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Determination of Collagen Content and Antioxidant Activity in *Sesbania grandiflora* (L.) Extracts

Sathaporn Satsue[1,*], Wilailak Suksai[2], Kanaporn Tansriprapasiri[2], Kritsana Pachuen[3], Napasorn Kummeekorn[3], Watcharin Phuttasanbundid[3] and Sunisa Sanlad[3]

[1]*Department of Thai Traditional Medicine, Kanchanabhishek Institute of Medical and Public Health Technology, Nonthaburi 11150, Thailand.*
[2]*Professional Pharmacist, Kanchanabhishek Institute of Medical and Public Health Technology, Nonthaburi 11150, Thailand.*
[3]*Thai Traditional Medicine, Kanchanabhishek Institute of Medical and Public Health Technology, Nonthaburi 11150, Thailand*

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ABSTRACT

This study investigated the phytochemical screening and biological activity of *Sesbania grandiflora* (L.), locally named as "Khae ban", bark extractions, which were soft and hard barks. The phytochemical screening was carried out on the extraction of each particular bark with 95% Hexane, 95% Ethyl acetate, and 95% Ethanol for Alkaloids, Steroids, Tannins, Flavonoids, and Terpenoids. The anti-inflammatory activity was also evaluated for scavenging of nitric oxide free radicals (NO), and the collagen extent was determined by Hydroxyproline assay. The results from phytochemical screening indicated that the 95% of ethanol extraction of hard bark provided a more positive result than those of others. The hard bark's extraction showed IC_{50} value ranging from 45.83 ± 14.95 to 254.86 ± 7.58 microgram per milliliter, which was a significantly statistical difference ($P < 0.05$) from scavenging of nitric oxide free radicals (NO), and higher activity than that of soft bark. However, the highest activity of soft bark's extraction was found in 95% of Ethyl acetate with IC50 value of 470.24 ± 3.63 microgram per milliliter. In hard bark's extractions, the 95% ethanol extraction not only showed the highest activity (IC50 value of 45.85 ± 47.78 microgram per milliliter), but at the low concentration of extraction had collagen content of 49.89 microgram per milliliter, which was higher than that in other solvents. This research indicated the Khae ban's hard bark extraction in 95% of ethanol and potentially able to be developed as a cosmeceutical product or mouth sore product treating mouth ulcer.

***Corresponding author**: Satha.arm@gmail.com **doi:** 10.14456/scitechasia.2019.14

Keywords: Phytochemical; Biological activity; *Sesbania grandiflora* (L.); Anti-inflammatory; Collagen

1. Introduction

Khae ban (*Sesbania grandiflora* (L.) Desv.) is a soft-wooded tree generally planted in settled areas, such as a garden, and found in the northeastern region of Thailand. It grows between 3 and 10 meters in height, has rough thick brownish bark, is fast growing, and well adapted to hot, humid environments in tropics. Green leaves are alternate and compound; pinnate, entire-rounded leaflets with an obtuse leaf apex. It has flower clusters that are pea like, with scented-white flowers hanging at axillary. Its legumes are long and narrow with brown seeds [1]. According to the "Five Class-Planting Project" of His Majesty the King, which introduced the concept of the *New Theory*, Khae ban requiring less light and heat resistance was recommended to be planted as the third class [2]. Department of Agricultural Extension and Land Development Department campaigned for planting Khae ban due to its fast growth period, high-tolerance of Thailand's terrain with a hot and arid climate. Besides that, it not only had several non-trivial compounds, for example, fiber, calcium, phosphorus, iron, beta-carotene, vitamin A, vitamin B1, vitamin B2, vitamin C, protein, carbohydrate, and fat, but also its flowers, leaves and shoots were able to be cooked for sour Khae ban flower curry, fried Khae ban flower or parboiled Khae ban flower with chili paste [3]. Its bark, seed, pod and root have been used as a traditional remedy treating dysentery in which stools contain visible red blood, and external and internal astringents, including wound cleansing [1]. In addition, the old leaves could be used as fertilizer to adjust the soil. In Northeast Asia and India, various parts of Khae ban have been empirically used as a traditional remedy in folk medicine for laxative, diuretics, fever treatment and tonic. Furthermore, they have been applied for bruise, dysentery, painkillers, astringent, sore throat, and catarrh or mouth sores [4]. In ancient India, Khae ban was also used to treat various diseases including anemia, bronchitis, eye diseases, leprosy, gout and rheumatoid arthritis. It has properties of anti-sedative and anti-epileptics as well as preventing liver toxicity [4].

