M. hortensis* leaf and stem bark extract that affected antibacterial and antiinflammatory activities.

2. Materials and methods

2.1 Plant materials and preparations

The leaf and stem bark of *M. hortensis* were collected at Chiang Mai and Lamphun Province, Thailand, in January 2022. The leaf and stem bark samples were washed thoroughly with distilled water and dried at 45- 50°C for two days to ensure they lost most of their moisture content. Then, the dried samples were powdered with a mean particle size ranging from 10 to 200 μm and stored at room temperature in an airtight dark container for further use.

2.2 Extraction of plant

Dry powders of *M. hortensis* leaf and stem bark (250 grams) were macerated with 1 liter of 95% ethanol for 3 days at room temperature or soaked with distilled water at 45°C in a water bath for 3 hours to maintain the crude extract from contamination. Then, the suspension of plant extracts was filtered through filter paper Whatman No.1 (Whatman, USA). A rotary evaporator concentrated the filtrate to remove the solvent and dried it with a lyophilizer to obtain crude extracts. Next, the dried extract was reconstituted by 10% dimethylsulfoxide (DMSO, Labscan, Ireland) using an aseptic condition for stock preparation of each crude extract as 500 mg/ml and stored in an amber glass bottle at 4°C before investigating further.

2.3 Antibacterial activites

Test organisms such as *Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii* and *Proteus mirabilis* used in the experiment were obtained from Department of biology, Faculty of Science and Technology, Chiang Mai Rajabhat University, Chiang Mai, Thailand. The antibacterial activities of the aqueous and ethanol extracts were evaluated by agar disc diffusion and dilution method. After reconstituting *M. hortensis* leaf and stem bark extracts with 10% dimethylsulfoxide (DMSO), the two-fold serial dilution of the extracts was used on the test organisms. Serial dilution of the extract was made using 5 concentrations (500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, and 31.25 mg/ml) of crude extract of *M. hortensis* leaf and stem bark were prepared using 10% DMSO. Subsequently, agar discs of 6 mm diameter were filled with 25 µl of clude extract and placed on top of a sterile Mueller Hinton agar plate surface. The plates were incubated at 37° for 24 hours. A disc containing the same volume of DMSO (10%) as the negative control, and Gentamicin (10 mg/ml) was used as the positive control. Three replicates were carried out for each extract against each test organism. The inhibition zone were measured to millimeters (mm). Data were expressed as mean±standard deviation.

The minimum inhibitory concentration (MIC) was determined for each test organism. The MIC of these extracts was performed by broth dilution method with the stocks concentration of 500 mg/ml of *M. hortensis* leaf and stem bark extracts, which were resuspended in 10% DMSO to produce two-fold serial dilutions using 5 concentrations (500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml) of crude extract of *M. hortensis* leaf and stem bark. The inoculum preparation was performed by broth culture method using Mueller Hinton broth. Each tested bacteria culture is adjusted with sterile Mueller Hinton broth to give a turbidity equivalent to the McFarland 0.5 standard. For the inoculation of tubes, a volume of bacterial suspension equal to the volume of diluted antimicrobial solution of *M. hortensis* leaf and stem bark extract, is added to each tube, were incubated at 35–37 °C for 24 hours. The lowest concentration of an antibacterial agent, under defined *in vitro* conditions, where no turbidity was observed by looking through the paper with black lines, was determined and noted as the MIC value. Finally, The MIC value of the clude extract was confirmed by testing the minimum bactericidal concentration (MBC) value using the spread plate technique. All samples were tested in triplicates.

2.4 Cell cytotoxicity testing (MTT assay)

Cytotoxicity of *M. hortensis* leaf and stem bark extract was investigated on macrophages RAW 264.7 cells. The extracts were serially two-fold diluted with Minimum Essential Medium (MEM) as serial dilution ranging from 0.078 to 10.000 mg/ml. Each extract concentration was added to quadruplet wells onto a 96-well tissue culture plate. After that, the clude extracts at different concentrations were added to each well, and incubation was carried out at 37°C for 48 hours. Then, 15 µl of MTT (5 mg/ml) was added to each well and incubated at 37°C in a 5% CO_2 incubator for 4 hours. Finally, DMSO was added to each well to form an MTT-formazan product. Determination of cell viability was performed using an ELISA reader by measuring the absorbance at 540 nm with reference wavelength at 630 nm.

