Phytochemical Screening, Total Phenolic and Flavonoid Contents, Antioxidant, Tyrosinase Inhibitory, and HaCaT Cell Cytotoxic Activities of Leaf Extracts from *Bouea oppositifolia* (Roxb.) Meisn.

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Abstract

Natural medicines from plants have proved to display effective activity, especially through phytochemicals that can prevent or protect against various chronic diseases caused by free radicals. *Bouea oppositifolia* (Roxb.) Meisn., commonly known as Marian plum or Ma-Yong-Chid in Thai, is a perennial tree belonging to the family Anacardiaceae. It is closely related to plum mango that has been reported to possess significant biological activities, notably, with strong antioxidant activity in its leaf extract. In this study, the researchers aimed to investigate the phytochemical screening, total phenolic, and flavonoid contents, as well as the antioxidant, tyrosinase inhibitory, and HaCaT cytotoxic activities of leaf extracts from *B. oppositifolia*. Successive extraction of the dried leaf powder was carried out using three different solvents (hexane, ethyl acetate, and methanol) via the maceration technique. The preliminary phytochemical screening of *B. oppositifolia* extracts revealed the presence of phenolics, saponins, flavonoids, phytosterols, glycosides, anthraquinones, diterpenes, triterpenoids, and tannins. Among the three extracts (hexane, ethyl acetate, and methanol), the methanol extract had the highest total phenolic and flavonoid contents (74.25±3.31 mg GAE/g, dried leaf and 71.48±1.94 mg QE/g, dried leaf, respectively.), along with the strongest antioxidant capacity with IC_{50} values of 1.39±0.40 µg/mL and 21.97±0.31 µg/mL for DPPH and ABTS assays, respectively, and a FRAP value of 505.55±24.32 mg $FeSO_4$/g, of dried leaf. On the other hand, the ethyl acetate extract exhibited the strongest tyrosinase inhibitory activity, with an IC_{50} value of 242.90±7.48 µg/mL, and showed the lowest cytotoxicity toward HaCaT cells (%cell viability of 59.04±0.15%, at the concentration of 1,000 µg/mL). This study suggests that leaf extracts from *B. oppositifolia* are abundant in potential antioxidant and biological activities and could be further applied in pharmaceutical and cosmetic industries.

Keywords: *Bouea oppositifolia* (Roxb.) Meisn., Antioxidant activity, Tyrosinase inhibitory, Cytotoxicity

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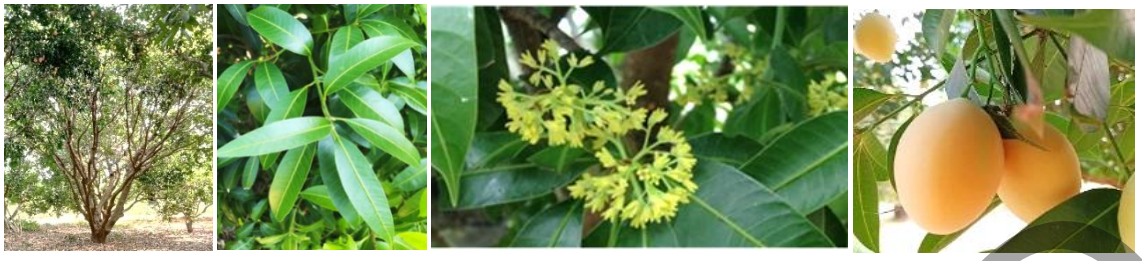
Introduction

Plants play an important role in life and act as natural resources. They function as indicators of biological abundance, provide essential habitats within ecosystems, and constitute a source of natural compounds with a long history of use in traditional medicine (Phong et al., 2022). Notably, phytochemicals are secondary metabolite compounds produced in many parts of plants including roots, leaves, twigs, flowers, fruits, seeds, and stems to protect themselves from insects and natural enemies. Phytochemicals show health benefits in humans (Yoo et al., 2018). Several studies have revealed that the consumption of phytochemicals can decrease the risk of some chronic diseases, such as cardiovascular disease, brain and memory disorders, rheumatism, dermatitis, and cancer (Kumar et al., 2023; Moghadamtousi et al., 2013). Many chronic diseases are caused by free radicals of oxidation reaction, especially reactive oxygen species (ROS), which exist in the environment. For example, ultraviolet radiation, smoking, X-ray radiation, high-fat food, and heavy metals can cause free radicals to occur in the body (Phaniendra et al., 2015). Free radicals are molecules or atoms with unpaired electrons in the outer orbitals of electron configuration that are unstable. Due to the instability of electron configuration, free radicals can rapidly react to biomolecules, such as lipid, protein, DNA, and tissue, leading to cell damage and initiating various diseases (Sisein, 2014). The best choice for preventing chronic diseases caused by free radicals is consumption of antioxidants that can normally be found in nature. Numerous studies have revealed that

phytochemicals, including phenolics, flavonoids, carotenoids, alkaloids, anthraquinones, terpenoids, and tannins have been recognized as having strong antioxidant activity and have the ability to potentially decrease the risk of several diseases (Forni et al., 2019).

