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Antityrosinase, antioxidative activities, and brine shrimp lethality of ethanolic extracts from *Protium serratum* (Wall. ex Colebr.) Engl.

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ABSTRACT

Objective: To evaluate all parts of *Protium serratum* (Wall. ex Colebr.) Engl. (*P. serratum*) for their phytochemistry and biological activities including antityrosinase, antioxidant, and brine shrimp lethality.

Methods: Nine ethanolic extracts from different *P. serratum* parts such as twig, whole fruit, pericarp, and root were investigated for their phytochemical screening and biological activities in terms of tyrosinase inhibition, antioxidant against 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radicals, and lethality to brine shrimp larvae.

Results: Phytochemical screening also revealed the presence of flavonoids, condensed tannins, alkaloids, triterpenoids, steroids, and sugars in *P. serratum*. The root extract was the most effective for antityrosinase activity with IC_{50} of (21.63 ± 0.31) μg/mL, and the leaf extract exhibited the highest antioxidation activity using 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging methods, with IC_{50} of (4.34 ± 0.09) and (119.80 ± 1.01) μg/mL, respectively. The essential oil extracted from the whole fruit displayed the highest toxicity against brine shrimp, with LC_{50} of (3.57 ± 1.82) μg/mL.

Conclusions: This study indicates that ethanolic extracts from each *P. serratum* part have differences in phytochemistry and biological activities (antityrosinase, antioxidant, and brine shrimp lethality). Some parts of the plant should be considered in the further study.

1. Introduction

There has been growing interest in use of traditional medicines including plants, animals, and mineral for new drug development, as the medicinal properties of natural materials have been proved for effectiveness against disease. Natural products play a key role in medicinal therapy such as artemisinin from *Artemisia annua* for malaria [1], reserpine from *Rauvolfia serpentina* for hypertension [2], and taxol from *Taxus brevifolia* for cancer [3]. Thus, natural materials are studied to protect the human body from diseases through their anticancer, antioxidant, and antityrosinase inducing abilities.

In Thailand, there are a large number of biodiversity, with approximately 12000 recorded species of vascular plants. Some flora are indigenous, some are rare, and many may have as yet unknown medicinal properties [4]. It is interesting to study these plants for foods and medicines. Burseraceae is a family of trees and shrubs. There are 16 genera and 550 species in the world [5]. Several biological activities of the plants in this family were examined such as anti-inflammatory, antioxidation, antimalaria, and neuroprotective. Previous studies indicated that methyl-3,4,5-trihydroxybenzoate from the hexane extract of *Dacryodes edulis* stem bark inhibited 3D7 (chloroquine-susceptible) and Dd2 (multidrug-resistant) strains of *Plasmodium falciparum* [6]. Moreover, sesquiterpenes such as myrrhterpenoids K and N from the resin of *Commiphora myrrha* showed neuroprotective effects against 1-methyl-4-phenyl-pyridinium-induced neuronal cell death in dopaminergic neuroblastoma SH-SY5Y cells [7]. Additionally, triterpenoid compounds such as boscartene A-K from *Boswellia carterii* gum resin exhibited different hepatoprotective activities against D-galactosamine-induced human hepatic (HL-7702) cell injury [8]. Sesquiterpenoid compound such as guaia-4β, 7β, 10α-trihydroxy-5 ene from the resinous exudates of *Commiphora opobalsamum* showed cytotoxicity against human cervical epithelioid carcinoma (HeLa) and liver

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hepatocellular (HepG2) cell lines with IC_{50} of 15.4 and 8.7 μmol/L, respectively [9]. Moreover, two new prenylated flavonoids such as 6-(3,3-dimethylallyl)-2,3-dihydrokaempferol-3-β-O-glucoside and 6-(3,3-dimethylallyl)-naringenin-7-β-O-glucoside were isolated from stem wood of *Commiphora opobalsamum* [10]. Ethanolic extract from *Protium serratum* (Wall. ex Colebr.) Engl. (*P. serratum*) leaf exhibited over 50% inhibition of plaque formation in herpes simplex virus type 1 and poliovirus [11]. Methanolic extract from *P. serratum* leaf showed inhibition of protein denaturation at 1 000 μg/mL and thrombolytic activity [12].

