Agr. Nat. Resour. 53 (2019) 621–628

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Antioxidant and anti-HIV properties and isolation of bioactive compound of *Hymenodictyon orixense* (Roxb.) Mabb.

Melissa Soun-udom[a], Kiattawee Choowongkomon[b], Srunya Vajrodaya[a], Siriluk Ratanabunyong[b], Rungcharn Suksungworn[a,c], Nattawut Srisombat[a], Sorawit Bapia[a], Sutsawat Duangsrisai[a,c,*]

[a] *Phyto-Chemodiversity and Ecology Research Unit, Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

[b] *Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

[c] *Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok 10900, Thailand*

Article Info

Article history:
Received 21 Mar 2018
Revised 9 June 2018
Accepted 19 June 2018
Available online 25 December 2019

Keywords:
Anti-HIV activity,
Antioxidant activities,
Fractionation,
Hymenodictyon orixense (Roxb.) Mabb

Abstract

Hymenodictyon orixense (Roxb.) Mabb., or "Som Kob" or "U-Lok" in Thai, is known as a folk medicinal plant. This plant has been used against different dermal diseases, fever and chicken pox, indicating different bioactivity potentials. Methanolic extracts from three parts (bark, wood, fruit) of *H. orixense* were tested for their antioxidant activities using five assays (total phenolic content, total flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power, nitric oxide radical activity). Bark crude extract had the highest antioxidant activity, so this part was chosen for further fractionation. The fractions were ranked based on from antioxidant and anti-HIV bioactivities. Fraction 2 had the highest level for total flavonoid content (323.63 mg of quercetin (QE) per gram) and nitric oxide radical activity (half maximal inhibitory concentration (IC_{50}); 88.38 µg/mL). Fraction 4 contained the highest amounts of phenolic compounds (32.89 mg of gallic acid equivalent/g), while Fraction 5 had the highest activity of DPPH radical activity (IC_{50}; 192.76 µg/mL) and ferric reducing antioxidant power (88.08 mg of trolox equivalent/g). For anti-HIV activity, all fractions produced a percentage inhibition greater than 50%, with Fraction 3 being the highest (89.80%) compared to the positive control (97.06%). In addition, scopoletin was isolated from the chloroform extract of the *H. orixense* bark. Isolation and structural elucidation of other crude chloroform extracts will be further analyzed. The current study demonstrated the potential of developing *H. orixense* as an antioxidant and anti-HIV agent.

Introduction

Free radicals affect human health by causing severe diseases such as cancer, cardiovascular disease and macular degeneration (Machlin and Bendich, 1987; Pham-Huy et al., 2008). These free radicals can cause lipid peroxidation, cell damage and organelle disruption (Bailly and Cotelle, 2005). Many of the antioxidants can be found in natural products. Therefore, investigating chemical compositions of natural products is critical to the search of new antioxidants that will help cure non-communicable diseases.

* Corresponding author.
E-mail address: fscissw@ku.ac.th (S. Duangsrisai)

https://doi.org/10.34044/j.anres.2019.53.6.09

Apart from these non-communicable diseases, many communicable diseases, such as AIDS caused by HIV (human immunodeficiency virus), continue to be a major global public health issue. In 2015, it was estimated that 36.7 million people were living with HIV, but only 18.2 million people had accessed the antiretroviral therapy (UNAIDS, 2017). In Thailand, 440,000 people are living with HIV, but only 64% of adult patients are on antiretroviral treatment (AVERTing HIV and AIDS, 2016). Therefore, developing an effective antiviral drug is important. Volberding and Deeks (2010) discussed issues associated with six major types of drugs are currently used to treat HIV patients. Each group targets different steps in HIV replication such as entry inhibitors, reverse transcriptase inhibitor or integrase inhibitor, as most of the current HIV drugs are expensive and have side effects. Consequently, it is important to find a new and better source for treatments. In 1989, the World Health Organization declared the necessity of developing and integrating natural products in modern drugs to lessen the side effects and to increase the affordability for those undergoing long-term treatment (Kurapati et al., 2016).

A large number of medicinal plants have played important roles in many traditional systems of medication in Asian countries (Sheng-Ji, 2001). *Hymenodictyon orixense* (Rubiaceae), known in Thai as "som kob" or "u-lok", is one of traditional medicinal plants in Thailand that have many bioactivities against various diseases; the bark extract of *H. orixense* has been used to cure dermal diseases (Dhiman et al., 2012), fever (Neamsuvan and Tanthien, 2015) and folliculitis (Jagtap et al., 2009) while the wood has been used to treat herpes (Akter et al., 2014) and the leaves can be used to treat chickenpox sweating fever (Manilal and Remesh, 2009) and toothache irritation (Sakong et al., 2011). However, there has been little information regarding the antioxidant and anti-HIV activities of different parts of *H. orixense*. Therefore, the purpose of the present study was to investigate the antioxidant activity of three parts (stem bark, wood, fruit) of this plant using different assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power, nitric oxide radical activity, total phenolic content and total flavonoid content. Parts showing the greatest potential were further fractionated, isolated and evaluated for their antioxidant and anti-HIV activities.

Materials and Methods

Plant collection

Hymenodictyon orixense (Roxb.) Mabb. was collected from Wang Man Sub-district, Wat Sing district, Chai Nat province, Thailand. The plant specimens were identified using the key and description from Flora of China (Tao and Taylor, 2011). The voucher specimens were deposited in the Phyto-Chemodiversity and Ecology Research Unit, Kasetsart University, Bangkok, Thailand. Samples of the stem bark, wood and fruit of this plant were cleaned, air-dried at room temperature and separately powdered using an electric grinder.