Skin is an important organ of the largest size, which covers the body. The roles of skin are prevention and protection from dirt and various germs, as well as regulating the body's temperature to maintain balance (Zagórska-Dziok et al., 2020). If skin has an abnormality, it will affect many systems of daily life (Evers et al., 2008). The abnormality frequently found are uneven skin tone, blemishes, freckles, and dark spots. The causes of these problems come from tyrosinase (Jin et al., 2024). Normally, tyrosinase is an enzyme that is produced to protect skin from long-term exposure to ultraviolet rays and it can be found in various parts of mammals, such as hair, eyes, and skin (Onar et al., 2012). However, if humans have higher free radicals and imbalance in body, it will lead to over-melanogenesis and accumulation of excessive melanin, and the dark spots around the skin will occur (Tada et al., 2014). Tyrosinase is a copper protein enzyme species relating to the oxidation reaction of the amino acid named L-tyrosine, which contains copper (II) ions at the active site (Akyilmaz et al., 2010). The copper (II) ions will cooperate with molecules of L-tyrosine and decompose the monophenol groups of L-tyrosine into *O*-diphenols and will be oxidized into the final product of *O*-dopaquinone (Lai et al., 2018). These processes will be synthesized in melanosome, which is a part of the pigmentation process. If any substances can inhibit this oxidation reaction or compete with copper atoms, it will terminate the over-melanogenesis process (Song et al., 2022). The substances that have been known as strong enzyme inhibitors from oxidative stress and safety are kojic acid, arbutin, coumarin, and other polyphenol compounds (Lee et al., 2016).

Bouea oppositifolia (Roxb.) Meisn. (Homotypic synonym: *Bouea burmanica* Griff.), also known as Marian plum or Ma-Yong-Chid in Thai, is a perennial tree belonging to the Anacardiaceae family closely to *Mangifera indica* (mango), *Spondias mombin* (olive), and *Bouea macrophylla* Griffth (plum mango). It is a native plant in Asia that can be found in tropical and humid landscapes in both South Asian and Southeast Asian countries, such as India, Sri Lanka, Malaysia, Indonesia, and especially Thailand. It is an economic plant of Thailand that can generate great income for farmers. *B. oppositifolia* is similar to the plum mango, it is a dicotyledonous plant, 8–15 m high (Mavuso & Yapwattanaphun, 2017). The fruit is bigger than that of plum mango, unripe fruit is green and turns to yellow-orange when ripe. Ripe fruit has a sweet-sour flavor, which can vary depending on the cultivar. However, *B. oppositifolia* can be produced only once a year, and generates income for farmers on an annual basis. Moreover, there is limited research on the biological activities of various parts of *B. oppositifolia*. Seekhaw et al. (2022) evaluated the use of the *B. oppositifolia* leaf for aqueous fabric dye and assessed its acute toxicity, skin irritation, and corrosion potential using a rabbit model. The results indicated that the dye was non-toxic to rabbit skin under all tested conditions. The phytoconstituents of aqueous fabric dye, such as phenolics, anthraquinones, diterpenes, and triterpenes were found. Recently, phytochemical screening of the extracts from *B. oppositifolia* twigs revealed the presence of phenolics, flavonoids, steroids, anthraquinones, diterpenes, triterpenes, and tannins. The methanol extract exhibited strong antioxidant activity, with IC_{50} values of 1.14±0.16 µg/mL and 47.86±1.39 µg/mL for DPPH and ABTS assays, respectively. Additionally, the ferric reducing antioxidant power (FRAP) assay yielded a value of 227.87±3.85 mg $FeSO_4$ equivalent/g dry weight (Lamkhwan & Seekhaw, 2025). Several studies have reported on the biological activities of plants which belong to the Anacardiaceae family, especially plum mango. For instance, the leaf extracts of plum mango have shown significant biological activities, including strong antioxidant properties, antibacterial effects against both Gram-positive and Gram-negative bacteria, and anticancer activity. The leaf extract was found to exhibit inhibitory effects on HeLa and HCT116 cancer cell lines, with IC_{50} values of 24±0.8 and 28±0.9 µg/mL, respectively. Several bioactive compounds, potentially responsible for the biological activities of the extract, including polyphenols, flavonoids, caryophyllene, phytol, and trans-geranylgeraniol, were identified (Nguyen et al., 2020). This research aimed to investigate preliminary phytochemical screening, total phenolic and flavonoid contents, to assess the antioxidant and tyrosinase inhibitory activities, as well as HaCaT cell cytotoxicity of *B. oppositifolia* leaf extracts. These findings may contribute to the potential development of pharmaceutical products.



(A) (B) (C) (D)

Fig. 1 Botanical characteristics of *Bouea oppositifolia* (Roxb.) Meisn. (A) Tree; (B) Leaf; (C) Inflorescence; (D) Fruit

Materials and methods

1. Chemical reagents

All chemicals used in this study were of analytical grade. Aluminium chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), sodium carbonate (Na_2CO_3), ferric chloride ($FeCl_3$), potassium persulfate ($K_2S_2O_8$), ferrous sulfate ($FeSO_4$), L-ascorbic acid (99.5%), and Folin-Ciocalteu's phenol reagent were purchased from Loba Chemie, India. Sodium acetate (CH_3COONa) and hydrochloric acid (HCl) were obtained from RCI Labscan, Thailand. Additionally, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), tyrosinase enzyme, gallic acid (98%), and quercetin (95%) were purchased from Sigma-Aldrich, USA. All reagents were used as received, without further purification.