In 2009, Vijay, D and Wagh, KV. studied phytochemical screening and pharmacological action of Khae ban in ethanol extract. Most of the elements found were Sterols, Saponin and Tannin. However, components found in Khae ban were different depending on the part of the tree, for example, Leucocyanidin, Cyanidin and Saponin found in the seed; Oleanolic acid, Methyl ester and Kaemferol-3-rutinoside were found in the flower; Tannin and its gum were found in Khae ban's bark. Generally, bitter Khae ban's bark is used for tonic, antipyretics, smallpox, rash fever, astringent, mouth sores, and peptic ulcer [5]. Treatment practice was carried out in several ways such as covering scabies with pounded bark, and drinking decoction from boiled bark for a cough, diarrhea, or dysentery. Ethanol bark extract showed pharmacological activity of being an antioxidant preventing gall stones in the urinary tract [6], anti-cancerous [7], anti-inflammatory, antibacterial [8] astringent healing of acute peptic ulcers, stress reliever, and nonsteroidal anti-inflammatory agents-induced acute lesions [9] by collagen formation to repair and recovery. Collagen is a protein found in connective tissue being a main component of dermis. Collagen content is approximately 30% of the total protein in the body, and mostly found in skin, tendons, bones and cartilage [10]. It is able to make

skin healthy, elastic and heal damage [11]. Sources of collagen are fruits and vegetables which contain collagen- stimulating substances such as vitamin C, vitamin E, vitamin A, and anthocyanins. These vitamins act as antioxidants playing an important role in healing [12] and enhancing collagen formation [13]. The aim of this study was the determination of collagen content and antioxidant activity in *Sesbania grandiflora* (L.) extracts. The study had a potential to be brought for developing as a cosmeceutical product, or pharmaceutical product healing mouth sores.

2. Materials and Methods

2.1 Equipment and chemicals

Chloramine- T, Ethyl alcohol, Hexana, Sodium acetate trihydrate, Citric acid, Perchloric acid, n- propanol, Sodium hydroxide, Acetic acid, HCL, Sodium Nitroprusside, Sulphanilamide, N- 1-naphthylenediamine, Phosphoric acid, Ascorbic acid, Ibuprofen, Ethyl Acetate, p-dimethylaminobenzaldehyde,

2.2 Plant and extraction

Khae ban's fresh bark, including soft and hard bark, was washed and cut into small pieces. Then they were dried at 60 degrees Celsius (°C) for 24 hours or until entire bark pieces were dried. They were extracted with each solvent: 95% of hexane, 95% of ethyl acetate, and 95% of ethanol. Solubility was 4 to 1 (4:1) of herbal powder 25 grams (g) in 100 milliliters (ml) of each solvent. Each extract was macerated at room temperature, done in triplicate, and shaken twice a day for 3 days. Then they were filtered with filter paper (Whatman No.1). The filtered extract solutions were vaporized by rotary evaporation set at 40 °C for crude extract.

2.3 Phytochemical screening by chemical reaction modified from [14]

2.3.1 Alkaloid investigation method

We prepared 1,000 micrograms per milliliter (µg/ml) of each extract, and took 1 ml to be evaporated. We dissolved that trace by 5 ml of 5% sulfuric acid, then filtered it, and divided it into two.

Part one: We placed a droplet of filtered solution on a slide, dropped one droplet of Dragendorff solution onto that extraction, and observed the precipitation. The positive result showed as an orange precipitation.

Part two: We adjusted the pH of the filtered extraction to be alkaline by a gradual deposit of ammonium hydroxide concentrate. Then we checked the alkaline status with the litmus paper changing from red to blue. After that, we added the dichloromethane into the mixture, applied one drop of the dichloromethane extract onto the filter paper, and then sprayed with Dragendorff solution. A positive result showed orange.