Plant extraction, fractionation and isolation

Samples (10 g each) of powdered bark, wood and fruit were macerated in methanol (J.T. Baker, USA) at room temperature for 5 d, followed by re-maceration for 5 d. After filtration, the filtrate was evaporated at 45ºC under a reduced pressure in a rotary evaporator (R-205; Büchi, Thailand) to give a syrupy mass of crude methanol extract of stem bark, wood and fruit, respectively. The crude methanol extract samples of bark, wood and fruit were used for antioxidant screening testing. Bark was chosen to be further fractionated and isolated. The powdered stem bark (7.8 kg) was extracted using the protocol above. Methanol extract was re-extracted with 300 mL of $CHCl_3$ (J.T. Baker, USA) mixed with 50 mL H_2O repeated three times to give 103.61 g of crude chloroform extract of bark.

The crude chloroform extract of bark (45.17g) was applied to column chromatography using a 0.2–0.5 mm silica gel (Merck, Germany) column (350 g, 5 cm × 90 cm) and eluted with the mixtures of n-hexane:$CHCl_3$, $CHCl_3$:Me_2CO (J.T. Baker, USA) as follows: n-hexane:$CHCl_3$ (1:1), n-hexane:$CHCl_3$ (3:7), n-hexane:$CHCl_3$ (1:9), $CHCl_3$:Me_2CO (9:1), $CHCl_3$:Me_2CO (7:3), $CHCl_3$:Me_2CO (1:1) and $CHCl_3$:Me_2CO (3:7) and pure Me_2CO and MeOH, with 250 mL of each elution solvent providing 10 mL for each fraction. Thin layer chromatography (TLC) was applied and every fraction was spotted on a silica gel-coated plate and placed in the closed chamber with the mobile phase which was mixed solvents. The fractions were investigated under ultraviolet light at two different wavelengths (254 nm and 365 nm). Fractions that had similar TLC profiles were combined and named as the combined fraction.

Three techniques were used for isolation and purification: a preparative TLC technique with silica gel (GF_{254}, 0.2 mm; Merck, USA), sephadex (LH-20 100G, GE; Sigma-Aldrich, Germany) column chromatography, and recrystallization. After pure compound had been obtained, its structure was elucidated using spectral data from nuclear magnetic resonance (NMR; Bruker Avance III HD 400 Hz NMR spectrometer; USA) and mass spectrometry (Bruker/microtof-Q III high resolution mass spectrometer; USA).

Antioxidant activities

Total phenolic content

The phenolic compounds assay followed the protocol of Folin and Ciocalteu (1927) as described in Khlift et al. (2013). The plant sample (1 mg/mL, 0.25 mL) was added with Folin-Ciocalteu (0.2 N, 1.25 mL; Merck, USA) and incubated for 5 min at room temperature. Then, sodium carbonate (75 g/L in water, 1 mL; Merck, Germany) was added and the mixture was incubated for 1 hr. The absorbance was measured at 765 nm. Gallic acid (Merck, USA) was used as the standard. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

Total flavonoid content

The flavonoids were evaluated using the Dowd method as described in Khlift et al. (2013) with some modification. Aluminum trichloride (Univar, New Zealand) in methanol (2% weight per volume (w/v), 0.8 mL) was added to the plant sample (1 mg/mL, 0.8 mL), and incubated for 15 min at room temperature. The absorbance was measured at 415 nm. The standard was quercetin acid (Sigma-Aldrich, Germany). The results were expressed as quercetin equivalent (QE) per gram of extract.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The DPPH assay followed the protocol in Khlift et al. (2013) and Gourine et al. (2010) with some modifications. Various concentrations of plant sample (0.625–10 mg/mL, 1 mL) were mixed with 2,2-diphenyl-1-picrylhydrazyl (DPPH; Merck, USA) in methanol and incubated for 30 min in the dark. Then, the mixture was measured for absorbance at 517 nm. The free radical scavenging activity was expressed as the percentage inhibition using Equation 1:

$$\text{Inhibition (\%)} = (A_{blank} - A_{sample}) / A_{blank} \times 100 \quad (1)$$

where A_{blank} is the absorbance of all reagents without the sample, while A_{sample} is the absorbance of the sample. The percentage inhibition was plotted against the concentration of the plant samples to determine the half maximal inhibitory concentration (IC_{50}) values.

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was evaluated using the modified method from Iqbal et al. (2015) and Gourine et al. (2010). The fresh FRAP reagent was prepared by mixing sodium acetate buffer (Univar, New Zealand) (300 mM, 100 mL, pH 3.6) with 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ; Fluka, Switzerland) in 40 mM HCL (10 mM, 10 mL) and $FeCl_3.6H_2O$ (20 mM, 10mL; Chem-supply, Australia). A plant sample (0.1 mg/mL, 0.15 mL) was added in the fresh FRAP reagent (2.850 mL) and incubated for 30 min in the dark. The absorbance was measured at 593 nm. Trolox (Sigma-Aldrich, Germany) was used as the standard. The results were expressed as trolox equivalent (TE) per gram of extract.

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging (NO) assay followed the protocol in Ferreres et al. (2017). Various concentrations of plant sample (50–500 µg/mL, 0.25 mL) were mixed with sodium nitroprusside (Himedia, India) in phosphate buffer saline (10 mM, 1.25 mL) and incubated for 150 min in the dark. Then, the mixture was added with Griess reagent (1.5 mL; (1% (w/v) sulfanilamide (Carlo Erba, France), 2% (w/v) H_3PO_4 (Macron Fine Chemicals, China) and 0.1% (w/v) naphthaylethylenediamine hydrochloride (AppliChem Panreac, Germany)). The absorbance was measured at 546 nm. The nitric oxide radical scavenging activity was expressed as the percentage inhibition using Equation 1.

