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Antioxidant, xanthine oxidase inhibitory and antibacterial activities of selected galactogogue Thai medicinal plant water and ethyl acetate extracts

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ABSTRACT: Water and ethyl acetate extracts of sixteen galactogogue Thai medicinal plants collected in Northeastern Thailand were evaluated for the antioxidant activity in terms of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging property and total phenolic content (TPC), xanthine oxidase inhibitory and antibacterial activities. Among the extracts, both of the water and ethyl acetate extracts from the stem bark of *Caesalpinia sappan* (CS) and *Ochna integerrima* (OI) exhibited potent DPPH radical scavenging capacity (IC_{50} 9.47 ± 0.59 – 13.25 ± 0.52 µg/mL) as compared to ascorbic acid (IC_{50} 5.39 ± 0.32 µg/mL) and high TPC (632.89 ± 10.18 – 985.34 ± 0.76 mg gallic acid equivalent (GAE)/g of extract). The strongest xanthine oxidase inhibitory property was detected in the ethyl acetate extract from the root of *Clausena harmandiana* (CH) followed by CS and OI extracts showing %inhibition of 99.22 ± 0.22, 78.00 ± 0.79 and 68.67 ± 1.14, respectively, at the tested concentration of 50 µg/mL and referenced to allopurinol. The water extracts of CS and OI also possessed good antibacterial activity against eight tested pathogenic bacteria especially Gram-positive, *S. aureus* (MSSA) DMST 2933, *S. aureus* (MRSA) DMST 20651 and *Bacillus cereus* ATCC 11778, with the MIC and MBC values ranging from 0.39 – 1.56 mg/mL. The antibacterial property of OI extract and xanthine oxidase inhibitory activity of CH and OI extracts were first reported.

KEYWORDS: Antioxidant activity; xanthine oxidase inhibition; antibacterial activity; galactogogue; DPPH; total phenolic content.

1. INTRODUCTION

Plants are a rich source of bioactive compounds with large variety of biological activities. Numerous of drugs or potential candidate drugs are produced by plants [1]. Although research on biologically active compounds from plants and medicinal plant properties have been studying extensively a large number of plants including their biological activities are still unexplored [2].

In our continuous search for bioactive natural products from local medicinal plants, we are interested in biological property of galactogogue medicinal plants due to the wide distribution and utilization of these plants in Northeastern Thailand. This type of plants is used to induce, maintain and increase milk production [3]. Similarly to other medicinal plants, many of the galactogogue plants are also traditionally used for other treatments as well. From literature survey, research study related the galactogoge medicinal plants to their antioxidant, xanthine oxidase inhibitory and antibacterial activities is limited.

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Plants are natural sources for producing polyphenol group of compounds. These polyphenols are main compounds responsible for the antioxidant activity which play an important role in the prevention of chronic and degenerative diseases [4-6]. They are natural phytochemical antioxidants not only due to their ability of scavenge free radicals but also their capabilities to inhibit the xanthine oxidase enzyme. The activity of the phenolic acids act as both antioxidants and xanthine oxidase inhibitor has been reported [7]. The xanthine oxidase catalyses the oxidation of xanthine and hypoxanthine to uric acid and superoxide radicals ($O^{2\bullet}$) during purine metabolism [8]. Overproduction and insufficient excretion of uric acid associated with kidney disease, gout, hypertension, type 2 diabetes, nephrolithiasis and hyperuricemi due to the formation of uric acid crystals in kedney, joints, other tissues and blood [9-12]. Xanthine oxidase inhibitor is needed to reduce the activity of xanthine oxidase resulting to the reduction of the risk of those diseases. Allopurinol has been used to reduce the activity of xanthine oxidase but its saide effects have been reported and concerned [13]. Thus, searching for plant natural products which having antioxidant and xanthine oxidase inhibitory activities is important.

The phenolic compounds which including flavonoids have been studied for their potential properties and many of them may also exhibited antibacterial activity [14, 15]. The antimicrobial capacity of polyphenols and flavonoids from plants has been investigated against a wide range of microorganisms. For example, antibacterial properties of flavonols toward Gram-positive bacteria; *Staphylococcus aureus*, *Lactobacillus acidophilus* and *Actinomyces naeslundii*, and Gram-negative bacteria; *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* [16], and tannins type of compounds such as proanthocyanidins against *E. coli*, *S. mutans*, and *S. aureus* [17] have been reported.

In this study, sixteen species of the galactogogue Thai medicinal plants from six families collected in Northeast Thailand were selected to evaluate for their antioxidant, xanthine oxidase inhibitory and antibacterial activities. The aim is to search for potential local plants which might be utilized as antioxidant, xanthine oxidase inhibitory and antibacterial sources in food, beverage, pharmaceutical and healthcare applications. Information of classes of isolated compounds and biological activities of these sixteen plant species published since the year 2000 is summarized and given in Supplementary Material (Table S1).