2.3.2 Steroid investigation method

Steroid investigation by using Liebermann- Burchard's method: We prepared the extract concentration of 1000 µg/ml, took 5 ml of the concentration to be evaporated, and then dropped with three drops of acetic anhydride and one drop of sulfuric acid concentration. The positive result showed as blue-green color.

Bubble Detection: We put 0.005 g of extract into a tube, then added 5 ml of hot water and shook it vertically for 10 minutes. We noticed the appearance of the bubble, measured its height, and then dropped one droplet of 5% of hydrochloric acid. If the bubble was still stable, did not disappear, and had a honeycomb- liked structure, it meant that the sample contained saponins.

Result Interpretation: Blue- green positive result from Liebermann- Burchard's method and stable- bubble appearance indicated that the extract contained steroidal saponins. However, if the result provided a stable- bubble appearance, but showed red,

pink, or magenta by Liebermann-Burchard's method, it meant that extraction contained triterpenoid saponins.

2.3.3 Tannin investigation method

Prepared 5 ml - the extract concentration of 1000 µg/ml.

Part one: We evaporated one milliliter of extraction to be dried, and then added two to three droplets of 10% sodium chloride solution. After filtering and discarding its sludge, the filtered solution was divided into 3 tubes;

Tube No. 1 added with 1 % of gelatin

Tube No. 2 added with gelatin salt

Tube No. 3 presented as a control

The positive result was precipitation appearing in Tube No. 1 and Tube No. 2.

Part two: We brought four milliliters of extract divided into four tubes;

Tube No. 1 presented as a control

Tube No. 2 added 1% of ferric chloride which positive result showed blue or green.

Tube No. 3 added bromine water which positive result showed light-orange-sediment

Tube No. 4 added liquid lime water which the positive result showed grayish.

Result interpretation:

1) Providing negative result with gelatin and gelatin salt, and showing no color with 1% of ferric chloride which means that extract did not have tannins.

2) Providing positive result with gelatin and gelatin salt, being green with ferric chloride, positive result with bromine water, but providing negative one with lime water. It meant that extracts contained condensed-tannins.

3) Providing positive result with gelatin and gelatin salt, showing green-bluish, black-bluish with ferric chloride, being positive with lime water, and being negative with bromine water. It meant that the extract contained hydrolysable tannins.

2.3.4 Flavonoid investigation by Shinoda's method

We prepared three milliliters of extract concentration of 1,000 µg/ml, then dropped 1 ml of hydrochloric acid, and added about 5 to 8 magnesium ribbons into it. When the reaction ended, the red-orangish color appeared.

2.3.5 Terpenoid investigation by Shinoda's method

We prepared five milliliters of extract concentration, then added 2 ml of chloroform (CHCl3), followed by gradually adding 2 ml of concentrated sulfuric acid. The positive result was brown-reddish interfaced between solutions, meaning the extraction contained terpenoids.

2.4 Evaluation for scavenging of nitric oxide free radicals (NO) [14]

We prepared the Griss reagent, consisting of 1% of sulphanilamide in 5% of phosphoric acid and 0.1% of N-1-naphthylenediamine, and 0.1 molar of sodium nitroprusside. Then we brought 50 µl of Khae ban bark extraction concentrated 10 to 1,000 µg/ml mixing with 50 µl of 0.1 molar of sodium nitroprusside and incubated those in the dark at room temperature for 3 hours. After that, we added 100 µl of Griss reagent, and measured the light absorption (A) at wavelength of 550 nm by using ascorbic acid as a reference standard for calculating the value of half maximal inhibitory concentration (IC50) : % Inhibition = (A control - A sample/ A control) x 100

2.5 Total phenolic content (TPC)

Total phenolic content (TPC) was estimated using the personalized Folin–Ciocalteau technique8 with few modifications. The plant sample (0.05 g) was ground with acetone : water (1 : 1, v/v) at 4°C. Next, 9 µl of the extract aliquot was mixed with Folin–Ciocalteau reagent (109 µl) and left for 3 min at 25°C. Thereafter, Na_2CO_3 solution (180 µl; 7.5%, w/v) was

added to the extract and mixed thoroughly. The solution thus obtained was allowed to stand for 5 min at 25°C and the absorbance was measured at 760 nm. TPC was calculated by preparing a standard curve of gallic acid and expressed as mg/g gallic acid equivalents (GAE)