HIV-1 reverse transcriptase inhibition assay (Anti-HIV-1 RT)

The HIV-1 reverse transcriptase inhibition assay (anti-HIV-1 RT) assay followed the fluorescence method from Silprasit et al. (2011) with modifications. A reverse transcriptase assay kit was purchased from EnzChek®, Molecular Probes, Inc., the Netherlands. To measure HIV-1 RT activity with inhibitors, 5 µL of 25 nM/µL purified recombinant HIV-1 RT and 5 µL of 50 µg/mL of each plant extract sample were added into the wells for sample testing reaction (RT_{Sample}). The addition of 5 µL of Tris buffer instead of plant extract sample served as the control reaction ($RT_{Control}$). The blank reaction ($RT_{Background}$) was prepared by adding 2 µL of 0.2 M EDTA and 5 µL of Tris buffer. Nevirapine (GPO, Thailand) was added instead of plant extract sample for the positive control inhibition reaction. The plate was gently mixed before adding 15 µL of primer/template polymerization buffer into all wells. Reactions were started by incubation at 25°C for 30 min and then stopped by the addition of 2 µL of 0.2 M EDTA. Three independent experiments were performed. The fluorescence of the reaction was measured in a microplate reader with an excitation wavelength of 502 nm and an emission wavelength of 523 nm. The inhibitory effect on HIV-1 RT activity was expressed as the percentage of relative inhibition, using Equation 2 (Silprasit et al., 2011):

$$\text{Relative Inhibition (\%)} = \{[(RT_{Control} - RT_{Background}) - (RT_{Sample} - RT_{Background})] / (RT_{Control} - RT_{Background})\} \times 100 \quad (2)$$

Results

Antioxidant activities of crude extract of Hymenodictyon orixense

The total phenolic content (TPC) values for bark, wood and fruit are shown in Fig. 1A. The fruit extract had the highest TPC whereas the wood had the lowest. The highest TFC was found in the wood extract (7.82 mg QE/g), followed by the fruit and the bark extracts. Bark extract had the highest NO (IC_{50}; 18.28 mg/mL) and the highest FRAP (242.85 mg of TE/g). Although the wood extract showed the highest DPPH (IC_{50}; 0.09 mg/mL), it had the lowest NO (IC_{50}; 121.32 mg/mL) and FRAP (92.64 mg of TE/g). Fruit extract exhibited the lowest DPPH (IC_{50}; 1.04 mg/mL) as shown in Fig. 1C–E.

From the ranking of the five antioxidant activities (Table 1), the bark had the highest rank in overall antioxidant activity. Wood extract ranked first in TFC and DPPH, while it had the lowest ranking for the three others. Fruit extract was ranked second, because it showed only the highest ranking in TPC, with second rankings in TFC, FRAP and NO, and the lowest ranking in DPPH. Because the bark extract exhibited the highest antioxidant activity, it was subject to further fractionation and bioactivity tests.

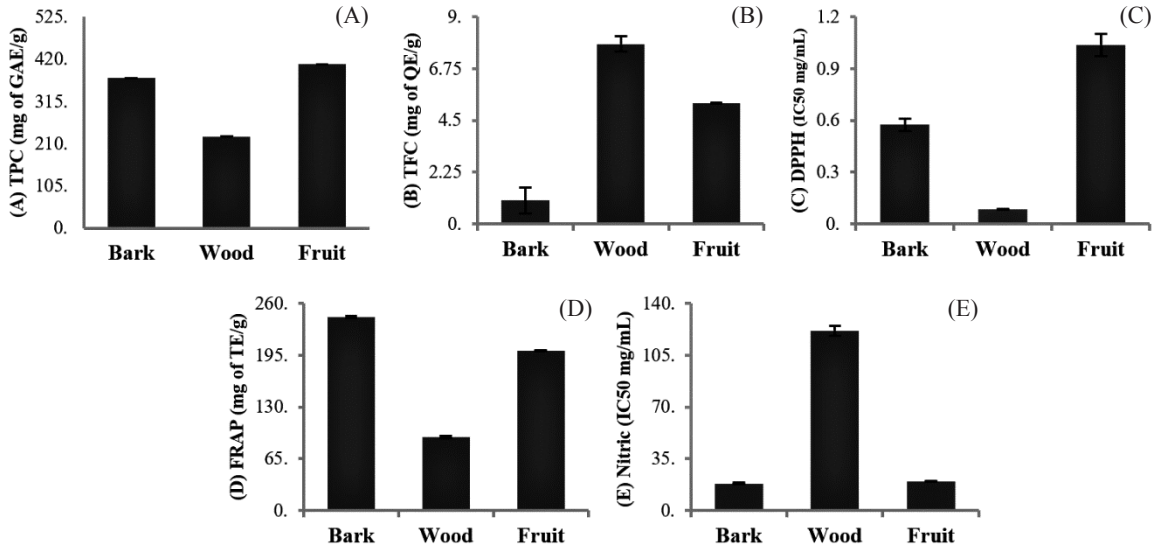

Fig. 1 Antioxidant activities of stem bark, wood and fruit methanolic extract of *H. orixense* : (A) total phenolic content (TPC); (B) total flavonoid content (TFC); (C) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; (D) ferric reducing antioxidant power (FRAP); (E) nitric oxide radical activity, where data are derived from 3 replicates (n = 3) with error bar showing SD, GAE = gallic acid equivalent ; QE = quercetin ; IC50 = half maximal inhibitory concentration ; TE = trolox equivalent

