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Comparative study of semi-purification methods of *Caesalpinia sappan* L. extract: Thin layer chromatography and free radical scavenging activity

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Keywords: *Caesalpinia sappan* L.; Brazilin; Protosappanin A; DPPH; TLC fingerprint

Objectives: The purpose of this study was to compare thin layer chromatography (TLC) fingerprints and free radical scavenging activities of semi-purified *Caesalpinia sappan* L. extracts.
Methods: Sappanwood crude extract was prepared by maceration of heartwood with 95% ethanol. The crude extract was semi-purified by partition method and ion-exchange chromatography. For partition method, the crude extract was partitioned with deionized water, dichloromethane and ethyl acetate, respectively. For ion-exchange chromatography, Diaion® HP-20 was used to semi-purify the extract. Brazilin and protosappanin A reference standards were isolated from ethyl acetate fraction by vacuum liquid chromatography on silica gel and Shephadex LH20. The identification of brazilin and protosappanin A were performed by HR-ESIMS and NMR. All extracts and standards was evaluated for TLC fingerprint. The free radical scavenging activity of all extracts was determined using DPPH assay.
Results: Ethyl acetate fraction had the highest percentage of yield (71.05%) followed by Diaion® HP-20 fraction (69.5%), water fraction (12.69%) and dichloromethane fraction (5.02%), respectively. The isolation and identification of protosappanin A and brazilin were approved by NMR and MS data with a previous report. All semi-purified extracts showed that brazilin was a major band, whereas protosappanin A was a minor band in TLC fingerprint. The water fraction had the highest IC_{50} value of DPPH scavenging activity (1.19 ± 0.02 µg/ml) followed by Diaion® HP-20 (2.08 ± 0.02 µg/ml), ethyl acetate fraction (2.03 ± 0.04 µg/ml), crude extract (2.72 ± 0.03 µg/ml) and dichloromethane fraction (3.95 ± 0.05 µg/ml), respectively.
Conclusion: The ethyl acetate fraction and Diaion® HP-20 fraction had high percentage of yields and similar DPPH scavenging activity. Both fractions showed improved antioxidant activity when compared to crude extract. The TLC finger print showed that brazilin was a major compound of sappanwood. In further study, brazilin content of various sappanwood extracts will be evaluated by using high pressure liquid chromatography.

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Introduction

Caesalpinia sappan L. (Fabaceae) or commonly known Sappanwood is a small tree widely found in Thailand. The heartwood of sappanwood is used in Thai traditional medicine for wound healing, diarrhea, epilepsy, blood disease and menstrual disorder etc. Moreover, red color from heartwood is widely used in foods, beverages, fabrics and cosmetics. Sappanwood was composed of various phenolic compounds such as brazilin, protosappanin A-E, sappanchalcone, sappanone B, brazilein, and 3-deoxysappanchalcone[1,2]. Brazilin is the major component of sappanwood. Its content was 8.7-22.2% w/w of the extract[3]. Maceration is a common method for phenolic compound extraction. It is simple for both initial and bulk extractions. However, this method also yielded some unwanted compounds such as sugars, fats, terpenes and waxes[4]. Therefore, the extract obtained from this method may have decreased activity. Purification of a crude extract is essential to remove impurity[5] and to increase its activity when compared with a crude extract[6]. In this study, partition method and ion-exchange chromatography were used to semi-purify the crude extract of sappanwood and compared by evaluation for a free radical scavenging activity and TLC fingerprint of each semi-purified extract.

Materials and methods

Chemicals DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbic acid, gallic acid and Diaion HP-20 were supplied from Sigma Aldrich (USA). Ascorbic acid was obtained from Carlo Erba (Italy). Absolute ethanol were purchased from Honey-well (USA). Shephadex® LH20 and silica gel 60 GF_{254} were supplied from Merck (USA).

Plant material *Caesalpinia sappan* heartwood was collected from Band dong bung, Prachinburi province, Thailand in July, 2014. The heartwood was cut into small pieces, then dried in a hot air oven at 60°C for 6 hours and grounded to coarse powder.

Preparation of crude extract The sappanwood powder was macerated with 95% ethanol for 24 hour. The maceration was repeated three times with the same volume of solvent. Ethanol was removed by using rotary evaporator at 40°C under vacuum to obtain the crude extract.