2.6 Assay for collagen content by Hydroxyproline assay [14]

A normal standard curve was initially prepared by dissolved Hydroxyproline standard in concentrations ranging from 1 to 5 µg/ml. Then 20 µl of Hydroxyproline standard solution was mixed with 20 µl of 2N NaOH, and 90 µl of chloramine-T. These were incubated for 25 minutes at room temperature; then 100 µl of Ehrlich's aldehyde reagent was added before incubating at 65 ° C for 20 minutes. After that, we measured those solutions at the wavelength of 550 nm. Then we took 20 to 30 µg/ ml of Khae ban bark extraction to analyze the collagen content by mixing extracted solution with 20 µl of 2N NaOH. This was then autoclaved at 121 ° C for 20 minutes, and later mixed with 90 µl of chloramine- T for 25 minutes at room temperature. Next, we added 100 µl of Ehrlich's aldehyde reagent, then incubated at 65 °C for 20 minutes. We then measured the light absorption at the wavelength of 550 nm for calculating the collagen content in the unit of µg/ ml from the Hydroxyproline standard curve, which was mentioned above, by this equation: Collagen content = (Hydroxyproline concentration (µg/ ml) x 100) / 13.5

Statistical analysis was presented as an average value (Mean) ± standard deviation (SD) . The statistical values by ANOVA analysis showed $p<0.05$ as significantly different.

3. Results

Table 1. Phytochemical components found in Khae ban bark extracts, soft bark and hard bark, varied based on the usage of 95% of hexane, 95% of ethyl acetate, or 95% of ethanol.

Solvents	Alkaloids		Saponins	Tannins		Steroids	Flavonoids	Terpenoids
	A.1	A.2		T.1	T.2			
Extraction of Khae ban's hard bark								
95% Hexane	+	+	+	++	++	++	+	+
95% Ethyl acetate	+	+	+	+	+	+	+	+
95% Ethanol	+	+	+	++	+++	+++	+	++
Extraction of Khae ban's soft bark								
95% Hexane	+	+	+	++	++	+	+	+
95% Ethyl acetate	+	+	+	++	+++	++	+	+
95% Ethanol	+	+	+	+	+	+	+	+

Remark: Positive (+) means dark color and having precipitation. Number of plus sign showed strong reaction.

Negative (-) means no reaction occurred, or no precipitation with testing solution.

Alkaloid investigation: A.1 = testing with Dragendorff ;
A.2 = spraying with Dragendorff.

Tannin investigation: T.1 = testing with 1% of gelatin and gelatin salt;
T.2 = testing with 1% of ferric chloride, bromine, and lime water.

Table 1 shows that bark extracts in ethanol were found to have more phytochemicals of alkaloids, steroids, flavonoids, terpenoids and tannins than those in other solvents. In hard bark extraction, the table indicates higher concentration of phytochemicals content in 95% of ethanol than those in 95% hexane and 95% ethyl acetate. While, the soft bark extraction showed higher phytochemicals content in 95% ethyl acetate than those in 95% hexane and 95% of ethanol.

Table 2. Evaluation for scavenging of nitric oxide free radicals (NO) and Total phenolic content (TPC) in hard bark and soft bark extraction at the concentration of 10 to 1,000 µg/ml (n=5).

Types of bark	Solvents	IC_{50} (µg/ml) ± SD	Total phenolic content (TPC) (mg/g GAE)
Hard bark	95% Hexane	91.95 ± 2.10	12.37 ±1.32
	95% Ethyl acetate	254.86 ± 1.20	4.68 ±0.91
	95% Ethanol	45.83 ± 1.80	22.49 ±0.54
Soft bark	95% Hexane	497.33 ± 2.20	3.05 ±1.60
	95% Ethyl acetate	470.24 ± 2.0	3.17 ±1.83
	95% Ethanol	743.04 ± 1.60	2.65 ±0.80
	Ascorbic acid	8.19 ± 1.55	-
	Ibuprofen	11.34 ± 1.70	-