Table 1 Ranking of antioxidant activity of bark, wood and fruit of H. orixense

Plant Part	Antioxidant activity					Ranking
	TPC (mg GA/g)	TFC (mg QE/g)	DPPH (IC_{50} mg/mL)	FRAP (mg TE/g)	NO (IC_{50} mg/mL)	
Bark	372.57 ± 0.02	1.03 ± 0.56	0.57 ± 0.04	242.85 ± 1.29	18.28 ± 0.50	1
Wood	227.57 ± 0.01	7.82 ± 0.33	0.09 ± 0.01	92.64 ± 1.45	121.32 ± 3.23	3
Fruit	406.86 ± 0.03	5.25 ± 0.01	1.04 ± 0.06	200.78 ± 0.66	19.93 ± 0.37	2

TPC = total phenolic content; TFC = total flavonoid content; DPHH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; FRAP = ferric reducing antioxidant power; NO = nitric oxide radical activity; GAE = gallic acid equivalent ; QE = quercetin ; IC50 = half maximal inhibitory concentration ; TE = trolox equivalent. Values are shown as mean ± SD (n= 3 replicates).

Fractionation of bark extract of Hymenodictyon orixense

The crude chloroform extract of *H. orixense* bark was applied to column chromatography and eluted to collect fractions. In total, 223 fractions were obtained, which were further combined according to their similarity in the TLC profiles. The combined Fractions 1, 2 and 3 consisted of the fractions which were eluted with hexane:chloroform (1:1 ratio). Combined Fraction 4 was obtained from several solvent systems (hexane:chloroform using 1:1 to 1:9 ratios). Combined Fraction 5 was eluted from the solvent system chloroform:acetone (from 9:1 to 3:7 ratios).

Antioxidant activities of bark chloroform fractions of Hymenodictyon orixense

The TPC values of the five fractions were in the range 22.01–32.89 mg GAE/g (Fig. 2A). Fraction 4 had the highest TPC. The TFC values were in the range 317.94–323.63 mg QE/g (Fig. 2B). The radical scavenging ability in DPPH assay showed a wide range in the IC_{50}, (192.76–306.24), with Fraction 5 having the highest DPPH (lowest IC_{50} values), but this was not significantly higher than that of Fraction 4. FRAP values were in the range 83.50–88.08 mg of TE/g and was the highest in Fraction 5 (Fig. 2C and 2D).

Similar to DPPH, NO was expressed as IC_{50}. The IC_{50} was in the range 88.38–191.91 µg/mL. Fraction 5 had the highest NO, while Fraction 2 had the lowest activity (Fig. 2E). Among the five fractions, Fraction 5 had the greatest performance in DPPH and FRAP, while Fraction 2 had the highest activity in NO.

Anti HIV-1 reverse transcriptase activity of bark chloroform extract of Hymenodictyon orixense

The HIV-1 RT inhibition activity of the bark extract fractions was in the range 52.54–89.80% relative inhibition (Fig. 3). Fraction 3 had the highest inhibition activity, which was not significantly different from that of the positive control (nevirapine), Fraction 1 and Fraction 2 showed significantly lower inhibition activity than Fraction 4 and Fraction 5.

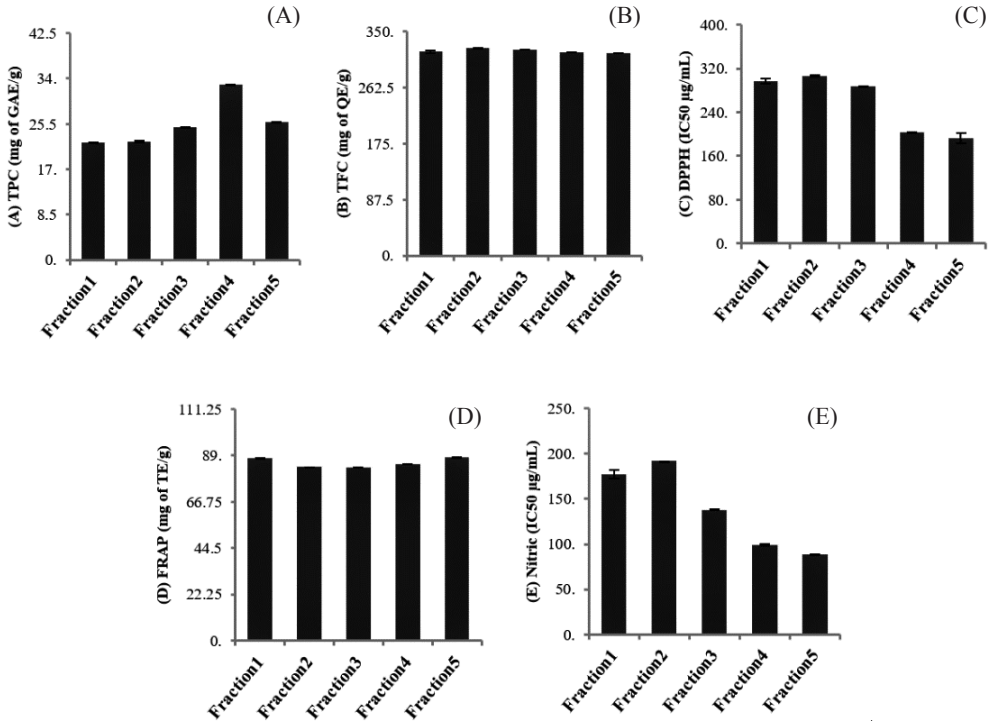

Fig. 2 Antioxidant activities of *Hymenodictyon orixense* bark chloroform extract: (A) total phenolic content (TPC); (B) total flavonoid content (TFC); (C) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; (D) ferric reducing antioxidant power (FRAP); (E) nitric oxide radical activity, where data are derived from 3 replicates (n = 3) with error bar showing SD, GAE = gallic acid equivalent ; QE = quercetin ; IC50 = half maximal inhibitory concentration ; TE = trolox equivalent