2. RESULTS

2.1. Extraction and yield of extraction

From our study, the extraction yield varied between 0.98 – 4.29 and CS displayed the highest extraction yield of 4.29% (Table 1). In our experiment, the water extraction was performed due to the traditional use of these plants was to boil in water and drink as a decoction.

Table 1. Weight of the water and ethyl acetate extracts and the extraction yield.

Plant species (Code)	Weight of extract (mg)		Extraction yield* (%)
	Water [a]	Ethyl acetate [b]	
Hubera cerasoides (HC)	1,161	132	2.59
Polyalthia debilis (PD)	708	72	1.56
Polyalthia evecta (PE)	647	84	1.46
Polyalthia suberosa (PS)	426	88	1.03
Uvaria rufa (UR)	563	80	1.28
Caesalpinia sappan (CS)	548	1,596	4.29
Celastrus paniculatus (CP)	567	84	1.30
Salacia chinensis (SCh)	728	100	1.65
Salacia verrucosa (SV)	605	92	1.39
Siphonodon celastrineus (SCe)	920	100	2.00
Clausena harmandiana (CH)	583	136	1.44
Glycosmis pentaphylla (GP)	612	112	1.45
Micromelum minutum (MM)	637	132	1.54
Naringi crenulata (NC)	371	116	0.98
Diospyros ehretioides (DE)	607	56	1.33
Ochna integerrima (OI)	93	232	2.17

[a] mg of the extract/100 mL of extracted aqueous solution. [b] mg of the extract/400 mL of partitioned ethyl acetate layer.

2.2. Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging was reported as 50% inhibitory concentration (IC_{50}; µg/mL) while the total phenolic content (TPC) was expressed as gallic acid equivalent (GAE)/g of extract. The least of the IC_{50} value and high TPC indicate good antioxidant activity.

All of the extracts showed dose-dependent DPPH radical scavenging activity (%RSA) and their IC_{50} values are shown in Table 2. The ethyl acetate extracts of each plant showed better scavenging activity than their water extracts, except for CS and OI which the activities of the ethyl acetate extracts were slightly lower than and in the same level of its water extract. This can be explained by the solubility and purity of organic substances in the ethyl acetate after the partition especially small molecules of phenolic compounds which are known to be good antioxidants. The results indicated that the extracts of CS, SCh, SV and OI possessed good DPPH radical scavenging capacity (IC_{50} 9.17 – 24.59 µg/mL) when compared to standard ascorbic acid (IC_{50} 5.39 ± 0.32 µg/mL). The activity of the water extracts decreased in the order of CS > OI > SCh > SV while the activity of the ethyl acetate extracts was SCh > CS = SV = OI (Table 2). Interestingly, the radical scavenging abilities of the water extracts of both CS and OI were not much different from their ethyl acetate extracts. These activities were almost in the same level of the ascorbic acid activity.

Table 2. DPPH radical scavenging activity of the water and ethyl acetate extracts.

Plant species (Code)	DPPH radical scavenging activity (IC_{50}; µg/mL)	
	Water extract	Ethyl acetate extract
Hubera cerasoides (HC)	$35.53 \pm 0.43^{e,H,I}$	$25.03 \pm 0.35^{f,F}$
Polyalthia debilis (PD)	$150.89 \pm 0.41^{j,Q}$	$32.67 \pm 0.38^{g,G}$
Polyalthia evacta (PE)	$118.75 \pm 0.82^{i,P}$	$36.57 \pm 0.84^{i,I}$
Polyalthia suberosa (PS)	$153.90 \pm 0.17^{n,U}$	$38.44 \pm 0.43^{k,K}$
Uvaria rufa (UR)	$96.67 \pm 0.84^{h,O}$	$15.39 \pm 0.63^{d,D}$
Caesalpinia sappan (CS)	$9.47 \pm 0.59^{a,A}$	$9.85 \pm 0.84^{b,B}$
Celastrus paniculatus (CP)	$138.35 \pm 0.29^{l,S}$	$17.13 \pm 0.67^{c,C}$
Salacia chinensis (SCh)	$22.76 \pm 0.80^{c,E}$	$9.17 \pm 0.30^{a,A}$
Salacia verrucosa (SV)	$24.59 \pm 0.13^{d,F}$	$10.40 \pm 0.17^{b,B}$
Siphonodon celastrineus (SCe)	$252.94 \pm 0.84^{o,V}$	$36.50 \pm 0.16^{i,I}$
Clausena harmandiana (CH)	$254.46 \pm 0.92^{p,W}$	$36.57 \pm 0.84^{l,L}$
Glycosmis pentaphylla (GP)	$83.66 \pm 0.05^{f,M}$	$27.72 \pm 0.32^{h,H}$
Micromelum minutum (MM)	$145.66 \pm 0.74^{m,T}$	$43.66 \pm 0.67^{j,J}$
Naringi crenulata (NC)	$128.89 \pm 0.77^{k,R}$	$43.33 \pm 0.47^{j,J}$
Diospyros ehretioides (DE)	$93.54 \pm 0.16^{g,N}$	$26.83 \pm 0.88^{e,E}$
Ochna integerrima (OI)	$10.86 \pm 0.10^{b,B}$	$13.25 \pm 0.52^{b,B}$
standard	**DPPH radical scavenging activity (IC_{50}; µg/mL)**	
Ascorbic acid	5.39 ± 0.32	