In addition, there are 5 genera and 20 species of the Burseraceae family in Thailand. These genera comprise *Canarium, Santiria, Dacryodes, Garuga*, and *Protium* [13]. *P. serratum* was found in evergreen forest, 1 500 m above sea level in the north and the northeast of Thailand [13]. This fruit of this plant tastes slightly sweet and sour and is used as a food source. Also, the roots are used for their detoxicity and antipyretic activity [14]. However, little research has previously been conducted on the biological activity and chemical constituents of this plant. Therefore, this study investigated the antityrosinase, antioxidative properties, the brine shrimp lethality and screening of the phytochemicals of *P. serratum*.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), tyrosinase enzyme, 2-deoxyribose, and kojic acid were obtained from Sigma–Aldrich, USA. Ascorbic acid was purchased from Ajax Finechem, Australia. Ethanol from Labscan was commercial grade which was redistilled before use.

2.2. Plant sample

P. serratum was collected from Chiang Rai Province on August 15, 2014. This plant was identified by J.F. Maxwell at Biological Herbarium, Department of Biology, Faculty of Science, Chiang Mai University in April, 2015. The voucher specimen is No. 26.

2.3. Plant extraction

All fresh parts of *P. serratum* were washed, dried, and chopped at room temperature. Five grams of dried plant material were macerated with ethanol three times. The filtrate was then collected, and the solvent evaporated using a rotary evaporator. Five grams of fruit pericarp (rind) were macerated with petroleum ether three times, and the macerated solution was filtered and dried. Essential oil from the fruit (whole fruit) was extracted with steam distillation.

2.4. Preliminary phytochemical screening

The phytochemical tests were evaluated using the methods of Kar [15] and Evans [16]. An ethanolic solution of each sample was prepared for the testing of flavonoids, sugars, and phenolic compounds. To test for tannins, the samples were dissolved in distilled water. The dried samples were investigated for alkaloids, triterpenoids, and steroids.

2.5. Biological activities

2.5.1. Tyrosinase inhibitory assay

The assay technique for dopachrome formation with anti-tyrosinase activity followed the methods of Mapunya *et al.* [17] and Potduang *et al.* [18] with some modifications. Twelve millimoles per liter of L-dopa were used as the substrate. Ethanolic extracts were prepared with 100, 50, 10, 5, and 1 μg/mL in triplicate. Mushroom tyrosinase enzyme was assayed as 333 unit/mL. The testing was divided into four groups A, B, C, and D using a 96-well plate. Group A included 30 μL of mushroom tyrosinase enzyme and 70 μL of phosphate buffer saline (PBS; pH 6.5). Group B contained 100 μL of PBS. Group C consisted of 30 μL of tyrosinase enzyme and 70 μL of sample. Group D was composed of 30 μL of PBS and 70 μL of sample. After 10 min incubation, 110 μL of L-dopa was added to each group. The absorbance was measured at a wavelength of 492 nm using microplate reader (BioTek Synergy H1, USA). The percentage inhibition was calculated to evaluate the activity using positive control of kojic acid. Fifty percent of the inhibitory concentration was evaluated using probit analysis.

$$\%\ \text{Inhibition} = \frac{(A-B)-(C-D)}{A-B} \times 100$$

2.5.2. Antioxidative activities

2.5.2.1. DPPH free radical scavenging activity

The DPPH assay followed the methods of Chung *et al.* [19] and Molyneux [20] with slight modifications. DPPH was prepared in ethanol at 75 μg/mL. The ethanolic extracts were dissolved in 1% dimethyl sulfoxide (DMSO) and serial dilutions were 100, 50, 10, 5, and 1 μg/mL in ethanol. All concentrations were performed in triplicate. The ratio of ethanolic extract and DPPH solution for each concentration was 1:1. After 60 min incubation, absorbance was read by UV–vis spectrophotometer (JASCO V-630 PC, Japan) at a wavelength of 518 nm. Ascorbic acid was used as the positive control. Negative controls were ethanolic extracts without DPPH or ascorbic acid without DPPH. The scavenging activity, calculated by the following equation, was analyzed as 50% of the inhibitory concentration.