2. Plant collection

Bouea oppositifolia (Roxb.) Meisn. fresh mature leaves were collected in October 2024 from the area of Krainok subdistrict, Kongkrailas district, Sukhothai province, Thailand. Reference samples were deposited in the plant specimen collection room at the Faculty of Science and Technology Pibulsongkram Rajabhat University (Voucher specimen No. PSRU 1010).

3. Preparation of plant extracts

B. oppositifolia leaves were washed with tap water to remove dust, and then cut into small pieces. Thereafter, the leaves were dried in a hot air oven at 50°C for 12 h and ground into powder. The powdered leaf material (500 g) was extracted successively using 1,500 mL of different organic solvents with varying polarity (hexane, ethyl acetate, and methanol) through a maceration technique for 7 d, with 2 extraction cycles for each solvent. After extraction, the residue was filtered out, and each extract was subjected to removal of the solvent using a rotary evaporator (Buchi Rotavapor® R-300, Buchi Labortechnik, Switzerland) at 45°C to obtain the *B. oppositifolia* leaf extracts. The extracts were stored in light-protected bottles at 4°C for further tests.

4. Preliminary phytochemical screening of *B. oppositifolia* leaf extracts

The leaf extracts from *B. oppositifolia* were determined for the presence of phytoconstituents, including phenolics, saponins, flavonoids, phytosterols, alkaloids, glycosides, anthraquinones, diterpenes, triterpenoids, and tannins using the previous methods described by De Silva et al. (2017); Shaikh and Patil (2020).

4.1 Detection of phenolics

5 mg of the extracts were dissolved in 1 mL of 95% ethanol. Subsequently, 3–4 drops of 5% w/v ferric chloride solution were added. The presence of phenolics is indicated by a color change to blue or black.

4.2 Detection of saponins

5 mg of the extracts were dissolved in 1 mL of 95% ethanol. 2 mL of purified water were added then the mixture was shaken. The formation of foam indicates the presence of saponins.

4.3 Detection of flavonoids

200 mg of the extracts were dissolved in 5 mL of 50% ethanol. Subsequently, 2–3 fragments of magnesium ribbon were added, followed by 5–6 drops of concentrated hydrochloric acid. The presence of flavonoids is indicated by a color change to a pink to crimson colored solution.

4.4 Detection of phytosterols

1 mg of the extract was dissolved in 1 mL of chloroform. 1 mL of concentrated sulfuric acid was carefully added down the side of the test tube. The presence of phytosterols is indicated by a color change to red in the bottom layer.

4.5 Detection of alkaloids

5 mg of the extracts were dissolved in 1 mL of 95% ethanol. 2–3 drops of Wagner's reagent were added to the solution. The formation of a brown precipitate indicates the presence of alkaloids.

4.6 Detection of glycosides

500 mg of the extracts were dissolved in 5 mL of purified water. 2 mL of concentrated acetic acid were added to the solution, followed by 2–3 drops of 5% w/v ferric chloride solution. Subsequently, 1 mL of concentrated sulfuric acid was carefully added down the side of the test tube. The formation of a brown ring or a purple ring indicates the presence of glycosides.

4.7 Detection of anthraquinones

100 mg of the extracts were dissolved in 5 mL of chloroform, followed by filtration. Then, 2 mL of 10% v/v ammonia solution was added and mixed well. The presence of anthraquinones is indicated by a color change to pink or orange in the upper layer.

4.8 Detection of diterpenes

100 mg of the extracts were dissolved in 5 mL of purified water, followed by heating for 10 min and filtration. Then, 2–3 drops of 10% w/v copper acetate solution were added. The presence of diterpenes is indicated by a color change to emerald green.

4.9 Detection of triterpenoids

100 mg of the extracts were dissolved in 5 mL of chloroform, mixed well and then filtered. Subsequently, 2 mL of concentrated sulfuric acid was added, and the solution was shaken for 2–3 min. The presence of triterpenoids is indicated by a color change to red-brown in the bottom layer.

4.10 Detection of tannins

100 mg of the extracts were dissolved in 5 mL of purified water. 2–3 drops of 1% w/v gelatin solution were added to the solution. The presence of tannins is indicated by the formation of white precipitate.

5. Evaluation of total phenolic content of *B. oppositifolia* leaf extracts

The total phenolic content of each extract was assessed by using Folin-Ciocalteu reagent following the method described by Majhenič et al. (2007). Briefly, each extract was prepared at a concentration of 0.1 mg/mL in methanol. A 12.5 µL aliquot of the extract solution was added to a 96-well plate, followed by 12.5 µL of distilled water and 12.5 µL of Folin-Ciocalteu reagent. The mixture was allowed to stand for 6 min at room temperature before 100 µL of distilled water and 125 µL of 7.5% (w/v) Na_2CO_3 were added. The reaction was incubated at room temperature in the dark for 90 min. The absorbance was then measured at 760 nm using a microplate reader (SPECTROstar Nano, BMG Labtech, Germany). Gallic acid was used as the reference standard, and the results were expressed as mg of gallic acid equivalents (GAE)/g of dried leaf (mg GAE/g, dried leaf).