Different superscript lower case letters indicate statistically significant difference within the same column and superscript upper case letters indicate statistically significant difference between the two columns (ANOVA, post-hoc; p <0.005).

The TPC (standard gallic acid linear regression $r^2 = 0.9993$) of all extracts is shown in Table 3. The highest TPC of water and ethyl acetate extracts was detected in CS (682.67 ± 6.11 mg GAE/g extract) and OI (985.34 ± 0.76 mg GAE/g extract). The ethyl acetate extracts of each plant had higher TPC than its water extracts. High TPC was detected in the extracts of CS, SCh, SV and OI which was in good agreement with the radical scavenging capacity results of these four plants when compared to others. Among this group, the TPC of the water extracts dropped in the sequence of CS > OI > SV > SCh while the TPC of the ethyl acetate declined in the order of OI > CS > SCh > SV. The TPC the water extracts of both CS and OI was considerably 3.8 – 4.7 folds higher than SV and SCh.

2.3. Xanthine oxidase inhibitory activity

The xanthine oxidase inhibitory activity (%) of the water and ethyl acetate extracts is shown in Table 4. The ethyl acetated extract from the root of CH exhibited the highest %xanthine oxidase inhibition of 99.22 ±

0.22 and decreased in the following order of CS (78.00 ± 0.79)> OI (68.67 ± 1.14)> DE (64.07 ± 1.78) extracts respectively. The activity of all ethyl acetate extracts was much better than activity of their water extracts.

Table 3. Total phenolic content (TPC) of the water and ethyl acetate extracts.

Plant species (Code)	TPC (mg GAE/g extract)	
	Water extract	Ethyl acetate extract
Hubera cerasoides (HC)	129.33 ± 2.31^{e,O}	402.67 ± 10.41^{e,G}
Polyalthia debilis (PD)	26.67 ± 0.00^{k,S}	419.56 ± 0.77^{d,e,F}
Polyalthia evacta (PE)	39.56 ± 0.77^{i,R}	254.67 ± 28.38^{h,J}
Polyalthia suberosa (PS)	33.33 ± 0.00^{j,R}	264.89 ± 0.77^{h,J}
Uvaria rufa (UR)	60.44 ± 0.77^{h,P,Q}	426.22 ± 6.58^{d,F}
Caesalpinia sappan (CS)	682.67 ± 6.11^{a,C}	891.56 ± 6.30^{b,B}
Celastrus paniculatus (CP)	20.00 ± 0.00^{l,S}	403.56 ± 22.48^{e,G}
Salacia chinensis (SCh)	145.33 ± 0.00^{d,N}	632.00 ± 4.00^{c,D}
Salacia verrucosa (SV)	165.33 ± 2.31^{c,M}	615.56 ± 7.58^{c,E}
Siphonodon celastrineus (SCe)	17.78 ± 0.77^{l,S}	299.11 ± 1.54^{g,I}
Clausena harmandiana (CH)	36.00 ± 0.00^{j,R}	195.56 ± 2.04^{j,L}
Glycosmis pentaphylla (GP)	64.00 ± 0.00^{g,P,Q}	308.89 ± 5.39^{g,I}
Micromelum minutum (MM)	59.11 ± 0.77^{h,Q}	235.56 ± 0.77^{i,K}
Naringi crenulata (NC)	62.67 ± 1.33^{f,P}	261.78 ± 2.04^{h,J}
Diospyros ehretioides (DE)	34.67 ± 0.00^{J,R}	375.11 ± 4.29^{f,H}
Ochna integerrima (OI)	632.89 ± 10.18^{b,A}	985.34 ± 0.76^{a,D}

Different superscript lower case letters indicate statistically significant difference within the same column and superscript upper case letters indicate statistically significant difference between the two columns (ANOVA, post-hoc; $p < 0.005$). Linear regression of gallic acid had r^2 0.9993.