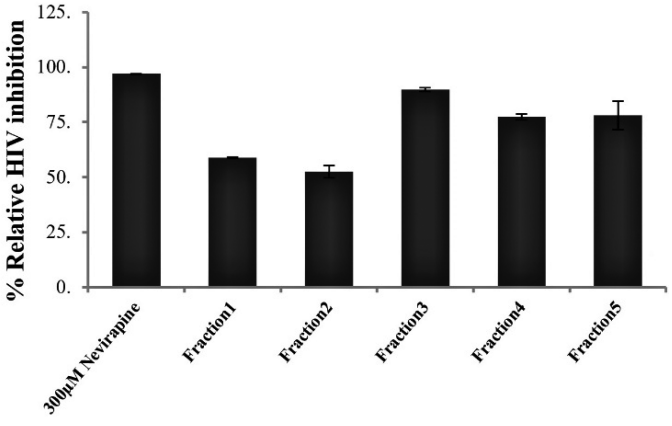

Fig. 3 Inhibition of HIV-1 RT by fractions from *H. orixense* bark chloroform extract, where nevirapine was used as the positive control for the HIV-1 reverse transcriptase assay and data are derived from 3 replicates (n = 3) with error bar showing SD

Isolation and purification of bark extract of Hymenodictyon orixense

The combined Fraction 3, 4 and 5 were targeted for isolation of pure compounds due to their high antioxidant activity and anti HIV-1 RT activity (Table 2). Greenish crystals were recovered from Fraction 62 in the combined Fraction 4. This crystal was recrystallized with hexane and chloroform. In total, 39.1 mg of greenish crystal (Compound 1) were obtained and produced a blue fluorescence under ultraviolet light at 365 nm. The chemical structures of peaks were identified using the comparison of the ^{1}H-NMR and ^{13}C-NMR data with the literature data (Table 3). The ^{1}H-NMR spectrum of Compound 1 showed one methoxy group at chemical shift (δ_H) 3.91 and four aromatic protons (δ_H 6.26, 6.86, 6.94, 7.62) combined with 10 carbons from the ^{13}C-NMR spectrum. According to the electrospray ionization mass spectrometry data, the molecular weight was 193.0511 m/z. Due to atmospheric pressure chemical ionization which is a soft ionization technique that affects the result shown in [M+H$^+$], the exact molecular weight was actually 192.0511, which was similar to the calculated molecular weight of $C_{10}H_8O_4$ (MW = 192). The results confirmed that this compound was 7-hydroxy-6 methoxycoumarin, or scopoletin (Fig. 4).

Table 2 Ranking of antioxidant activity and anti-HIV-1 reverse transcriptase activity of *H. orixense* bark chloroform extract

Fraction	Antioxidant activity					Anti-HIV1-RT (% Relative inhibition)	Ranking
	TPC (mg GA/g)	TFC (mg QE/g)	DPPH (IC_{50} mg/mL)	FRAP (mg TE/g)	NO (IC_{50} mg/mL)		
1	22.01 ± 0.02	318.53 ± 2.07	297.40 ± 4.92	87.67 ± 0.22	177.39 ± 4.81	58.88 ± 0.42	5
2	22.15 ± 0.17	323.63 ± 0.20	306.24 ± 1.35	83.50 ± 0.02	81.00 ± 0.11	52.54 ± 2.83	4
3	24.86 ± 0.03	322.06 ± 0.52	287.05 ± 0.14	83.25 ± 0.15	138.08 ± 0.33	89.80 ± 0.81	3
4	32.89 ± 0.03	317.94 ± 0.81	202.85 ± 0.21	85.00 ± 0.08	98.99 ± 0.92	77.35 ± 1.28	2
5	25.86 ± 0.05	316.76 ± 0.62	192.76 ± 9.88	88.08 ± 0.18	88.38 ± 0.26	78.09 ± 6.46	1

anti-HIV-1-RT = anti-HIV-1 reverse transcriptase activity; TPC = total phenolic content; TFC = total flavonoid content; DPHH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; FRAP = ferric reducing antioxidant power; NO = nitric oxide radical activity; GAE = gallic acid equivalent ; QE = quercetin ; IC50 = half maximal inhibitory concentration ; TE = trolox equivalent. Values are shown mean ± SD (n= 3 replicates).