2.5 Antiinflammatory activity

The macrophage cell line, RAW 264.7, was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) containing 4 mM L-glutamine, antibiotics (1% penicillin/streptomycin), and 10% fetal bovine serum at 37°C in 5% CO_2. RAW 264.7 cells (3×10^4 cells/well) were exposed to the indicated concentrations of extracts for 24 h. Then, an additional 24 hours of incubation with lipopolysaccharide (LPS) (1 ug/ml) was performed. After that, each culture supernatant was mixed with the Griess reagent to determine the nitric oxide (NO) production by RAW 264.7 cells. The mixture's optical density at 540 nm was determined using a spectrophotometer microplate.

2.6 GC-MS analysis

GC-MS was performed using a GC-TurboMatrix Headspace HS instrument. Gas chromatograph fitted with Initial temp 60°C for 5 min, ramp 7°C/min to 250°C, hold 5 min, InjBauto=250°C and volume 0 µL. An electron ionization system with an ionization energy of 70 eV was used for GC-MS detection. Helium was used as the carrier gas at a 1 ml/min flow rate. The injector and transfer line temperatures were set at 250 °C, respectively. The column temperature was initially kept at 10 °C for 1 minute and was then gradually

increased to 200 °C at a rate of 7 °C/min; finally, it was raised to 250 °C at a rate of 1 °C/min. The crude extract's relative amount of individual bioactive compounds is expressed as a percentage peak area close to the total peak area.

3. Results and discussion

This study evaluated the aqueous and ethanol extract of *M. hortensis* leaf and stem bark against antibacterial and antiinflammatory activities. Additionally, this research has elucidated bioactive substance's results in the effective *M. hortensis* extract by GC-MS profiling. These analyses revealed the presence of natural bioactive compounds related to various biological activities.

3.1 Crude extracts and antibacterial activities

This study depicted the occurrence of crude extracts against gram-positive tested bacteria. The agar disc diffusion susceptibility test of *M. hortensis* leaf and stem bark extracts evaluated an inhibitory effect on 9 tested bacteria strains. Their zone of inhibition against most of the bacterial microbes at 500 mg/ml concentrations was very effective (Table 1). There were 4 gram-positive bacteria (*Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, and *Bacillus cereus*) were found high inhibited by ethanol extract of *M. hortensis* stem bark (Table 1). In addition, The MIC and MBC of ethanol extract of *M. hortensis* stem bark were the highest value at 500 mg/ml against gram-positive bacteria such as *E. faecalis*, *E. faecium*, and *B. cereus* (Table 2). The gram-positive bacteria, *E. faecalis*, and *E. faecium*, causes urinary tract infection [15]. On the other hand, *B. cereus* causes crucial foodborne [16]. These results indicated the ethanol extract of *M. hortensis* stem bark had a broad spectrum of antibacterial activity. This result, correlated with essential oil extracted from *M. hortensis* flowers, showed an inhibitory effect on gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* [16]. In addition, the prior reports of polar extract as an aqueous alcohol fraction of *M. hortensis* leaf have broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria, yeasts such as *Candida albicans* and *Saccharomyces cerevisiae*, and an actinomycete strain *Nocardia* sp. [17].

Table 1. The inhibition zone diameters of *M. hortensis* leaf and stem bark extracts against tested bacteria

Bacteria	Gram	Diameter of inhibition zone (mm)				Gentamicin (10 mg/ml)
		Aqueous extract (500 mg/ml)		Ethanol extract (500 mg/ml)		
		Leaf	Stem bark	Leaf	Stem bark	
Enterococcus faecalis	+	nz	nz	nz	14.4 ± 0.8	19.5 ± 0.7
Enterococcus faecium	+	nz	nz	8.4 ± 0.7	11.5 ± 1.1	18.3 ± 1.0
Staphylococcus aureus	+	nz	7.2 ± 1.3	7.4 ± 0.5	10.7 ± 1.0	22.5 ± 0.8
Bacillus cereus	+	6.5 ± 1.0	7.3 ± 0.9	7.8 ± 0.8	11.8 ± 0.9	20.6 ± 0.9
Escherichia coli	-	nz	nz	nz	nz	23.1 ± 0.7
Pseudomonas aeruginosa	-	nz	nz	nz	8.8 ± 0.5	18.5 ± 1.2
Klebsiella pneumoniae	-	nz	nz	nz	nz	20.4 ± 0.6
Acinetobacter baumannii	-	nz	nz	nz	8.6 ± 1.2	18.6 ± 0.8
Proteus mirabilis	-	nz	nz	nz	nz	19.5 ± 1.0

