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EVALUATION BIOLOGICAL ACTIVITIES OF *Piper* Sp INTENDED FOR SKIN FORMULATION PRESERVATIVE

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Graphical abstract

Abstract

Preservatives are substances added into product to prevent their premature spoilage. Preservative helps to prolong the product's shelf life by protecting the product from microbial growth and oxidation process. In this study, the potential of a *Piper* sp. as preservative for cosmetic products is being investigated. The exploration begins with the screening of major phytochemical groups; flavonoid, steroid, alkaloid, saponin and triterpene. Evaluation of biological activities exhibited the plant possessed antimicrobial property towards two strains of *Staphylococcus aureus* and free radical scavenging capacity. The minimum inhibitory concentration values (MIC) for both *S. aureus* ATCC 25923 and *S. aureus* ATCC 6538 is 2500 µg/mL and 312.5 µg/mL respectively. Free radical scavenging capacity of the plant was determined *via* Superoxide scavenging assay and DPPH radical scavenging assay and the capacity values are 76.4±0.35% and 59.7±0.75% respectively. Cytotoxicity study was also carried out to determine the safety of *Piper* sp. where median inhibitory concentration (IC_{50}) value for normal kidney cell lines (Vero) is 62.96 µg/mL and normal liver cell lines (WRL-68) is 69.91µg/mL. The extract of *Piper* sp. was formulated as cosmetic preservative ingredient especially for topical product. The specification of ingredient's characteristic and performance were described based on colour, odour, pH value as well the solubility in product formulation and stability at 4°C, room temperature and 40°C.

Keywords: Plant based preservative, *Piper* sp.

Abstrak

Pengawet adalah bahan yang ditambahkan ke dalam produk untuk menghalang kerosakan pramatang. Pengawet membantu memanjangkan jangka hayat produk dengan memelihara produk daripada pencemaran mikorganisma dan proses pengoksidaan. Dalam kajian ini, potensi *Piper* sp. sebagai bahan pengawet produk kosmetik telah dikaji. Kajian dimulakan dengan penyaringan kumpulan fitokimia utama; flavonoid, steroid, alkaloid, saponin dan triterpena. Penilaian aktiviti biologi menunjukkan tumbuhan ini mempunyai ciri antimikrob terhadap dua strain *Staphylococcus aureus* dan mempunyai keupayaan memerangkap radikal bebas. Nilai kepekatan perencatan minimum (MIC) bagi *S. aureus* ATCC 25923 dan *S. aureus* ATCC 6538 masing-masing adalah 2500 µg/mL dan 312.5 µg/mL. Keupayaan memerangkap radikal bebas bagi tumbuhan ini ditentukan melalui cerakin 'Superoxide scavenging' dan 'DPPH radical scavenging' dan nilai kapasiti masing-masing adalah 76.4±0.35% dan 59.7±0.75%. Kajian sitotoksik turut dijalankan bagi menentukan tahap keselamatan *Piper* sp. di mana nilai kepekatan perencatan median (IC_{50}) bagi titisan sel buah pinggang normal (Vero) adalah 62.96 µg/mL dan titisan sel hati normal (WRL-68) adalah 69.91 µg/mL. Ekstrak *Piper* sp. telah diformulakan menjadi pengawet produk kosmetik terutamanya untuk kegunaan luaran. Pengspesifikasian bahan pengawet ini diterangkan berdasarkan warna, bau, nilai pH, keterlarutan dalam formulasi produk serta kestabilan pada 4°C, suhu bilik dan 40°C.

Kata kunci: Pengawet berasaskan ekstrak tumbuhan, *Piper* sp.

1.0 INTRODUCTION

Preservatives are chemical substances that are added into product to prevent microorganism growth, ensure the product's stability and shelf life [1]. Plant based preservative are more likeable by consumers [2]. They can be in form of essential oils [3] or organic extracts and besides having preservative properties, plant based preservative may exhibit a wide range of biological activities. Other advantages of plant based preservative are low mammalian toxicity and less environmental effects. Plant based preservative can be used in raw and processed food, pharmaceuticals, alternative medicine and natural therapies [4].

In discovering potentials in Malaysian medicinal plant, Natural Product Division, FRIM is committed by carrying phytochemical screening and bioactivity evaluation annually. Based on these works, a wild *Piper* sp. was discovered to exhibit significant antimicrobial and antioxidant properties. According to Mastura *et al.*, (2014), exploration and development of anti-microbial products, which includes preservative, involved three phases; research, development and commercialization [5]. Research stage involves bioprospecting initiatives in identifying suitable active ingredient. While, development stage is more on focusing on initiatives on determining the safety and efficacy of preservative. The present work focus on both research and development of plant based preservative specifically for cosmetic product.