Table 3 Nuclear magnetic resonance spectral data (400 Hz) of compound 1 and 7-hydroxy-6 methoxycoumarin or scopoletin

Position	Compound 1		7-hydroxy-6 Methoxycoumarin (Sutthivaiyakit et al., 2008)	
	δ_H	δ_C	δ_H	δ_C
1	-	161.49	-	161.85
2	6.26	113.40	6.23	112.84
3	7.62	143.34	7.58	143.67
4	6.94	103.20	6.87	103.18
5	-	150.26	-	150.22
6	-	144.04	-	144.55
7	6.86	107.57	6.82	107.79
8	-	149.73	-	150.22
9	-	111.50	-	111.33
OCH_3	3.91	56.42	3.91	56.38

δ_H = hydrogen chemical shifts; δ_C = carbon chemical shifts

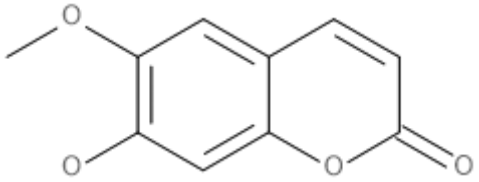

Fig. 4 Structure of 7-hydroxy-6 methoxycoumarin or scopoletin

Discussion

Antioxidant activities of crude extract

Based on the results of all the antioxidant assays, the bark extract of *H. orixense* had the highest antioxidant activity. This was consistent with the ethnobotanical information that indicated a number of uses of the bark of *H. orixense* in treatments of many diseases (Swe and Myint, 2006; Jagtap et al., 2009; Nareeboon et al., 2009; Dhiman et al., 2012; Kar et al., 2013; Akter et al., 2014; Neamsuvan and Tanthien, 2015; Rahman, 2015). Therefore, the crude methanolic extract was subjected to further fractionation to investigate the bioactivity and bioactive compounds in more detail.

Antioxidant activities and anti HIV-1 reverse transcriptase activity of bark chloroform extract

Five combined Fractions were obtained from column chromatography using various solvent systems. Fraction 5, which was eluted with a high polar solvent, had the highest FRAP, DPPH and NO values and was ranked first, with TPC ranked second and TFC ranked third. Fraction 5 still had high anti-HIV activity. It was possible that the most active compounds have high polarity, and most are likely dissolved in methanol which is more polar than chloroform. Previous studies on the extracts of mulberry juice and cacao husk showed that a high polar extract gave better anti-HIV activity (Sakagami et al., 2007; Sakagami et al., 2008). In addition, many researchers have used methanol extract to test activity and found that the methanol extract gave potent anti-HIV and antioxidant activities (Xu et al., 1996; Yu et al., 2012; Abarca-Vargas et al., 2016).

Fraction 3 had moderate antioxidant activities but had the highest anti-HIV-1 RT activity (89.98%). It is possible that the observed results may have been due to the synergistic effect, an effect arising between two or more substances that gives an effect greater than the individual effects. Many studies reported a synergistic effect in HIV inhibition. Hammer and Gillis (1987) observed synergistic activity of GM-CSF and AZT that inhibited HIV in the U-937 monocytic cell line. Chiappetta et al. (2011) showed the synergy of mixed polymeric micelles was more effective than the anti-HIV drug efavirenz. Dang et al. (2014) reported there was a synergistic/additive activity of tenofovir and nevirapine for enhanced prevention HIV with the vaginal route.

Bailly and Cotelle (2005) reported that the abundance of reactive oxygen species could lead to decreasing amounts of vitamins C and E including promoting lipid peroxidation and DNA damage which can link to oxidative stress and may support HIV disease pathogenesis

by decreasing the inflammatory immune response and increasing apoptosis. Therefore, plant extracts that contain high antioxidant activities may have potential for anti-HIV treatment. This may explain the obtained result showing that Fractions 3, 4 and 5, which had high antioxidant activities also had high anti-HIV activities. It can be assumed that antioxidant may play a role in the anti-HIV-1 RT mechanism.

Most chemotherapeutic agents are directed to the HIV-1 RT, a key enzyme that plays an essential and multifunctional role in the replication of the HIV-1 (Castro et al., 2006). A natural product that inhibits HIV-1 RT can be a target for drug design in the future.

Fraction 5 has the highest antioxidant activities, with good anti-HIV activity, but not the highest. It should be noted that only HIV-1 RT inhibition activity was tested, while there are many other methods to test for anti-HIV activity, such as using a protease inhibitor, integrase inhibitor and HIV-2 inhibition. Kapewangolo et al. (2013) tested two HIV-1 enzymes: protease (PR) and reverse transcriptase (RT) of *Plectranthus barbatus* leaves and found that they had good inhibition of HIV-1 PR, but were poor for HIV-1 RT. Some compounds have special attributes that only allow them to perform better in a certain anti-HIV test. For example, calanolide selectively inhibits HIV-1-RT, but not HIV-2-RT (Matthee et al., 1999). To obtain a complete picture of anti-HIV activity, it is suggested that different anti-HIV activity tests, targeting different stages during the HIV life cycle, should be conducted.

Pure compound of bark chloroform extract

The plants have been as a remedy by thousands of years. Several bioactivity compounds or so-called secondary metabolites exist in plants such as alkaloids, glycosides, flavonoids and coumarins (Bernhoft, 2010). Scopoletin, was isolated from the extract of *H. orixense* and is one of the coumarin derivatives found in various plants (Simoes et al., 2009; Darmawan et al., 2012). Scopoletin was reported to have several interesting properties such as antihypertensive, antitubercular, anti- inflammatory, anti-allergy, antibacterial, antifungal, antioxidant and anti-cancer (Gnonlonfin et al., 2012; Venugopala et al., 2013). Interestingly, the derivative of this compound was previously reported as a potent HIV RT inhibitor (Spino et al., 1998). In addition, the coumarin derivatives of scopoletin exhibited potent activity against HIV in different stages in the HIV replication cycle (Yu et al., 2003). For example, glycocoumarin and lipopyranocoumarin blocked viral adsorption. Calanolides and analogs inhibited reverse transcription, while 3-substituted-4-hydroxycoumarins had protease inhibition activity and tetramericcoumarins inhibited integration step. The reported activities of scopoletin suggest its role in anti-HIV1-RT activity in *H. orixense* extract.