Data are reported as Mean ± SD; nz = No inhibition zone

3.2 Cell cytotoxicity analysis

The cytotoxicity testing was widely used in *in vitro* toxicology studies. In the present study, the cytotoxic effect of ethanol extract of *M. hortensis* leaf and stem bark against macrophage RAW264.7 cell line were determined by MTT assay. The results elucidated that ethanol extracts of *M. hortensis* leaf and stem bark at concentrations of ≤0.625 mg/ml and aqueous extracts of *M. hortensis* and stem bark at concentrations of ≤5 mg/ml had cell viability of more than 80% (Table3). Then, *M. hortensis* extracts are poor cytotoxic agents against the macrophage RAW264.7 cell line. This research showed that the corresponding cytotoxic analysis, the

aqueous and ethanol extract of *M. hortensis*, were not cytotoxicity of the RKO colon cancer cell line, was assessed by MTT reduction assay [18].

In this research, a comparison of vehicle control, in which dimethyl sulfoxide (DMSO) was used as the solvent for the ethanol extracted, and DI (Deionized water) was used as the solvent for the aqueous extract. The results revealed that cell viability was 97.04% and 103.39%, respectively. Therefore, these extract concentrations were used to analyze the activity of inflammatory mediators through the secretion of nitric oxide by stimulating RAW264.7 cell cultures.

Table 2. The MIC and MBC (mg/ml) of *M. hortensis* leaf and stem bark extracts against tested bacteria

Bacteria	Concentration (mg/ml)							
	Aqueous extract				Ethanol extract			
	Leaf		Stem bark		Leaf		Stem bark	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Enterococcus faecalis	nd	nd	nd	nd	nd	nd	500	500
Enterococcus faecium	nd	nd	nd	nd	>500	nd	500	500
Staphylococcus aureus	nd	nd	>500	nd	>500	nd	>500	nd
Bacillus cereus	nd	nd	>500	nd	>500	nd	500	500
Pseudomonas aeruginosa	nd	nd	nd	nd	nd	nd	>500	nd
Acinetobacter baumannii	nd	nd	nd	nd	nd	nd	>500	nd

nd = Not detected

Table 3. The cytotoxicity analysis of *M. hortensis* leaf and stem bark extracts in macrophage RAW264.7 cell line by MTT assay

Tested concentration (mg/ml)	Cell viability (%)[1]			
	Aqueous extract		Ethanol extract	
	Leaf	Stem bark	Leaf	Stem bark
Cell Control (CC)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Vehicle control (VC)	103.39 ± 1.65	103.39 ± 1.65	97.04 ± 2.16	97.04 ± 2.16
0.078	88.34 ± 1.60	108.38 ± 1.48	104.64 ± 6.38	103.28 ± 9.82
0.156	94.74 ± 3.29	94.73 ± 3.46	106.11 ± 2.15	102.28 ± 9.48
0.312	96.82 ± 9.76	86.51 ± 6.63	109.38 ± 2.66	104.38 ± 7.68
0.625	92.90 ± 3.64	98.05 ± 9.43	107.50 ± 7.95	104.60 ± 7.09
1.250	108.74 ± 4.03	104.66 ± 1.94	68.15 ± 5.98	2.45 ± 0.12
2.500	104.40 ± 5.47	104.10 ± 7.08	4.90 ± 2.20	6.07 ± 1.32
5.000	106.21 ± 5.15	102.89 ± 6.97	10.71 ± 4.67	8.96 ± 1.66
10.000	78.80 ± 2.03	10.77 ± 4.71	18.95 ± 2.46	7.30 ± 1.68

1 = Analysis was performed in triplicated

3.3 Effect of *M. hortensis* leaf and stem bark crude extracts in LPS-stimulated RAW264.7 cells.

This study determined the effects of aqueous and ethanol extracts of *M. hortensis* leaf and stem bark on the production and secretion of inflammatory mediators. The secretion of nitric oxide from RAW264.7 cell culture was stimulated by lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 at a 1 μg/ml concentration. The results indicated that the ethanol extract of *M. hortensis* stem bark at a concentration of 0.625 mg/ml had the highest adequate nitric oxide secretion as 100 % inhibition (Table 4). The efficiency of nitric oxide inhibitory activity was decreased with the reduced concentration of extracts (Table 4). Interestingly, The ethanol extract of *M. hortensis* stem bark and leaf extracts were the most effective in inhibiting nitric oxide secretion, with IC_{50} values of 0.25 and 0.41 mg/ml, respectively. (table 5). The activated macrophage RAW264.7 cells can produce increased expression of inflammatory mediators such as nitric oxide (NO). Then, LPS-stimulation, RAW264.7 cells could generate NO production, which plays an important role in inflammatory response. Therefore, in this research, the ethanol extract of *M. hortensis* stem bark showed an antiinflammatory effect by attenuating NO generation.