2.0 EXPERIMENTAL

2.1 Plant Materials, Sample Preparation and Fractionation

Piper sp. was collected from Hutan Hulu Selangor. 212 g of air-dried leaves were soaked in ethanol at room temperature. After three days, the sample was filtered. Then the filtrate was concentrated using rotary evaporator and produce 12.3 g of ethanolic crude extract. The crude extract was subjected to solvent-solvent partitioning using petroleum ether and ethyl acetate.

Fresh samples were subjected for six main phytochemicals screening tests, namely, alkaloid, saponin, triterpene, steroid, flavonoid and tannin [6].

2.2 Detection of Alkaloid

Fresh sample (2 g) was cut into small pieces and mashed with 5 mL ammonical chloroform. The solvent was filtered and diluted with 10 drops of 2N sulphuric acid and shaken. The aqueous layer was tested with Meyer's reagent. Precipitate reaction occurred indicates alkaloid presence [6].

2.3 Detection of Saponin

Fresh sample (2 g) was cut into small pieces, ground in methanol and left to stand overnight. The next day, the mixture was filtered and the filtrate was evaporated to dryness. The extract was triturated with ether. Water was added to the ether insoluble material and was shaken vigorously using hand. The formation of froth indicated the presence of saponin constituent [6].

2.4 Detection of Triterpenes/Steroids

Fresh sample (2 g) were cut into small pieces, ground in ethanol and left overnight to stand after which the mixture was filtered. The filtrate was evaporated to dryness and triturated with ether. Then ether was evaporated and subjected with few drops of Liebermann-Bouchardt reagent (50% acetic acid anhydride-sulphuric acid, v/v). Formation of bright purple indicates presence of triterpenes. Formation of red or pink colour indicates the presence of steroids [6].

2.5 Detection of Flavonoid

Fresh sample (2 g) were cut into small pieces and ground with chloroform. After that, the extract was filtered and evaporated to dryness. The extract was dissolved with ether and shaken well with an equal amount of ammonia. Strong yellow colour in the ammonia layer indicated the presence of flavonoid [6].

2.6 Detection of Tannin

Fresh sample (1 g) was cut into small pieces, ground in methanol and left overnight to stand. The next day, the mixture was filtered and the filtrate was evaporated to dryness. The extract was then dissolved in methanol and 1% of ferric chloride was added. Blue black colour in the lower layer indicates the presence of hydrolysable tannins while brownish green colour indicates the condensed tannin [6].

All result for phytochemicals screening are shown in Table 1.

2.7 Antimicrobial Activity Evaluation

Stock solution of extract (10 µL) was pipetted to the well labeled as A, prior addition of 200 µL microbial inoculums. Inoculums (100 µL) was added into wells labeled as B until H. Inoculum and plant fractions in well A were mixed thoroughly and 100 µL of the mixture was transferred into well B to create serial dilution, the same procedure was repeated for inoculums mixture in well B until H. Then the microtiter plates were incubated at 30°C for 24 hours. Mixture

which shown turbidity indicates of growth thus the lowest concentration which remained clear after macroscopic evaluation will be taken as Minimum Inhibitory Concentration value (MIC). Results are shown in Table 2.

2.8 Antioxidant Activity Evaluation

2.8.1 Xanthine Oxidase Superoxide Scavenging Activity

Stock solutions of the test sample at a concentration of 50 mg ml^{-1} were prepared by dissolving the extract in ethanol. The reaction mixture was prepared by dissolving sodium carbonate (Na_2CO_3) (pH 10.2) (0.53 g), ethylene diaminetetraacetic acid (EDTA) (4.0 mg) and xanthine (2.0 mg) in 0.025 mM 4-nitro blue tetrazolium chloride (NBT). NBT solution (100 mL of 4.1 mM/L) was prepared by adding trizma hydrochloride (TrisHCl) (3.15 g), magnesium chloride ($MgCl_2$) (0.1 g), 5-bromo-4-chloro-3-indolyl phosphate (15.0 mg) and 4-nitro blue tetrazolium chloride (34.0 mg) to distilled water (100 mL). The mixture was kept refrigerated at 4°C.

The stock solution (5 µL) was mixed with reaction mixture (995 µL) in a microcuvette. The microcuvette was placed in the cell holder of a spectrophotometer and the reading was set zero. The reaction was then initiated by the addition of xanthine oxide (XOD) (1×10^{-3}U/mL) 0.1 µL). The absorbance of the resulting mixture was measured at 560 nm for 120 s. The absorbance of the negative control was obtained by replacing the stock solution with the reaction mixture (5 µL). Superoxide dimutase was used as a positive control in this assay. All experiments were performed in triplicates. The percentage of inhibition was calculated.