6. Evaluation of total flavonoid content of *B. oppositifolia* leaf extracts

The total flavonoid content of each extract was determined using the aluminium chloride colorimetric method, as described by Kaur et al. (2017). The extracts were prepared at a concentration of 0.1 mg/mL by dissolving in methanol. A volume of 125 µL of each extract was combined with 12.5 µL of 5% (w/v) $NaNO_2$ and incubated at room temperature for 5 min. Subsequently, 37.5 µL of 10% (w/v) $AlCl_3$ was added to the solution. The absorbance was then measured at 510 nm using a microplate reader (SPECTROstar Nano, BMG Labtech, Germany). Quercetin was used as the reference standard, and the results were expressed as mg of quercetin equivalents (QE)/g of dried leaf (mg QE/g, dried leaf).

7. Evaluation of antioxidant activities of *B. oppositifolia* leaf extracts

7.1 DPPH radical scavenging activity

The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, as described by Sriraksa and Seekhaw (2024) with some modifications. Each extract was dissolved in methanol and diluted to various concentrations. In a 96-well plate, 100 µL of 0.2 mM DPPH in methanol was mixed with 100 µL of the extract. The mixture was then incubated at room temperature for 30 min in the dark. After 30 min, the absorbance was measured at 517 nm using a microplate reader (SPECTROstar Nano, BMG Labtech, Germany). The control was the reaction mixture that did not contain test compounds. L-Ascorbic acid was used as a reference standard to compare with all samples. The data was expressed as the half-maximal inhibitory concentration (IC_{50} value) by plotting the %DPPH radical scavenging activity against different concentrations of the extract and making a calculation using the following equation:

$$\%\text{DPPH radical scavenging activity} = [(A_{\text{Control}} - A_{\text{Sample}}/A_{\text{Control}}) \times 100]$$

Where, A_{Control} is the absorbance of DPPH (without mixing with the sample); A_{Sample} is the absorbance of DPPH that reacted with the sample.

7.2 ABTS radical scavenging activity

In accordance with the method of Pang et al. (2018) with some modifications, the $ABTS^{\bullet+}$ stock solution was prepared by mixing 7 mM ABTS with 2.4 mM $K_2S_2O_8$ aqueous solution in a 4:6 mL ratio, then the mixture was allowed to stand in the dark at room temperature for 12 h. After incubation, the $ABTS^{\bullet+}$ stock solution was diluted with methanol (1:50 mL ratio) to provide an absorbance range of 0.70-0.71 at 734 nm using a SPECTROstar Nano (BMG Labtech microplate reader, Germany). Subsequently, 20 µL of each extract solution (ranging from 1.0 to 0.001 mg/mL) was added to a 96-well plate, followed by 180 µL of the $ABTS^{\bullet+}$ solution. The mixture was incubated in the dark at room temperature for 6 min, after which the absorbance was measured at 734 nm. The control was the reaction mixture that did not contain test compounds. L-Ascorbic acid was used as a reference standard to compare with all samples. The data was expressed as the half-maximal inhibitory concentration (IC_{50} value) by plotting the %ABTS radical scavenging activity against different concentrations of the extract and making a calculation using the following equation:

$$\%\text{ABTS radical scavenging activity} = [(A_{Control} - A_{Sample}/A_{Control}) \times 100]$$

Where, $A_{Control}$ is the absorbance of ABTS (without mixing with the sample); A_{Sample} is the absorbance of ABTS that reacted with the sample.

7.3 Ferric reducing antioxidant power

The reducing antioxidant power (FRAP) was carried out according to Benzie and Strain (1996). FRAP reagent was prepared by mixing a 300 mM acetate buffer (pH 3.6), 20 mM $FeCl_3$, 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl (in a 10:1:1 mL ratio). A 180 µL of FRAP reagent was mixed with 20 µL of each extract solution (0.05 mg/mL), and the mixture was incubated in the dark at room temperature for 6 min. The absorbance was then measured at 593 nm using a SPECTROstar Nano (BMG Labtech microplate reader, Germany). The $FeSO_4$ solution was used as a reference standard curve to compare with all samples. The data was expressed as mg $FeSO_4$/g, of dried leaf.

8. Evaluation of tyrosinase inhibitory activity of *B. oppositifolia* leaf extracts

The tyrosinase inhibitory activity using the Dopachrome method was carried out according to Masuda et al. (2005) with some modifications, by using L-DOPA as the substrate. Each extract solution was prepared at 0.1-1.0 mg/mL by dissolving in DMSO. Each solution of tyrosinase (46 units/mL) and 2.5 mM L-DOPA was prepared in 0.05 M phosphate buffer, pH 6.8. Furthermore, 40 µL of each extract solution was added to a 96-well plate, followed by 80 µL of 46 units/mL tyrosinase. The mixture was then allowed to stand in the dark at room temperature for 10 min. Then, 80 µL of 2.5 mM L-DOPA was added, and the mixture was incubated in the dark at room temperature for 10 min. After incubation, the absorbance was measured at 475 nm using a SPECTROstar Nano, BMG Labtech microplate reader (Germany). Kojic acid was used as a standard solution to compare with all samples. The data was expressed as the half-maximal inhibitory concentration (IC_{50} value) by plotting the %tyrosinase inhibitory activity against different concentrations of extract calculated by the following equation:

$$\%\text{Tyrosinase inhibitory activity} = [(A_{Control} - A_{Sample}/A_{Control}) \times 100]$$

Where, $A_{Control}$ is the absorbance of the negative control (without the inhibitor); A_{Sample} is the absorbance of the tyrosinase enzyme reacting with the sample.