Table 4. The effect of *M. hortensis* leaf and stem bark crude extracts on nitric oxide (NO) production in lipopolysaccharides (LPS)-stimulated RAW264.7 cell

Tested concentration (mg/ml)	Inhibition (%)[1] Aqueous extract		Tested concentration (mg/ml)	Inhibition (%)[1] Ethanol extract	
	Leaf	Stem bark		Leaf	Stem bark
0.312	3.63 ± 2.75	2.25 ± 2.52	0.039	10.28 ± 5.87	0.00
0.625	9.74 ± 2.30	15.14 ± 0.94	0.078	12.77 ± 4.61	0.00
1.250	22.53 ± 2.41	47.93 ± 2.88	0.156	24.81 ± 1.86	11.11 ± 5.21
2.500	55.74 ± 4.98	89.22 ± 2.86	0.312	35.40 ± 4.95	74.90 ± 1.90
5.000	94.90 ± 2.50	97.85 ± 0.84	0.625	74.490 ± 3.11	100.44 ± 0.89

1 = Analysis was performed in triplicated

Table 5. The IC_{50} value of *M. hortensis* leaf and stem bark crude extracts on nitric oxide (NO) production in lipopolysaccharides (LPS)-stimulated RAW264.7 cells

The crude extract		IC_{50} (mg/ml)
Leaf	Aqueous extract	2.58
	Ethanol extract	0.41
Stem bark	Aqueous extract	1.46
	Ethanol extract	0.25

3.4 Gas Chromatography-Mass Spectrophotometry Analysis (GC- MS analysis)

GC–MS is a rapid and accurate technique to identify the phytochemical constituents that could contribute to the medicinal quality of the plant. The natural compounds were confirmed based on the peak area, retention time, and molecular formula. In this present study, the GC-MS technique analyzed the highest antibacterial and antiinflammatory activity as the ethanol extract of *M. hortensis* stem bark. The chromatogram of this extract confirms the presence of various substances with different retention times (RT), as shown in 18 peaks in Figure 1. The GC-MS profiling of this effective extract revealed eighteen compounds (Table 6). The most separated peak was identified to be 9,12-Octadecadienoic acid, ethyl ester (12.356%) followed by trans-13-Octadecenoic acid (9.284%) and oleic acid (5.956%), respectively (Table 7).

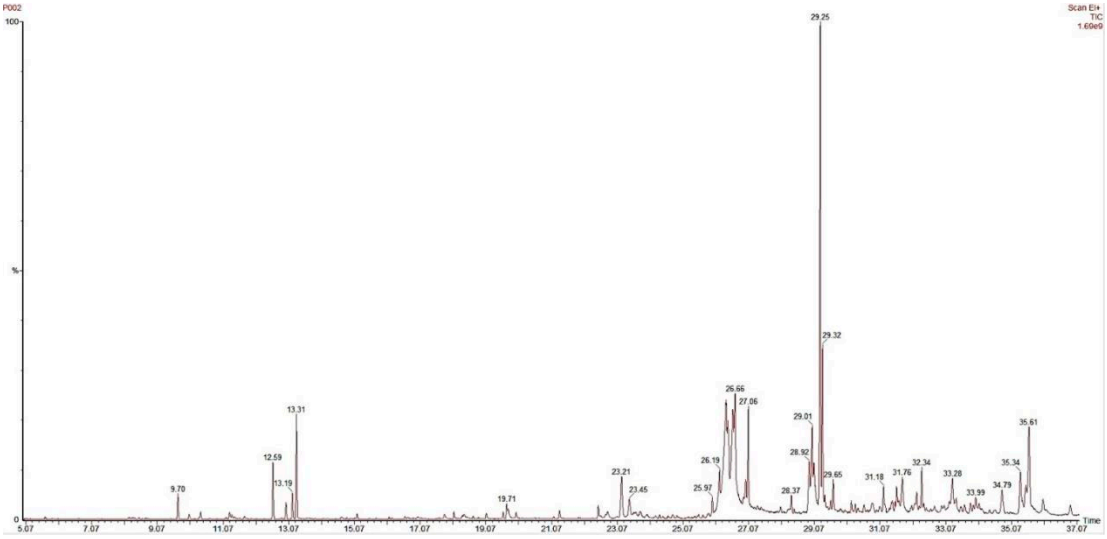

Figure 1. GC-MS chromatogram of the ethanol extract of *M. hortensis* stem bark

Table 6. The GC-MS profiling compounds identified in the ethanol extract of *M. hortensis* stem bark