Partition of crude extract The crude extract was dissolved in deionized water and partitioned with dichloromethane and ethyl acetate, respectively. The fractions of water, dichloromethane and ethyl acetate were obtained.

Ion-exchange chromatography The method was slightly modified from a previous study[7]. The crude extract was dissolved in 35% ethanol, then sample was loaded on Diaion® HP-20 column (1 g of crude extract per 60 g of resin) and eluted with 35% ethanol. The fractions were pooled and dried by using rotary evaporator at 40°C under vacuum to obtain semi-purified crude extract (Diaion® HP-20 fraction).

Isolation of brazilin and protosappanin A The ethyl acetate fraction was subjected to vacuum liquid chromatography on silica gel (hexane-ethyl acetate gradient) to give seven fractions (A-G). Fraction G was separated by silica gel column (hexane-ethyl acetate) to give eight fractions (G1-G8). Fraction G2 was purified by Shephadex LH20 to give Protosappanin A, 4.1 mg. Fraction G3 was subjected to silica gel column (hexane-ethyl acetate gradient) to give Brazilin, 3.1 mg. The identification of brazilin and protosappanin A was performed by high-resolution electrospray ionization mass spectrometry (HR-ESIMS) for structure mass calculation and nuclear magnetic resonance (NMR) for structural determination. Brazilin and protosappanin A were used as reference standards in this study.

Thin layer chromatographic densitometric method The sample and standard solution at concentration of 100 µg/ml were spot in band length 5.0 mm and 20 µl/spot with a Linomat V automatic sample spotter (CAMAG) under nitrogen flow. TLC plate was developed in chamber with mobile phase ethyl acetate-hexane (6:4). The densitometric scanning at a wavelength of 280 nm[7] was presented using TLC scanner 3 (CAMAG) with winCAT software.

DPPH radical scavenging activity Radical scavenging activity of each sample was determined by DPPH assay[8]. Briefly, a sample at concentration range between 0.2-10 µg/ml was added to DPPH solution in a 96-well plate. The mixture was incubated for 30 min at room temperature and protected from light. Its absorbance was measured at 510 nm with a microplate reader (VICTOR® 3, Model 1420-012). Each sample was determined in triplicate. Ascorbic acid and gallic acid were used as positive references. Percent of DPPH inhibition was calculated using equation 1. IC_{50} value (a concentration providing 50% inhibition) of each sample was determined.

$$\%\text{Inhibition of DPPH} = \frac{(A-B) - (C-D)}{(A-B)} \qquad (1)$$

Where A is absorbance of solution of ethanol and DPPH; B is absorbance of ethanol; C is absorbance of solution of a sample and DPPH; and D is absorbance of solution of a sample and ethanol.

Results and discussion

Identification of isolated compounds Chromatographic separation of ethyl acetate fraction resulted in the isolation of protosappanin A and brazilin. Identification of these isolates was carried out by comparison of their NMR and MS data with the reported values[9].

Protosappanin A. Colorless needles, $C_{15}H_{12}O_5$ HR-ESIMS: *m/z* 295.0582 $[M+Na]^+$. ^{1}H NMR (300 MHz, acetone-d_6) : δ 3.41 (2H, s, H-8), 4.47 (1H, s, H-6), 6.74 (1H, d, brs Hz, H-4), 6.75 (1H, brd, *J*= 7.8, 9.3 Hz, H-2), 6.77 (2H, s, H-12 and H-9), 7.13 (1H, d, *J*= 7.8 Hz, H-1) ; ^{13}C NMR (75 MHz, acetone-d_6): 48.8 (C-8), 78.4 (C-6), 108.7 (C-4), 113.0 (C-2), 117.1 (C-12), 117.2 (C-9), 124.8 (C-1a), 126.8 (C-8a), 131.4 (C-1), 131.4 (C-12a), 145.0 (C-11), 145.2 (C-10), 158.8 (C-3), 159.2 (C-4a).