2.8.2 DPPH Radical Scavenging Assay

This assay system evaluates the effect of plants fractions on 1,2-diphenyl-2-picrylhydrazyl (DPPH) radical) of the plant fractions(4 mL each) was added to 1 mL of 1mM DPPH. The mixture was shaken and left a room temperature for 10 minutes. The absorbance of resulting solution was measured spectrophotometrically at 520nm [7].

2.9 Cytotoxicity study

Normal cell lines were used in this study, WRL-68 (human liver) and Vero (African green monkey kidney). Both cell lines were obtained from ATCC. They were cultured in Dulbecco's Modified Eagle Medium (Sigma, USA) and supplemented with 5% foetal bovine serum (PAA Laboratories), 1% amphotericin B and penicillin-streptomycin (PAA Laboratories), and 0.5% gentamycin (PAA Laboratories). Cells were maintained in a humidified incubator containing 5% CO_2/ in 96-well plate to yield 10,000 cell/well. After 24 h of recovery period, cells were exposed to the plant fractions at concentrations ranging from 0.1 to 1000 µg/mL for 72 h. Paclitaxel was used for comparison and each treatment was conducted in triplicates. After 72 h of incubation, the toxic endpoints were determined using sulphorhadamine B (SRB) assay [8] with slight modification [9]. Results were expressed as percentage of cell survival and the median inhibition concentration (IC_{50}) was determined from the corresponding dose response curves.

2.10 Formulation Study

Formulation of plant based preservative was developed by determining the solubility of the extract and fractions in several solvents; deionised water, propylene glycol, isopropyl myristate and isostearyl isostearate. The solubility was tested by dissolving solute (1 g) in 1-1,000 mL solvents. The concentration was decreased accordingly until the extract/fraction was fully dissolved. The highest concentration was recorded. Characteristic of plant based preservative's solubility was described based on BP 2011[10] classification.

The formulated plant based preservative was tested for i) solubility in alcohol, water, mineral and vegetables oils, ii) stability in three different conditions (room temperature, 4 and 40°C) and iii) skin irritation test; using *in vitro* skin model EpiDerm™. As for skin irritation test, plant based preservative was send for service at SIRIM Berhad. The pH value, colour, and odour of the formulation were also observed and recorded.

3.0 RESULTS AND DISCUSSION

Phytochemical groups of *Piper* sp. leaves were detected as in Table 1 and they were flavonoid, steroid and triterpene. Based on chemical fingerprinting profiles of this species by fourier transform infrared spectroscopy (FTIR) with two dimensional correlation IR spectroscopy (2D-IR) and high performance liquid chromatography (HPLC), several compounds were detected [11]. These compounds were belonged to triterpene group and was used as markers in producing standardized extract [11].

Table 1 Phytochemical screening *Piper* sp. leaves

Phytochemical properties					
Flavonoid	Tannin	Alkaloid	Steroid	Saponin	Triterpene
+2	-ve	-ve	+3	-ve	+1

Note: -ve: not present, +1: low, +2: moderate, +3: high

Antimicrobial property towards two strains of *Staphylococcus aureus* exhibited only in petroleum ether fraction (Table 2). Our result exhibited that the MIC value (312.5 µg/mL) of petroleum ether fraction against *S. aureus* ATCC 6538 is comparable to the MIC value (310 µg/mL) of standard antibiotic compound,

oxacillin against *S. aureus* ATCC 33591[12]. The positive results on both strains indicate
that this plant fraction has potential to be developed as preservative for cosmetic product as *S. aureus* was a harmful microorganism that causes skin infections, bacteremia as well as pneumonia and empyema [12].

Table 2 Inhibitory potential expressed as MIC value (μg/mL) of extracts and fractions of *Piper* sp.

	MIC & MBC (μg/mL)							
	***S.aureus* ATCC 25923**		***S.aureus* ATCC 6538**		***E.coli* ATCC 8739**		***C.albicans* ATCC 10231**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ethyl acetate fraction	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
Petroleum ether fraction	2500	2500	312.5	312.5	>5000	>5000	>5000	>5000
Ethanol extract	NT	NT	>5000	>5000	>5000	>5000	>5000	>5000

Note: weak: MIC≥5000 μg/mLl, moderate; 1000μg/mLl<MIC<4900g/mLl, strong; MIC≤1000μg/mL

As described in Table 3, antioxidant properties of the plant were evaluated by radical scavenging capacities *via* Xanthine oxidase superoxide scavenging assay and DPPH radical scavenging assay. All fractions posed moderate to high activity.

Cytotoxicity study was also carried out to determine the safety of *Piper* sp. Median inhibitory concentration (IC_{50}) value for normal kidney cell lines (Vero) was 62.96 μg/mL and normal liver cell lines (WRL-68) was 69.91 μg/mL. According to Elmore *et al.*, 2002, substance that does not inhibit growth more than 50% at concentration of 1000 μg/mL can be considered nontoxic with the system tested [13].