9. Evaluation of HaCaT cells cytotoxic screening effects of *B. oppositifolia* leaf extracts

9.1 Cell culture

Normal human epidermal keratinocytes (HaCaT) cells were purchased from AddexBio Technologies, San Diego, CA, USA. HaCaT cells were cultured in optimized DMEM (Dulbecco's modified essential medium, Gibco) with 4 mM of L-glutamine (Gibco), supplemented with 5% (v/v) FBS (Fetal bovine serum, Gibco) and 1% (v/v) antibiotics (100 IU/mL Penicillin and 100 µg/mL Streptomycin, Gibco). HaCaT cells cultured were maintained at 37°C in a humidified, 5% CO_2 atmosphere for further tests.

9.2 Cell viability assay

The cytotoxicity of HaCaT cells were evaluated by using MTT assay, which was described according to ISO 10993-5:2009 (International Organization for Standardization, 2009) with some modifications. HaCaT cells were prepared by seeding a cell line at 1×10^5 cells/mL into a 96-well plate 100 µL/well and were cultured for 24 h at 37°C, 5% CO_2 atmosphere. Thereafter, the culture medium was removed and treated with 1,000 µg/mL of each extract 100 µL/well (the extract was dissolved in DMSO and diluted

with DMEM and 5% of FBS before being filtered through a 0.45 µm membrane, percentage of DMSO had not exceeded 1%). The plate was then incubated for another 24 h at 37°C, 5% CO_2 atmosphere. After incubation, 5 mg/mL of MTT solution 10 µL was placed into each well, and the plate was further incubated at 37°C, 5% CO_2 atmosphere for 4 h. Thereafter, MTT solution was discarded, and cells were washed with sterile PBS. The accumulation of dye in cells was extracted by formazan solution (100%DMSO and 10%SDS in a ratio 9:1 mL) 100 µL/well. The plate was then gently shaken for 5 min to form a homogeneous solution. The optical density (OD) was then measured at 570 nm using a microplate reader (SPECTROstar Nano, BMG Labtech, Germany). 1%DMSO was used as the negative control, and it was tested under the same experimental conditions as the extracts. The data was expressed as percentage cell viability (%cell viability) calculated by the following equation:

$$\%\text{Cell viability} = [(OD_{\text{Treatment}} / OD_{\text{Control}}) \times 100]$$

Where, $OD_{\text{Treatment}}$ is the absorbance of the treatment (test sample); OD_{Control} is the absorbance of the untreated control

10. Statistical analysis

The total phenolic and flavonoid contents, antioxidant, tyrosinase, and cytotoxic activities of *B. oppositifolia* leaf extracts were determined from triplicate experiments (n = 3) and are presented as the mean ± standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) with Tukey's HSD test ($P<0.05$) and a paired *t*-test were employed to analyze the variance of means and determine statistically significant differences using GNU PSPP software version 2.0.1.

Results and discussion

1. Extraction yield

B. oppositifolia leaf was extracted by 3 different polarity organic solvents (hexane, ethyl acetate and methanol) to identify the physical characteristic, percentage yield and phytochemical compositions of each extract. The physical characteristic and percentage yield of each extract from *B. oppositifolia* leaf are presented in Table 1. The experiment revealed that the methanol extract had the highest extraction yield (11.28%). Ethyl acetate extract showed higher yield than hexane extract (3.30% and 3.17%, respectively). These 2 extracts were viscous, dark green, and sticky, while the methanol extract was a viscous, brown, sticky substance.

Table 1 Physical characteristic and the percentage of *B. oppositifolia* leaf extracts

Samples	Physical characteristic	Yield (%)
Hexane extract	Viscous, dark green, sticky	3.17
Ethyl acetate extract	Viscous, dark green, sticky	3.30
Methanol extract	Viscous, brown, sticky	11.28

The extraction yield tended to decrease when the solvent used was less polar. Moreover, polyphenol compounds mostly found in nature have their functional groups, which are hydrophilic compounds and can easily be extracted in high polar solvents (Jaengklang et al., 2024).

2. Preliminary phytochemical screening of *B. oppositifolia* leaf extracts

Phytochemical screening of *B. oppositifolia* leaf extracts was carried out based on solubility, color reactions by characteristic reagents and precipitation tests. The results demonstrated that phenolics and anthraquinones were detected in the leaf extracts prepared with three solvents of different polarity. Diterpenes were found only in the fractions of hexane and ethyl acetate which had less polarity. Ethyl acetate and methanol extracts presented the presence of flavonoids, glycosides and tannins. Methanol is the highest polarity solvent of this experiment, which revealed the presence of saponins, phytosterols and triterpenoids as the composition of the methanol extract, as shown in Table 2.

Table 2 Preliminary phytochemical screening of *B. oppositifolia* leaf extracts

Phytoconstituents	Hexane extract	Ethyl acetate extract	Methanol extract
Phenolics	+	+++	+++
Saponins	–	–	+
Flavonoids	–	+	+++
Phytosterols	–	–	+
Alkaloids	–	–	–
Glycosides	–	+	++
Anthraquinones	+	++	+++
Diterpenes	+	++	–
Triterpenoids	–	–	++
Tannins	–	+	+++

Remark: (+++) Highly positive (significantly visible color change), (++) Moderately positive, (+) Mildly positive, (–) Negative (Phytoconstituents not detected).