Brazilin. Red crystals, $C_{16}H_{14}O_5$ HR-ESIMS: *m/z* 309.0738 $[M+Na]^+$,. ^{1}H NMR (300 MHz, CD_3OD): δ 2.77 (1H, d, *J* = 15.6 Hz, H-7), 3.02 (1H, d, *J* = 15.6 Hz, H-7), 3.69 (1H, d, *J*= 11.1 Hz, H-6), 3.93
(1H, d, *J* = 11.4 Hz, H-6), 3.96 (1H, s, H-12), 6.29 (1H, d, *J* = 2.4 Hz, H-4), 6.45 (1H, dd, *J* = 8.1,2.4 Hz, H-2),6.59 (1H, *s*, H-11), 6.70 (1H, s, H-8), 7.18 (1H, d, *J* = 8.1 Hz, H-1); ^{13}C NMR (75 MHz, CD_3OD): 42.9 (C-7), 51.0 (C-12), 70.8 (C-6), 78.0 (C-6a), 104.2 (C-4), 109.9 (C-2), 112.4(C-11), 112.8 (C-8), 115.5 (C-1a), 131.3 (C-7a), 132.2 (C-1), 137.4 (C-11a), 145.3 (C-10), 145.8 (C-9), 155.7 (C-3), 151.8 (C-4a).

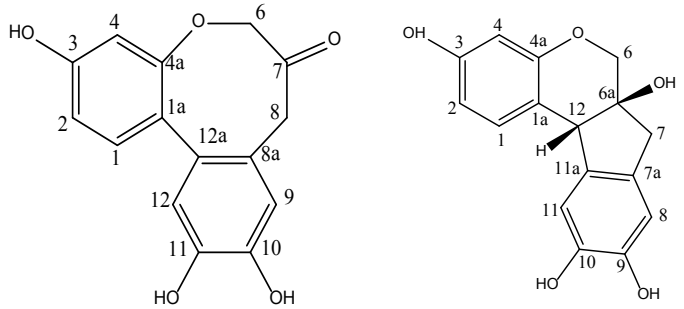

Figure 1. Structure of (1) protosappanin A and (2) brazilin

Semi-purification method of crude extracts Percentage yields of semi-purified extracts when compared to the content of initial crude extract are shown in Table 1. As for partition method, the ethyl acetate fraction had the highest yield followed by the water fraction and the dichloromethane fraction, respectively. The difference of percentage yields was as result of different properties of compounds in the extract such as solubility and polarity. Sappanwood extract contained many phenolic compounds[1,2]. Ethyl acetate is semi-polar solvent which was suitable for the extraction of phenolic compounds[4]. Partition method could be used for separation of chemical components in a sappanwood extract. The ion-exchange chromatography gave high yield of the extract similar to that of the ethyl acetate fraction. The previous study showed that ion-exchange chromatography could improve brazilin content in crude extract due to property of Diaion® HP-20 on absorption and elution of phenolic compounds with the appropriate mobile phase[7].

Table 1. Percentage yields of semi-purified extracts obtained from various semi-purification methods of the crude extract.

Method	Extraction solvent	%Yield
Partition	Water	12.69
	Ethyl acetate	71.05
	Dichloromethane	5.02
Ion-exchange Chromatography	Ethanol 35%	69.55

Thin layer chromatographic densitometric method TLC fingerprints of reference standards and various sappanwood extracts are showed in Figure 2. All extracts presented chromatographic band corresponding to that of standard brazilin. Moreover, we found very thin chromatographic band of protosappanin A in all sappanwood extracts (Figure 2B). In addition, the TLC densitogram in Figure 3 showed that brazilin was a major compound in the crude extract and all studied fractions, whereas protosappanin A was rarely found.