Table 2 shows that the hard bark extract in 95% ethanol had the highest capacity for Nitric Oxide scavenging (IC_{50}) of 45.83 ± 14.95, while the soft bark extract in 95% ethyl acetate had the highest capacity for Nitric Oxide scavenging (IC_{50}) of 470.24 ± 3.63. There were significant differences ($p<0.05$) in Nitric Oxide scavenging activity between hard and soft barks extracted in various solvents. When comparing to reference standard as ascorbic acid and ibuprofen, they still showed statistically significant differences ($p=0.05$) in Nitric Oxide scavenging activity as well. The total phenolic content of the samples showed large variations, between 2.65 ±0.80 and 22.49 ±0.54 mg/g GAE extract. The hard bark extract in 95% ethanol contained the highest total phenol content (22.49 ±0.54 mg/g GAE). The soft bark extract in 95% Ethyl acetate contained the highest total phenol content (3.17 ±1.83 mg/g GAE).

Table 3. Collagen content obtained from maceration of hard and soft barks in 95% of Hexane, 95% of Ethyl acetate and 95% of Ethanol by using Hydroxyproline assay.

Types of bark	Solvents	Collagen content at different concentration (µg/ml)	
		20 (Mean ± SD)	30 (Mean ± SD)
Hard bark	95% Hexane	41.32 ± 0.02	54.42 ± 0.02
	95% Ethyl acetate	17.64 ± 0.02	34.77 ± 0.01
	95% Ethanol	49.89 ± 0.02	44.34 ± 0.02
Soft bark	95% Hexane	34.77 ± 0.03	20.16 ± 0.02
	95% Ethyl acetate	4.54 ± 0.02	66.01 ± 0.02
	95% Ethanol	28,22 ± 0.02	24.19 ± 0.02

Table 3 indicates that 20 to 30 μg/ml of collagen content was found in hard bark extraction. At the concentration of hard bark extraction of 20 μg/ ml, the highest concentration of collagen content was found in 95% ethanol extraction at 49.89 ± 0.02 μg/ ml. It was statistically significantly different from others (P=0.05). Additionally, at the concentration of hard bark extraction of 30 μg/ ml, it showed the highest collagen content found in 95% hexane of 54.42 ± 0.02 μg/ml.

However, soft bark extraction showed different results. At the concentration of 20 μg/ ml of extraction, the highest collagen content was found in 95% hexane extraction with 37.77 ± 0.03 μg/ ml which was statistically significantly different from others ($P=0.05$), while at 30 μg/ ml of extraction, it showed the highest collagen content was found in 95% ethyl acetate of 66.01 ± 0.08 μg/ml.

4. Discussion and Conclusion

The study of soft and hard bark extraction in various solvents: 95% hexane, 95% ethyl acetate, and 95% ethanol indicated that each extraction provided the different phytochemical components. Soft bark in 95% ethyl acetate and hard bark in 95% ethanol extractions showed more positive results than those in other solvents. Besides that, different extracting solvents provided various capacity for Nitric Oxide scavenging. The khae ban bark had high phenolic content and antioxidant activity. At low concentrations of extract, the hard bark in 95% of ethanol provided anti-inflammatory activity and had collagen content higher than those in other solvents. Due to each type of bark containing different phytochemical compounds, they have different biological activity as well [15]. The soft and hard bark extraction in varieties of solvents had different extracting solvents provided various capacity for effect antioxidant activity. Because of varieties of solvents had appropriate. Therefore, depending on the properties of the plant. [16]

This research was relevant and supported the traditional Thai folk medicine using Khae ban hard bark for relief of inflammation and healing mouth sores. Furthermore, it corresponded to the research of [9] which specified that Khae ban bark was effective for wound healing by pathway of collagen formation. Additionally, a research study showed that Khae ban bark in 100% ethanol extraction, showed anti-inflammatory activity. Therefore, this study showed that Khae ban hard bark, in 95% ethanol, was one of the interesting plants which had a potential to be brought for developing as a cosmeceutical product, or pharmaceutical product healing mouth sores [8].

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