$$\%\ \text{Scavenging activity} = \frac{A_{DPPH} - \left(A_{sample+DPPH} - A_{sample}\right)}{A_{DPPH}} \times 100$$

where A_{DPPH} is the absorbance of DPPH, $A_{sample+DPPH}$ is the absorbance of the sample and DPPH solution, and A_{sample} is the absorbance of the sample solution without DPPH solution.

2.5.2.2. Hydroxyl radical assay

The method was adapted from Awah and Verla [21]. All reagents were prepared in phosphate buffer (pH 7.4) such as 10 mmol/L ferrous sulfate, 10 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L 2-deoxyribose. The extracts dissolved in 1% DMSO and ascorbic acid used as the positive control, were assayed as 1 000, 500, 100, 50, and 10 μg/mL in phosphate buffer. Each concentration of samples and positive control were tested in triplicate. The mixture solution contained 100 μL of 10 mmol/L ferrous sulfate, 100 μL of 10 mmol/L ethylenediaminetetraacetic

acid, 200 μL of 10 mmol/L 2-deoxyribose, 20 μL of extract, 1.38 mL of 50 mmol/L phosphate buffer, and 200 μL of 10 mmol/L H_2O_2, respectively. After incubation for 60 min at room temperature, the reaction was stopped with 1 mL of 2.8% trichloroacetic acid and 1 mL of 1% thiobarbituric acid. The mixture solution was then boiled at 100 °C for 15 min, before cooling in an ice bath. Absorbance was monitored using UV–vis spectrophotometer at a wavelength of 532 nm (JASCO V-630 PC, Japan). Fifty percent of the inhibitory concentration was calculated from the percentage of scavenging activity. The positive control was ascorbic acid, and the negative control was sample without 2-deoxyribose and ascorbic acid without 2-deoxyribose. All concentrations were carried out in triplicate. The hydroxyl scavenging activity was calculated following the equation:

$$\%\ \text{Scavenging activity} = \left[A_{control} - \left(A_{sample} - A_{sample\ without\ 2\text{-}deoxyribose}\right)\right] / A_{control} \times 100$$

where $A_{control}$ is the 2-deoxyribose oxidation without sample extracts or ascorbic acid, A_{sample} is the absorbance of the sample or ascorbic acid, and $A_{sample\ without\ 2\text{-}deoxyribose}$ is the sample without 2-deoxyribose or ascorbic acid without 2-deoxyribose.

2.6. Brine shrimp lethality

The general toxicity test was adapted from the test of Mclaughlin *et al.* [22] with some modifications. Artificial sea water (Jor Charoen aquarium; Thailand) was prepared at 38 g/L in a small tank. *Artemia salina* L. eggs (S. K. Trading; China) were then added to the tank. After 48 h, ten brine shrimp larvae were added into each vial of plant extract solution (dissolved in 1% of DMSO) and prepared at 1000, 100, 10 and 1 μg/mL in triplicate. After incubation for 24 h, the brine shrimp larvae were counted and the survivors were recorded. The percentage mortality was analyzed to be LC_{50}.

$$\%\ \text{Mortality} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

3. Results

3.1. Phytochemical screening

All parts of *P. serratum* were screened and analyzed; the results showed different chemical groupings. The roots, the twigs, and the pericarps (rind) were tested positively for alkaloids. Triterpenoids were found in petroleum ether extracted from the pericarps, the roots, the pericarps (rind), and the whole fruits. However, only the leaves gave a positive test for steroids. Positive tests for tannins were detected in the pericarps (rind), roots, twigs, and leaves. The petroleum ether extracted from the roots, twigs, the pericarps (rind), and leaves exhibited a positive test for flavonoids. Moreover, all parts of the plant were tested positively for sugars (Table 1).