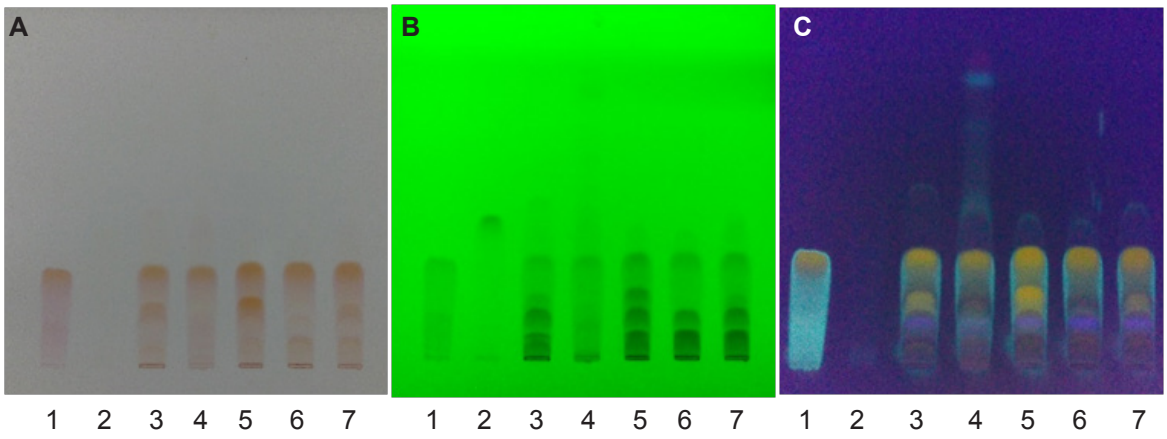

Figure 2. TLC fingerprints of 1 = standard brazilin, 2 = standard protosappanin A, 3 = crude extract, 4 = dichloromethane fraction, 5 = water fraction, 6 = ethyl acetate fraction, 7 = Diaion® HP-20 fraction. Stationary phase: silica gel GF_{245} .Solvent system: ethyl acetate-hexane (6:4). Detection: A = natural light, B = UV 254 nm, C = UV 366 nm.

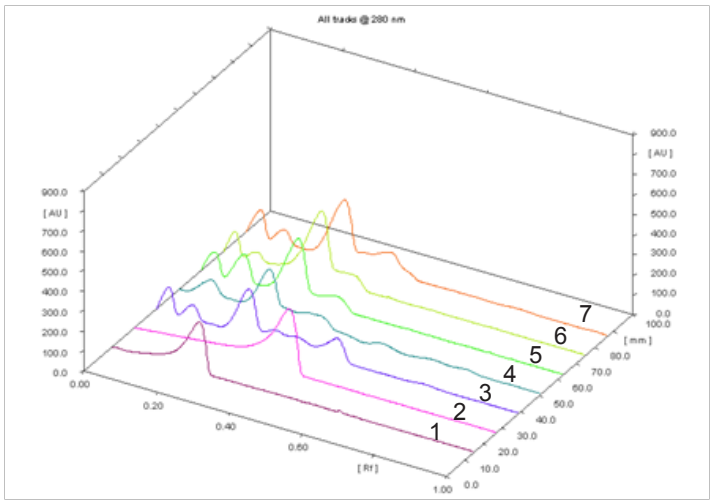

Figure 3. 3D TLC densitogram of 1 = standard brazilin, 2 = standard protosappanin A, 3 = crude extract, 4 = dichloromethane fraction, 5 = water fraction, 6 = ethyl acetate fraction, 7 = Diaion® HP-20 fraction.

DPPH radical scavenging activity The IC_{50} values of sappanwood extracts are shown in Table 2. All sappanwood extracts obtained had strong free radical scavenging activity. The water fraction showed the highest DPPH radical scavenging activity. In previous study, heartwood of sappanwood showed high content of phenolic compound (150

mg/g) and tannin (171 mg/g)[12]. Tannin is water soluble polyphenol[13]. It was reported the potential of free radical scavenging activity[14,15]. The highest IC_{50} value of water fraction may be explained that it had high tannin content. DPPH scavenging activity of Diaion® HP-20 fraction and ethyl acetate fraction were similar and better than the crude extract. The dichloromethane fraction had the lowest activity. However, its antioxidant activity was not only from brazilin but also various less polar compounds of sappanwood extract[11]. Different activities of all sappanwood extracts may be due to difference in content of bioactive compounds such as brazilin as shown on TLC densitogram (Figure 3). The result indicated that both methods could be used to semi-purify the crude extract and increase DPPH scavenging activity of the extract.

Table 2. DPPH radical scavenging activity of various sappanwood extracts and positive controls (n = 3, mean ± S.D.)

Sample	DPPH inhibition IC_{50} (μg/ml)
Crude extract	2.72 ± 0.03
Water fraction	1.19 ± 0.02
Ethyl acetate fraction	2.08 ± 0.02
Dichloromethane fraction	3.95 ± 0.05
Diaion® HP-20 fraction	2.03 ± 0.04
Ascorbic acid	1.58 ± 0.02
Gallic acid	0.63 ± 0.01

Conclusion

The present study was able to show that the partition with ethyl acetate and ion-exchange chromatography by Diaion® HP-20 gave the semi-purified extracts with high yield and similar DPPH scavenging activity. Crude extract and all semi-purified extracts had brazilin as a major component which could be used as a biomarker of sappanwood extract. However, determination of brazilin content in the extract will be studied further using a more precise method such as high pressure liquid chromatography.

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