Acknowledgements

The authors are grateful for the advice on the nuclear magnetic resonance experiment from Assistant Professor Dr Pakorn Wattana-Amorn from the Department of Chemistry, Kasetsart University, Bangkok, Thailand. This research was supported by a graduate scholarship provided by the National Research Council of Thailand (NRCT) as of fiscal year 2018, the Higher Education Research University Project of Thailand, Office of the Higher Education Commission and the Kasetsart University Research and Development Institute (KURDI).

References

Abarca-Vargas, R., Malacara, C.F.P., Petricevich, V.L. 2016. Characterization of chemical compounds with antioxidant and cytotoxic activities in *Bougainvillea* × *buttiana* Holttum and Standl, (var. Rose) extracts. Antioxidants. 5: 1–11.

Akter, R., Uddin, S.J., Grice, I.D., Tiralongo, E. 2014. Cytotoxic activity screening of Bangladeshi medicinal plant extract. J. Nat. Med. 68: 246–252.

AVERTing HIV and AIDS. 2016. HIV and AIDS in Thailand. https://www.avert.org/professionals/hiv-around-world/asia-pacific/Thailand, 18 August 2017.

Bailly, F., Cotelle, P. 2005. Anti-HIV activities of natural antioxidant caffeic acid derivatives: Toward an antiviral supplementation diet. Curr. Med. Chem. 12: 1811–1818.

Bernhoft, A. 2010. A brief review. In: Bernhoft, A. (Ed.). Bioactive Compounds in Plants—Benefits and Risks for Man and Animals. The Norwegian Academy of Science and Letters. Oslo, Norway, pp. 11–17.

Castro, H.C., Loureiro, N.I.V., Pujol-Luz, M., Souza, A.M.T., Albuquerque, M.G., Santosa, D.O., Cabral, L.M., Frugulhetti, I.C., Rodrigues, C.R. 2006. HIV-1 reverse transcriptase: a therapeutical target in the spotlight. Curr. Med. Chem. 13: 313–324.

Chiappetta, D.A., Facrorro, G., Celis, E.R., Sosnik, A. 2011. Synergistic encapsulation of the anti-HIV agent efavirenz within mixed poloxamine/poloxamer polymeric micelles. Nanomedicine: Nanomedicine. 7: 624–637.

Dang, N.T.T., Sivakumaran, H., Harrich, D., Shaw, P.N., Poynter, N.D., Coombes, A.G.A. 2014. Synergistic activity of tenofovir and nevirapine combinations released from polycaprolactone matrices for potential enhanced prevention of HIV infection through the vaginal route. Eur. J. Pharm. Biopharm. 88: 406–414.

Darmawan, A, Kosela, S., Kardono, L.B.S., Syah, Y.M. 2012. Scopoletin, a coumarin derivative compound isolated from *Macaranga gigantifolia* Merr. J. Appl. Pharm. Sci. 2: 175–177.

Dhiman, A., Singh, D., Bala, M., Sharma, K. 2012. Potential phytotherapeotic agents in design of ethosomes: a review. J. Pharm. Innov. 1: 26–30.

Ferreres, F., Duangsrisai, S., Gomes, N.G.M., Suksungworn, R., Pereira, D.M., Gil-Izquierdo, A., Valentao, P., Choowongkomon, K. et al. 2017. Anti-inflammatory properties of the stem bark from the herbal drug *Vitex peduncularis* Wall. ex Schauer and characterization of its polyphenolic profile. Food Chem. Toxicol. 106: 8–16.

Folin, O., Ciocalteu, V. 1927. On tyrosine and tryptophane determinations in proteins. J. Biol. Chem. 73: 627–650.

Gnonlonfin, G.J.B., Sanni, A., Brimer, L. 2012. Review: Sopoletin – a coumarin phytoalexin with medicinal properties. Plant Sci. 31: 47–56.

Gourine, N., Yousfi, M., Bombarda, I., Nadjemi, B., Stocker, P., Gaydou, E.M. 2010. Antioxidant activities and chemical composition of essential oil of *Pistacia atlantica* from Algeria. Ind. Crops Prod. 31: 203–208.

Hammer, S.M., Gillis, J.M. 1987. Synergistic activity of granulocyte-macrophage colony-stimulating factor and 3'-azido-3'-deoxythymidine against human immunodeficiency virus *in vitro*. Antimicrob. Agents Chemother. 31: 1046–1050.

Iqbal, E., Salim, K.A., Lim, L.B.L. 2015. Phytochemistry screening, total phenolics and antioxidant activities of bark and leaf extracts of *Goniothalamus velutinus* (Airy Shaw) from Brunei Darussalam. J. King Saud. Univ. 27: 224–232.

Jagtap, S.D., Deokule, S.S., Pawar, P.K., Harsulkar, A.M. 2009. Traditional ethnomedicinal knowledge confined to the Pawra tribe of Satpura hills, Maharashtra, India. Ethnobot. Leaflets. 13: 98–115.

Kapewangolo, P., Hussein, A.A., Meyer, D. 2013. Inhibition of HIV-1 enzyme, antioxidant and anti-inflammatory activities of *Plectranthus barbatus*. J. Ethnopharmacol. 149: 184–190.

Kar, B., Nepal, A., Kumar, R.B.S., Dolai, N., Bhattacharya, S., Mazumder, U.K. 2013. Antioxidant and anti-inflammatory properties *Hymenodictyon excelsum* bark. Orient. Pharm. Exp. Med. 13: 103–111.

Khlift, D., Hayouni, E.A., Valentin, A., Cazaux, S. 2013. LC-MC analysis, anticancer, antioxidant and antimalarial activities of *Cynodon dactylon* L. extract. Ind. Crops Prod. 45: 240–247.