Table 1
Phytochemical tests of ethanolic extracts from *P. serratum*.

Ethanolic extract	Flavonoids	Sugar		Phenolic compounds	Tannins				Alkaloids				Triterpenoids/ steroids
	Shinoda	Molisch	Keller-Kiliani	$FeCl_3$	Gelatin	Lead acetate	Lime water	Vanillin	Dragendorff	Wagner	Mayer	Mame	Liebermann-Burchard
Seeds	−	+	+	−	−	−	−	−	−	−	−	−	−
Pericarps (rind)	+	+	+	+	+	+	−	+	+	+	+	+	+ Violet
Pulp fruits (flesh)	−	+	+	−	−	+	−	−	−	−	−	−	−
Leaves	+	+	+	+	−	−	−	+	−	−	−	−	+ Green
Twigs	+	+	+	+	+	+	−	+	+	+	+	+	+ Violet
Whole fruits	−	+	+	−	+	+	−	+	−	−	−	−	+ Violet
Roots	+	+	+	+	−	+	−	+	+	+	+	+	+ Violet
Petroleum ether extract from pericarps	+	+	+	−	−	−	−	−	−	−	−	−	+ Violet
Essential oil from fruits	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

+: Indicates the present compound; + Green: Indicates steroids; −: Indicates the absent compound; + Violet: Indicates triterpenoids; ND: Not determined.

3.2. Tyrosinase inhibitory activity

The results of antityrosinase activity were classified into four groups: highly active ($IC_{50} \leq 15.00$ μg/mL), moderately active (IC_{50}: 15.01–50.00 μg/mL), weakly active (IC_{50}: 50.01–100.00 μg/mL), and inactive ($IC_{50} > 100.00$ μg/mL). Roots, twigs, and leaves showed moderate activity against mushroom tyrosinase. Kojic acid, a positive control, showed high activity against tyrosinase enzyme with IC_{50} of (12.21 ± 0.62) μg/mL (Table 2).

3.3. Antioxidative activities

3.3.1. DPPH free radical scavenging activity

The DPPH scavenging activity of all ethanolic extracts from *P. serratum* was divided into four groups: highly active ($IC_{50} \leq 5.00$ μg/mL), moderately active (IC_{50}: 5.01–50.99 μg/mL), weakly active (IC_{50}: 51.00–100.00 μg/mL), and inactive ($IC_{50} > 100.00$ μg/mL). The ethanolic extracts from leaves showed high activity. Moderate radical scavenging activity was found in roots, pericarps (rind), and twigs (Table 2). Ascorbic acid showed high activity with IC_{50} of (3.00 ± 0.14) μg/mL, however, the pulp fruits and essential oil from the fruits were inactive.

Table 2
IC_{50} values (μg/mL) and classified activities of ethanolic extracts from parts of *P. serratum*.

Ethanolic extract	Tyrosinase inhibitory assay		DPPH radical		Hydroxyl radical		Brine shrimp lethality	
	IC_{50}	Classified activity	IC_{50}	Classified activity	IC_{50}	Classified activity	IC_{50}	Classified activity
Seeds	> 200	I	865.27 ± 0.69	I	219.60 ± 0.13	W	ND	ND
Pericarps (rind)	102.77 ± 0.10	I	7.20 ± 0.35	M	176.31 ± 0.87	W	ND	ND
Pulp fruits (flesh)	ND	ND	> 1 000	I	350.37 ± 0.50	W	ND	ND
Leaves	34.48 ± 0.02	M	4.34 ± 0.09	H	119.80 ± 1.01	W	108.02 ± 0.00	W
Twigs	22.77 ± 0.30	M	9.29 ± 0.13	M	137.20 ± 0.59	W	27.10 ± 2.39	M
Whole fruits	> 200	I	28.96 ± 0.84	M	244.77 ± 0.63	W	145.72 ± 0.00	W
Roots	21.63 ± 0.31	M	5.35 ± 0.37	M	147.96 ± 0.61	W	28.15 ± 0.91	M
Petroleum ether extract from pericarps	> 200	I	> 1 000	I	159.13 ± 1.07	W	14.14 ± 1.76	M
Essential oil from fruits	> 200	I	> 1 000	I	113.48 ± 0.67	W	3.57 ± 1.82	H
Ascorbic acid (positive control)	–	–	3.00 ± 0.14	H	98.70 ± 0.08	M	–	–
Kojic acid (positive control)	12.21 ± 0.62	H	–	–	–	–	–	–