Table 3 Antioxidant and cytotoxicity activities of extract and fractions of *Piper* sp. was expressed as percentage (%) and IC_{50}, respectively

Plant extract/fraction	**Antioxidant activity (%)**		**Cytotoxicity** (IC_{50})	
	Xanthine oxidase superoxide scavenging activity	**DPPH radical scavenging activity**	**Normal kidney cell lines (Vero)**	**Normal liver cell lines (WRL-68)**
Ethyl acetate fraction	93.4±0.10	95.3±0.75	NT	NT
Petroleum ether fraction	76.4±0.35	59.7±0.75	NT	NT
Ethanol extract	93.70±0.57	75.79± 3.8	62.96 μg/mL	69.91 μg/mL
Paclitaxel (positive control)	-	-	0.03±0.02 μg/mL	0.02±0.01 μg/mL

Note: NT: not tested, low: 0-49%, moderate: 50-69%, high: 70-100%

All fractions and extract of *Piper* sp. were subjected to preservative formulation process despite their biological activity. The formulation of preservative was carried out by dissolving the fractions and extract of *Piper* sp. in typical cosmetic solvents in formulation process. Based on solubility characteristic described by BP 2011, petroleum ether fraction was found freely soluble in isopropyl myristate. Description of freely soluble was approximate 1-10 mL of isopropyl myristate can dissolve 1 g of solute. While, 1 g of ethanolic extract was sparingly soluble at approximate 30 -100 mL of propylene glycol.

Slightly soluble characteristic for ethyl acetate fraction in isopropyl myristate and isostearyl isostearate as well as petroleum ether fraction in isostearyl isostearate, respectively, means 1 g solute dissolve in 100 -1000 mL of solvent.

Description of very slightly soluble was 1 g of solute dissolve in 1000 -10,000 mL of solvent. As a result, formulation of petroleum ether fraction in isopropyl myristate was chosen as plant based preservative ingredient. The results are shown in Table 4.

The formulation of plant based preservative from *Piper* sp. was found stable for three months at room temperature, 4°C and 40°C, without any separation and no layered formation. This indicates that the preservative's shelf life can achieve two (2) years. pH value of formulated plant based preservative was in

range 5.0-6.5, the suitable pH value for skin. The formulated preservative has spicy odour and the observed colour was dark green.

Table 4 Solubility of *Piper* sp. extract and fractions in selected solvent for formulating plant based preservative

Plant fraction	Solubility in solvent			
	Deionised water	**Propylene glycol**	**Isopropyl myristate**	**Isostearyl isostearate**
Ethyl acetate fraction	Very slightly soluble	Very slightly soluble	Slightly soluble	Slightly soluble
Petroleum ether fraction	Very slightly soluble	Very slightly soluble	Freely Soluble	Slightly soluble
Ethanol extract	Very slightly soluble	Sparingly soluble	Very slightly soluble	Very slightly soluble

Solubility of formulated preservative in selected solvents that are normally used in cosmetic formulation was described as in Table 5. Based on the results, this plant based preservative was easily soluble in oil based cosmetic formulation like cream and lotion. However, in water based cosmetic formulation required addition of emulsifier.

Table 5 Solubility of plant based preservative in selected solvents

	Solubility in solvent			
	Deionised water	**Vegetables oils**	**Minerals oils**	**Alcohols**
Plant based preservative	Slightly soluble	Freely Soluble	Freely Soluble	Freely Soluble

Although the extract was considered as moderately cytotoxic, the formulated plant based preservative was tested for skin irritation to ensure the safety of the preservative to be used topically. Based on results in Table 6, plant based preservative was considered as non-irritant on human skin.

Work from this study has all significant elements in developing a cosmetic preservative, which are antimicrobial efficacy, non-toxic, non-irritant and compatibility with final product [14]. Nevertheless, further work on the efficacy of the preservative and product formulated with this preservative must be done.

Table 6 *In vitro* skin irritation test and the *in vivo* prediction of plant based preservative

	Mean of viability (%)	***In vitro* result**	***In vivo* prediction**
Plant based preservative	101.6±5.30	Mean tissue viability > 50%	Non-irritant
Negative control	100.0±2.07	Mean tissue viability > 50%	Non-irritant
Positive control (5% SDS Solution)	12.6 ±1.39	Mean tissue viability ≤ 50%	Irritant

4.0 CONCLUSION

As conclusion, result from this study showed that *Piper* sp. has potential to be developed into plant based preservative based on their biological properties, formulation study and safety evaluation. Although the results are preliminary, it can be used as foundation for further work. Concurrently, study on the efficacy of the plant based preservative has been carrying out. Nevertheless, in order to be able to penetrate market, public should be educated with the benefit of using the plant based preservative despite having its own natural colour and odour that maybe not favored by consumer.

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