The utilization of organic solvents with high polarity has significantly affected the extraction process which extracts polyphenol compounds, affecting their potential biological activity (Itsarasook et al., 2023). Similarly, the phytochemical composition is closely linked to extraction efficiency, influenced by the polarity index of solvents. Solvent polarity is a critical parameter for extracting significant levels of bioactive compounds, particularly polyphenol compounds. Additionally, the selective extraction of hydrophobic and hydrophilic polyphenols from plants varies depending on factors such as solvent polarity, plant species, extraction temperature, extraction duration, and the extraction methods (Mamoona et al., 2024). Widyawati et al. (2014) informed that methanol was the most effective solvent for extracting phytochemical compounds compared to other solvents. It also exhibited high levels of total phenolic content and strong antioxidant activity. This efficacy is attributed to the solvent's polarity and the hydroxyl (–OH) group of alcohol, which influences the extraction of polyphenols by interacting with their functional groups. In this study, *B. oppositifolia* leaf extracts were rich in secondary metabolites, such as phenolics, saponins, flavonoids, phytosterols, glycosides, anthraquinones, diterpenes, triterpenoids, and tannins. Several studies have reported the secondary metabolites found in plants, especially phenolic and flavonoid compounds which have strong antioxidants. Additionally, phenolics and flavonoids are widely known for their biological and pharmacological activities, acting as antibacterials against both Gram-positive and Gram-negative bacteria, with anticancer, antitumor, anti-inflammatory, cardiovascular protective, antidiabetic, and anti-aging properties (Huyut et al., 2017; Mutha et al., 2021).

In addition, other phytoconstituents have also been reported for their biological activities. For instance, saponins are natural substances with antifungal, antiviral, anti-ulcer, hemolytic, and hepatoprotective properties (Barbosa, 2014). Phytosterols are a compound commonly found in plants that exist as an important role in health benefits in humans by supporting the immune system and contributing to reduce low-density lipoprotein (LDL), also known as bad cholesterol (Xiao et al., 2022). Khan et al. (2020) described that glycoside derivatives have been considered for their therapeutic potential, including anti-inflammatory, anticancer, antifungal, analgesic, and antiplatelet effects. Anthraquinones have been used as a traditional medicine for decades. Their medicinal properties are discussed in supporting and modulating the immune system, as anti-malaria infection, antihyperlipidemic, and for anticancer effects (Patel et al., 2021). The biological activities of diterpenes and triterpenoids were reported in the study of Bhatti and Khera (2014). They highly display antibiotic, anti-inflammatory, anti-HIV and anti-tumor properties. Tannins are a type of polyphenol compound with several notable beneficial properties that contribute to antioxidant, antimicrobial, anti-inflammatory activities, mycotoxin reduction, HIV-inhibitor, and promotion of digestive health (Fraga-Corral et al., 2021).

3. Total phenolic and flavonoid contents of *B. oppositifolia* leaf extracts

Based on the linear calibration curve, total phenolic and flavonoid contents were calculated from gallic acid ($y = 2.8795x + 0.0843$; $R^2 = 0.9979$) and quercetin ($y = 5.0992x + 0.0689$; $R^2 = 0.9987$) standard curves. The results demonstrated that the methanol extract of *B. oppositifolia* leaf provided the significantly highest total phenolic content (74.25±3.31 mg GAE/g, dried leaf), followed by ethyl acetate and hexane extracts (6.02±0.62 and 2.00±0.31 mg GAE/g, dried leaf, respectively). The total phenolic content of the methanol

extract was statistically significantly higher (P<0.05) than that of the other extracts, while no significant difference was observed between the ethyl acetate and hexane extracts. The total flavonoid content, estimated by the aluminium chloride colorimetric method, was also highest in the methanol extract (71.48±1.94 mg QE/g, dried leaf), compared to the ethyl acetate extract (7.95±1.62 mg QE/g, dried leaf), as shown in Table 3. The methanol extract exhibited a statistically significantly higher flavonoid content than the ethyl acetate extract (P<0.05). On the other hand, flavonoid was not observed in the hexane extract.

Table 3 Total phenolic and flavonoid contents of *B. oppositifolia* leaf extracts

Samples	Total phenolic content (mg GAE/g, dried leaf)	Total flavonoid content (mg QE/g, dried leaf)
Hexane extract	2.00±0.31^{b}	–
Ethyl acetate extract	6.02±0.62^{b}	7.95±1.62^{b}
Methanol extract	74.25±3.31^{a}	71.48±1.94^{a}

Remark: (–) Phytochemical content was not observed. Values were presented as mean ± standard deviation and done in triplicate (n=3). The different superscript letters within a column denote a significant difference (P<0.05).

Flavonoid compounds are large molecules with hydroxy groups attached to aromatic rings which are normally considered to be soluble in polar solvents. Due to the hydroxy groups of flavonoids, they can form bonds with hydrogen atoms of water or polar organic solvents (Kumar & Pandey, 2013). Extraction of flavonoid composition was not detected in non-polar solvents as hexane in this study. Furthermore, alcohols are commonly used as solvents to extract bioactive compounds due to their efficacy. The polarity of alcohol effectively dissolved a wide range of polyphenols including phenolics, flavonoids, terpenoids, alkaloids, quinines, saponins, and phytosterols (Iloki-Assanga et al., 2015; Rafi et al., 2020).