Kurapati, K.R.V., Atluri, V.S., Samikkannu, T., Garcia, G., and Nair, M.P.N. 2016. Natural products as anti-HIV agents and role in HIV-associated neurocognitive disorders (HAND): A brief overview. Front. Microbiol. 6: 1–14.

Machlin, L.J., Bendich, A. 1987. Free radical tissue damage: Protective role of antioxidant nutrients. FASEB J. 1: 441–445.

Manilal, K.S., Remesh, M. 2009. An analysis of the data on the medicinal plants recorded in Hortus Malabaricus. SAMAGRA. 5: 24–72.

Matthee, G., Wright, A.D., Konig, G.M. 1999. Review HIV reverse transcriptase inhibitors of natural origin. Planta Med. 65: 493–506.

Nareeboon, P., Komkhunthot, W., Lekcharoen, D., Wetprasit, N., Piriyapolsart, C., Sutthivaiyakit, S. 2009. Acetylenic fatty acids, triglyceride and triterpenes from the leaves of *Hymenodictyon excelsum*. Chem. Pharm. Bull. 57: 860–862.

Neamsuvan, O., Tanthien, S. 2015. Medicinal plants used for women's healthcare from Khao Phanom Bencha National Park, Krabi province. Burapha Sci. J. 20: 118–132.

Pham-Huy, L.A., He, H., Pham-Huy, C. 2008. Free radicals, antioxidants in disease and health. J. Biomed. Sci. 4: 89–96.

Rahman, M.M. 2015. Evaluation of *Hymenodictyon excelsum* phytochemical's therapeutic value against prostate cancer by molecular Docking study. JUNDISHAPUR J. Nat. Pharm. Prod. 10: 1–4.

Sakagami, H., Asano, K., Satoh, K., Takahashi, K., Kobayashi, M., Koga, N., Takahashi, H., Tachikawa, R. et al. 2007. Anti-stress, Anti-HIV and vitamin C-synergized radical scavenging activity of mulberry juice fractions. In Vivo. 21: 499–506.

Sakagami, H., Satoh, K., Fukamachi, H., Ikarashi, T., Shimizu, A., Yano, K., Kanamoto, T., Terakubo, S. et al. 2008. Anti-HIV and vitamin C-synergized radical scavenging activity of Cacao Husk lignin fractions. In Vivo. 22: 327–332.

Sakong, P., Khampitak, T., Cha'on, U., Pinitsoontorn, C., Sriboonlue, P., Yongvanit, P., Boonsiri, P. 2011. Antioxidant activity and bioactive phytochemical contents of traditional medicinal plants in northeast Thailand. J. Med. Plants Res. 5: 6822–6831.

Sheng-Ji, P. 2001. Ethnobotanical approaches of traditional medicine studies: Some experiences from Asia. Pharm. Biol. 39: 74–79.

Silprasit, K., Thammaporn, R., Tecchasakul, S., Hannongbua, S., Choowongkomon, K. 2011. Simple and rapid determination of the enzyme kinetics of HIV-1 reverse transcriptase and anti-HIV-1 agents by a fluorescence based method. J. Virol. Methods. 171: 381–387.

Simoes, K., Du, J., Pessoni, R.A.B., Cardoso-Lopes, E.M., Vivanco, J.M., Stermitz, F.R., Braga, M.R. 2009. Ipomopsin and hymenain, two biscoumarins from seeds of *Hymenaea courbaril*. Phytochem. Lett. 2: 59–62.

Spino, C., Dodier, M., Sotheeswaran, S. 1998. Anti-HIV coumarins from *Calophyllum* seed oil. Bioorganic Med. Chem. Lett. 8: 3475–3478.

Sutthivaiyakit, S., Chuankamnerdkarn, M., Nareebun, P., Mongkolvisut W., Ukhan, J., Lhinhatrakul, T., Mongthum, I., Sika, C. 2008. Biologically active compounds from some Thai medicinal and pesticidal plants: final report. 74.

Swe, N.N., Myint, W. 2006. Comparative study on phytochemical and physic-chemical aspects of *Hymenodictyon orixense* (Roxb.) Mabb. and *Cinchona succirubra* Pav. Ex. Klotz. New Agriculturist. 17: 215–218.

Tao, C., Taylor, C.M. 2011. Hymenodictyon. http://flora.huh.harvard.edu/china/PDF/PDF19/Hymenodictyon.pdf/, 30 August 2017.

UNAIDS. 2017. Fact sheet–world aids day 2017 global HIV statistics. http://www.unaids.org/sites/default/files/media_asset/UNAIDS_FactSheet_en.pdf/, 30 August 2017.

Venugopala, K.N., Rashmi, V., Odhav, B. 2013. Review on natural coumarin lead compounds for their pharmacological activity. Biomed. Res. Int. 2013: 1–14.

Volberding, P.A., Deeks, S.G. 2010. Antiretroviral therapy and management of HIV infection. The Lancet. 376: 49–62.

Xu, H.X., Zeng F.Q., Wan, M., Sim, K.Y. 1996. Anti-HIV triterpene acids from *Geum japonicum*. J. Nat. Prod. 59: 643–645.

Yu, D., Suzuki, M., Xie, L., Morris-Natschke, S.L., Lee, K.H. 2003. Recent progress in the development of coumarin derivatives as potent anti-HIV agents. Med. Res. Rev. 23: 322–345.

Yu, S., Yan, R., Liang, R., Wang, W., Yang, B. 2012. Bioactive polar compounds from stem bark of *Magnolia officinalis*. Fitoterapia. 83: 356–361.