Number	Retention time (RT)	Name of compound	Molecular formula	Peak Area (%)
1	9.701	Eucalyptol	$C_{10}H_{18}O$	0.699
2	12.592	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	$C_{10}H_{16}O$	1.519
3	13.187	endo-Borneol	$C_{10}H_{18}O$	0.701
4	13.307	Cyclohexanol, 1-methyl-4-(1-methylethyl)-	$C_{10}H_{20}O$	2.586
5	19.710	Linoleyl methyl ketone	$C_{75}H_{134}O$	0.483
6	23.207	9-Octadecene, 1,1-dimethoxy-, (Z)-	$C_{20}H_{40}O_2$	1.846
7	26.190	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	9.284
8	26.588	Oleic Acid	$C_{18}H^{34}O_2$	5.956
9	26.664	Isopropyl palmitate	$C_{19}H_{38}O_2$	4.810
10	27.064	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O$	2.473
11	28.374	Ethyl 14-methyl-hexadecanoate	$C_{19}H_{38}O$	0.457
12	28.920	19,19-Dimethyl-eicosa-8,11-dienoic acid	$C_{22}H_{40}O_2$	2.019
13	29.255	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	12.356
14	29.325	(E)-9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O$	3.469
15	29.650	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	0.787
16	32.336	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	$C_{18}H_{32}O$	1.215
17	33.992	5à-Pregn-16-en-20-one	$C_{21}H_{32}O$	0.582
18	35.608	6-Hydroxy-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-1-phenanthrenemethanol, (1à, 4aá, 10a.alpha)-	$C_{20}H_{28}O_2$	4.037

Table 7. The chemical structure of high components identified in the ethanol extract of *M. hortensis* stem bark by GC-MS analysis

Name of compound	Chemical groups	Retention time (RT)	Chemical structure	Biological activities
9,12-Octadecadienoic acid, ethyl ester	Fatty acid ester	29.255		antimicrobial, hypocholesterolemic, nematicide antiarthritic, hepatoprotective anti androgenic, hypocholesterolemic nematicide, 5-Alpha reductase inhibitor, antihistaminic, anticoronary Insectifuge, antieczemic and antiacne [19]
trans-13-Octadecenoic acid	Unsaturated fatty acids	26.190		antiinflammatory, dermatitigenic, anaemiagenic, insecticides,flavor [20]
Oleic acid	Fatty acid	26.588		antifungal, antiinflammatory, antioxidants [21]

4. Conclusions

Millingtonia hortensis Linn. is a practical traditional medicinal plant that is a rich source of compounds with antibacterial and antiinflammatory activity. This research has been carried out on their bioactive substance and properties from GC-MS profiling to identify the highest potential natural compounds of *M. hortensis* crude extract. The results of this present study evaluated that the ethanol extract of *M. hortensis* stem bark could be used as the highest potential antibacterial and antiinflammatory activity. Further analysis can be extended to assess their efficient phytochemical and pharmacological properties. These effective crude extracts could be examed *in vivo* to confirm their safety and efficacy.

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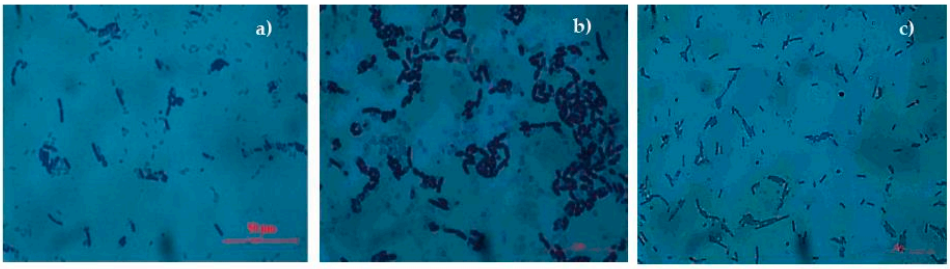

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4. Conclusions

In conclusion, this study reaffirms the hypothesis and highlights the key findings. The significant contributions to existing knowledge have been summarized, along with acknowledging the limitations of the research. The implications of the findings have been discussed, shedding light on potential future directions for further investigation. The conclusions are firmly grounded in the original research question and are

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