4. Antioxidant activities of *B. oppositifolia* leaf extracts

Relating to hydrogen atoms donating of the antioxidants, DPPH and ABTS methods were used to determine the IC_{50} values, representing the antioxidant activity of *B. oppositifolia* leaf extracts. A lower value of IC_{50} indicates of greater antioxidant property, as shown in Table 4.

Table 4 Antioxidant activities of *B. oppositifolia* leaf extracts

Samples	IC_{50} of DPPH (µg/mL)	IC_{50} of ABTS (µg/mL)	FRAP values (mg $FeSO_4$/g, dried leaf)
Hexane extract	11.95±2.42^{a}	372.30±12.22^{a}	28.27±0.74^{c}
Ethyl acetate extract	2.05±0.25^{b}	43.20±1.53^{b}	79.40±2.40^{b}
Methanol extract	1.39±0.40^{b}	21.97±0.31^{c}	505.55±24.32^{a}
L-Ascorbic acid	0.39±0.15^{b}	20.87±0.12^{c}	–

Remark: (–) Indicates did not test. Values were presented as mean ± standard deviation and done in triplicate (n=3). The different superscript letters within a column denote a significant difference (P<0.05).

The antioxidant activities of methanol, ethyl acetate, and hexane extracts were evaluated using both DPPH and ABTS assays, with L-ascorbic acid serving as a positive control. In the DPPH assay, the methanol extract exhibited the highest antioxidant activity (IC_{50} = 1.39±0.40 µg/mL), followed by the ethyl acetate extract (IC_{50} = 2.05±0.25 µg/mL) and the hexane extract (IC_{50} = 11.95±2.42 µg/mL). Statistical analysis revealed that the hexane extract demonstrated significantly lower DPPH scavenging activity compared to the other extracts and the positive control (P < 0.05). Notably, no statistically significant difference was observed among the IC_{50} values of the methanol, ethyl acetate extracts, and L-ascorbic acid (IC_{50} = 0.39±0.15 µg/mL).

Similarly, in the ABTS assay, the methanol extract demonstrated the strongest antioxidant capacity (IC_{50} = 21.97±0.31 µg/mL), followed by the ethyl acetate extract (IC_{50} = 43.20±1.53 µg/mL) and the hexane extract (IC_{50} = 372.30±12.22 µg/mL). While the methanol extract showed significantly higher ABTS scavenging activity compared to the ethyl acetate and hexane extracts (P<0.05), its activity was not statistically significantly different from that of L-ascorbic acid (IC_{50} = 20.87±0.12 µg/mL).

Furthermore, the reducing antioxidant power was assessed using the FRAP method, which is based on the reduction of Fe^{3+} to Fe^{2+}. The higher FRAP values represent stronger antioxidant power, also shown

in Table 4. The methanol extract also provided the maximum value for reducing antioxidant power (FRAP value 505.55±24.32 mg $FeSO_4$/g, dried leaf), significantly greater than the ethyl acetate and hexane extracts. FRAP values (79.40±2.40 and 28.27±0.74 mg $FeSO_4$/g, dried leaf, respectively).

According to Fathi Hafshejani et al. (2023), higher values of total phenolic and flavonoid contents tend to correlate with antioxidant properties, which generally donate their hydrogen atoms to free radicals in order to stabilize them.

5. Tyrosinase inhibitory activity of *B. oppositifolia* leaf extracts

The tyrosinase inhibitory activity was assessed by determining the IC_{50} values, where a lower IC_{50} value indicates better enzyme inhibition, as shown in Table 5. The ethyl acetate extract had significantly stronger tyrosinase inhibitory activity (IC_{50} = 242.90±7.48 µg/mL) than the methanol extract (IC_{50} = 474.30±19.40 µg/mL). Kojic acid was used as a standard for comparison in this experiment. Kojic acid had superior tyrosinase inhibition (IC_{50} = 9.60±1.65 µg/mL), which was significantly lower than both extracts (P<0.05).

Table 5 Tyrosinase inhibitory activity of *B. oppositifolia* leaf extracts

Samples	IC_{50} of Tyrosinase inhibitory (µg/mL)
Hexane extract	–
Ethyl acetate extract	242.90±7.48^{b}
Methanol extract	474.30±19.40^{a}
Kojic acid	9.60±1.65^{c}

Remark: (–) Indicates did not test. Values were presented as mean ± standard deviation and done in triplicate (n=3). The different superscript letters within a column denote a significant difference (P<0.05).

The results of this study corresponded to previous studies, which showed that plant extracts obtained from ethyl acetate extract had a high ability to inhibit tyrosinase (Zengin et al., 2015). Therefore, ethyl acetate is an organic solvent with moderate polarity that can extract bioactive compounds that might be effective against tyrosinase enzymes due to its chemical composition (Kiattisin et al., 2019). However, the extract from hexane was not tested due to a solubility problem. This could be attributed to solvent optimization, where medium to high polar solvents are more effective at extracting compounds with enzyme inhibitory potential (Dirar et al., 2019). Consequently, ethyl acetate was found to be suitable for *B. oppositifolia* leaves extraction to provide tyrosinase inhibitory activity.