H: High activity; M: Moderate activity; W: Weak activity; I: Inactive; ND: Not determined. The IC_{50} values are expressed as mean ± SD.

3.3.2. Hydroxyl radical activity

The detection of hydroxyl radical scavenging activity was classified into four groups: highly active ($IC_{50} \leq 50.00$ μg/mL), moderately active (IC_{50}: 50.01–100.99 μg/mL), weakly active (IC_{50}: 101.00–500.00 μg/mL), and inactive ($IC_{50} > 500.00$ μg/mL). Ethanolic extracts from all parts of *P. serratum* showed weak activity against hydroxyl radicals with IC_{50} of (113.48 ± 0.67)–(350.37 ± 0.50) μg/mL. However, ascorbic acid exhibited moderate activity (Table 2).

3.4. Brine shrimp lethality

The level of toxicity against brine shrimp was classified as four groups: highly toxic ($LC_{50} < 10.00$ μg/mL), moderately toxic (LC_{50}: 10.00–100.00 μg/mL), weakly toxic (LC_{50}: 100.00–1 000.00 μg/mL), and inactive ($LC_{50} > 1\,000.00$ μg/mL). Only the essential oil exhibited high toxicity with LC_{50} of (3.57 ± 1.82) μg/mL. Roots, petroleum ether extract from the pericarps, and twigs showed moderate toxicity, with LC_{50} of (14.14 ± 1.76)–(27.10 ± 2.39) μg/mL. Whole fruits and leaves were weakly toxic (Table 2). Nevertheless, the pulp fruits, pericarps (rind), and seeds were not determined.

4. Discussion

All parts of *P. serratum* are utilized for fruit, food, and traditional medicine. Secondary metabolite compounds in this plant are important for new drug discovery processes. Preliminary phytochemical screening can determine the biological activities; and results showed that each part of this plant contained different chemical compounds including flavonoids, alkaloids, triterpenoids, steroids, tannins, and sugars. The roots, twigs and pericarps (rind) all contained alkaloids which have been used as anticancer, antimalaria, anti-tussive, analgesic, and antispasmodic agents [23]. Condensed tannins were found in the pericarps (rind), roots, fruits, twigs, and leaves. Tannins are polyphenolic compounds which can be divided into two groups including true and pseudo tannins [23,24]. True tannins comprise condensed and hydrolyzable tannins. In terms of biological activities, tannins are used as antitussive, astringent, antitumor, and antioxidative agents [23,24]. Flavonoids, comprising benzo-γ-pyrone, were found in petroleum ether of pericarps, roots, twigs, the pericarps (rind), and leaves. It was found that flavonoids have been reported to have biological activities such as antioxidative, antiulcer, anti-inflammatory, and anti-viral agents [23,25]. Deoxy sugars with chemical structures of flavonoids, triterpenoids, and alkaloids were detected in all parts of the plant [24]. Steroids were mainly found in the leaf. Previous research indicated that the biological activities of steroids were anti-inflammatory, hormonal, and anti-fungal [24]. Triterpenoids were presented in *P. serratum* in the pericarps, whole fruits, and petroleum ether extract from pericarps. Triterpenoids exhibit biological activities such as antiseptic, anthelmintic, and antimicrobial agents. Coumarin and steroids (scopoletin, β-sitostenone, and β-amyrin) were isolated from the stem barks of *P. serratum* [26].

Skin color has an influence on both males and females, especially irregular dark skin. There are many causes of hyperpigmentation, such as inflammation and UV [27,28]. The causes of dark pigmentation are associated with abnormal melanin synthesis after acne and skin inflammation. In the general melanin process, L-dopa is an intermediate substance which is changed into dopaquinone and dopachrome by the tyrosinase enzyme [29]. Thus, this research may discover a new natural product to decrease hyperpigmentation, using tyrosinase inhibition. The tyrosinase inhibitory activities of the roots, twigs, and leaves extract from *P. serratum* were found to be weak compared with kojic acid. However, the ethanolic root extract showed the highest activity against the tyrosinase enzyme compared with other parts. Results indicated that tyrosinase inhibition of ethanolic extract from *P. serratum* roots and twigs could be associated with their chemical substances in phytochemical screening.

Oxidative stress relates to reactive oxygen species and other species which lead to macromolecular damages, and cardiovascular complaints, Alzheimer, and cancer as a result [30]. Antioxidants are agents that protect the human body [31]. DPPH assay for antioxidative agent screening is widely used because it is simple and rapid. The antioxidant gives the electron or proton to a stable nitrogen in DPPH radical. The method indicates ability of antioxidative agents [32]. The antioxidative activities of *P. serratum* extracts could result in the formation of new compounds to decrease free radical-induced oxidative stress. Four ninths of the plant extracts exhibited inactivity against DPPH

radicals. The ethanolic extract from leaves showed the highest activity against DPPH radicals, as compared with the other parts. However, the leaves extract was weak, as compared to ascorbic acid, a positive control. Previous study in India examining fruit and leaves extracts from *P. serratum* exhibited DPPH radical scavenging activity with IC_{50} of 29 and 52 μg/mL, respectively [33]. Reports of other species also showed that the methanolic extract from *Protium neglectum* leaves was composed of polyphenol, flavonoid, and tannin compounds. The extract exhibited 85.44% against DPPH radicals [34].

Moreover, Fenton's reaction which is caused by hydroxyl radicals related to biological activities such as anti-inflammatory, antiviral, and anti-arthritic activities [12]. In this study, the essential oil from fruit exhibited the highest activity against the hydroxyl radical. However, the scavenging ability of the hydroxyl radical of the essential oil from the fruit was weak, as compared to ascorbic acid. Previous research demonstrated that methanolic extracts of fruits and leaves from *P. serratum* exhibited hydroxyl radical scavenging activity with IC_{50} of 920 and 3460 μg/mL, respectively [33]. Other species have also shown hydroxyl radical scavenging activity. The ether extract from *Protium kleinii* or α-amyrin pentacyclic triterpene can inhibit 12-O-tetradecanoylphorbol-13-acetate which induced edema in mice ears [35]. Moreover, α-amyrin and β-amyrin in resin from *Protium heptaphyllum* at a dose of 400 mg/kg can decrease vascular permeability induced by acetic acid in mice [36]. These results demonstrated that effect of hydroxyl radicals on the human body was higher than that of the DPPH radicals. Therefore, scavenging activity of hydroxyl radical of this plant may predict antioxidative activity in *in vivo*, as compared with DPPH radicals.

The brine shrimp lethality assay was tested to predict the preliminary toxicity [23]. Results revealed that essential oil from the fruit has the highest toxicity. The other extracts showed various toxicities ranging from moderate to weak and inactive. Previous research indicated that the methanolic extract from *P. serratum* leaves, and the dichloromethane extract from *P. serratum* stem barks showed toxicity to brine shrimp with LC_{50} of 22.91 and 9.64 μg/mL, respectively [37]. This level of toxicity suggested that some parts of the plant should be further studied for antitumor activity.

The chemical components of all parts of *P. serratum* consisted of flavonoids, condensed tannins, alkaloids, triterpenoids, steroids, and deoxy sugars. The results suggested that all parts of the plant showed different biological activities. The biological activities determined in this experiment may suggest the usage of *P. serratum* roots in traditional medicine. Additionally, the leaves, twigs and roots of *P. serratum* are interesting for the isolation of bioactive compounds, and therefore worth further investigation.

Conflict of interest statement

I declare that I have no conflict of interest.

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