6. HaCaT cells cytotoxic screening effects of *B. oppositifolia* leaf extracts

The cytotoxicity of *B. oppositifolia* leaf extracts on human epidermal keratinocytes (HaCaT) cells was assessed using the MTT assay, with cell viability percentages as the indicator. The leaf extracts were determined at a concentration of 1,000 µg/mL and 1% DMSO was used as the negative control, as observed in Table 6. The results showed that ethyl acetate extract provided the highest cell viability (59.04±0.15%), which was significantly greater (P<0.05) than that of the methanol (40.33±0.03%) and hexane extracts (38.94±0.01%). However, all extracts exhibited significantly lower cell viability (P<0.05) compared to the negative control (97.70±0.23%).

Table 6 Percentages cell cytotoxic screening effects of HaCaT cells of *B. oppositifolia* leaf extracts at a concentration of 1,000 µg/mL

Samples	% Cell viability
Hexane extract	38.94±0.10^{c}
Ethyl acetate extract	59.04±0.15^{b}
Methanol extract	40.33±0.07^{c}
1% DMSO	97.70±0.23^{a}

Remark: Values were presented as mean ± standard deviation, the experiment was repeated on three separate plates and done in triplicate (n=3). The different superscript letters within a column denote a significant difference (P<0.05).

According to ISO 10993-5:2009 (International Organization for Standardization, 2009) the percentages of cell viability above 80% are considered as non-cytotoxicity. The range within 60–80% indicates weak cytotoxicity, between 40–60% indicates moderate cytotoxicity, and below 40% indicates considerably strong cytotoxicity. Based on this classification, the ethyl acetate and methanol extracts were categorized as having moderate cytotoxicity on HaCaT cells, while the hexane extract exhibited strong cytotoxicity. In this study, 1% DMSO served as the negative control when comparing with the test extracts. At this concentration, DMSO showed no cytotoxic effect on HaCaT cells, exhibiting a cell viability of 97.70±0.23%. Hajighasemi and Tajik (2017), reported that DMSO exhibited cytotoxic effects in human leukemic cell lines when the concentrations exceeded 2% at all time points tested (24, 48, and 72 h), and no significant differences were observed among the different leukemic cell types. While their study focused on leukemic cells, similar *in vitro* assays, using other human cell types often report a minimal cytotoxic effect at the concentrations below 2%. Therefore, DMSO at concentrations not exceeding 1% can be safely used as the negative control for comparing cytotoxic effects with extracts in this study.

Several studies have reported that plants in the Anacardiaceae family are the source of bioactive compounds with effective biological and pharmacological properties. Kumar et al. (2021) revealed that *Mangifera indica* leaf is rich in phytochemical compositions and considered the potential of antioxidants with anticancer, antidiabetic, antimicrobial, anti-obesity, lipid-lowering, hepatoprotection, and antidiarrheal properties. *Spondias mombin* leaf extracts also have biological activities, including anti-inflammatory, antioxidant, antibacterial, antiangiogenic, and antitumor effects (Rey-Blanes et al., 2020). In our study, the biological activity of *Bouea oppositifolia* (Roxb.) Meisn., was novel due to its antioxidant effects and other biological properties. The results in this study exhibited the leaf of *B. oppositifolia* extracts containing high amounts of phytochemicals, especially phenolics and flavonoids that correlate with the antioxidant activity, highlighting their role as significant contributors to this activity (Muflihah et al., 2021; Mehmood et al., 2022), and have the ability to inhibit tyrosinase with moderate cytotoxicity on HaCaT cells. These findings will be useful for further research, including green extraction techniques, *in vivo* or clinical investigations, to explore the broader biological properties of *B. oppositifolia*, such as anti-hyperpigmentation, anti-inflammatory, anti-aging, and anticancer effects with the potential for developing healthcare products in the future.

Conclusion

The findings of this study indicate that *B. oppositifolia* leaf extracts are abundant in phytochemicals with high amounts of total phenolic and flavonoid contents, particularly the methanol extract, which also has potential antioxidant activities involving DPPH, ABTS, and FRAP methods. In addition, the ethyl acetate extract showed the most potent tyrosinase inhibitory activity, along with moderate cytotoxicity on HaCaT cells, as indicated by the cell viability percentages. All these properties suggest the potential for further isolation and identification of its chemical composition that exhibited strong biological properties. Future research, including green extraction techniques, *in vivo* and clinical studies, will be essential to evaluate the safety and therapeutic applications of *B. oppositifolia* leaf extracts. Therefore, *B. oppositifolia* leaf is another natural resource that can be developed in the field of healthcare products.

Acknowledgments

The authors would like to express their appreciation to the Fundamental Fund Scholarship (Basic Research Fund 2022). Department of Chemistry and Science Center, Faculty of Science and Technology, Pibulsongkram Rajabhat University, and Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University for their kind support of this experiment in order for it to be successfully completed.

Conflicts of interest

The authors declare no conflict of interest.

Declaration of generative artificial intelligence in the writing process

The authors declare that no generative artificial intelligence or AI-assisted technologies were used in the preparation of this manuscript.

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Article info

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Received : 4 July 2025
Revised : 26 November 2025
Accepted : 10 December 2025