Table 4. Xanthine oxidase inhibitory activity of the water and ethyl acetate extracts.

Plant species (Code)	Xanthine oxidase inhibitory activity (%)	
	Water extract	Ethyl acetate extract
Hubera cerasoides (HC)	33.44 ± 0.75^{c,L}	51.82 ± 3.26^{f,g,h,i,H,G}
Polyalthia debilis (PD)	14.96 ± 0.59^{f,N}	59.73 ± 1.39^{d,e,E}
Polyalthia evacta (PE)	12.22 ± 1.46^{g,N}	54.94 ± 9.01^{e,f,g,h,F,G}
Polyalthia suberosa (PS)	20.88 ± 0.22^{e,M}	55.64 ± 0.52^{e,f,g,E,F,G}
Uvaria rufa (UR)	7.51 ± 0.07^{h,O}	36.75 ± 5.75^{k,L}
Caesalpinia sappan (CS)	45.16 ± 1.09^{a,I,J,K}	78.00 ± 0.79^{b,B}
Celastrus paniculatus (CP)	25.59 ± 1.74^{d,M}	48.81 ± 2.79^{h,i,j,H,I}
Salacia chinensis (SCh)	6.40 ± 0.29^{h,i,O}	57.69 ± 0.90^{e,f,F,E}
Salacia verrucosa (SV)	4.65 ± 0.55^{i,O}	56.06 ± 2.91^{e,f,g,E,F,G}
Siphonodon celastrineus (SCe)	33.36 ± 2.60^{c,L}	44.12 ± 1.79^{j,J,K}
Clausena harmandiana (CH)	21.59 ± 0.14^{e,M}	99.22 ± 0.22^{a,A}
Glycosmis pentaphylla (GP)	22.39 ± 1.83^{e,M}	50.04 ± 1.69^{g,h,i,j,H}
Micromelum minutum (MM)	41.41 ± 0.46^{b,K}	65.60 ± 2.04^{c,d,C,D}
Naringi crenulata (NC)	0.00 ± 0.00^{j,P}	48.25 ± 6.27^{i,j,H,I,J}
Diospyros ehretioides (DE)	21.65 ± 0.34^{e,M}	64.07 ± 1.78^{c,d,D}
Ochna integerrima (OI)	6.58 ± 0.60^{h,O}	68.67 ± 1.14^{c,C}
Standard	**Xanthine oxidase inhibitory activity (%)**	
Allopurinol	100	

Different superscript lower case letters indicate statistically significant difference within the same column and superscript upper case letters indicate statistically significant difference between the two columns (ANOVA, post-hoc; p <0.005).

2.4. Antimicrobial activity

The antimicrobial activity against eight pathogenic bacteria of the water extracts of the sixteen plant samples was investigated. The inhibition zone diameters of the water extracts were evaluated and data is shown in Table 5, while values of MIC and MBC were reported in Table 6. The results indicated that CS and OI extracts exhibited activity against all tested pathogenic bacteria. Beside these two active extracts, the antibacterial activity of SCh and SV against five and seven tested pathogenic bacteria was also observed. The CS, SCh, SV and OI water extracts possessed the inhibitory effect on three Gram-positive bacteria; (*S. aureus* (MSSA) DMST 2933, *S. aureus* (MRSA) DMST 20651), and three Gram-negative bacteria; (*V. cholerae* O1 DMST 9700, *S. flexneri* DMST 4423 and *S. dysenteriae* DMST 15110). Two strains of the staphylococci, *S. aureus* (MSSA)

DMST 2933 and *S. aureus* (MRSA) DMST 20651, were the most sensitive to CS, SCh, SV and OI water extracts when compared to other target bacteria. The water extract of CS showed the most active antibacterial activity with the MIC and MBC values ranging from 0.39 – 3.12 mg/mL. Among eight target bacteria, *S. aureus* (MSSA) DMST 2933, *V. cholerae* O1 DMST 9700, *B. cereus* ATCC 11778, *S. flexneri* DMST 4423 and *S. dysenteriae* DMST 15110 were inhibited potentially by the CS water extract with low MIC and MBC values in the range of 0.39 – 0.78 mg/mL. In addition, the water extract of OI displayed antibacterial activity with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging from 0.78 – 6.25 mg/mL against all test pathogenic bacteria (Table 6).

Table 5. Inhibition zone diameters of the water extracts.

Sample code	Inhibition zone diameters (cm)							
	S. aureus MSSA 2933	*S. aureus* MSRA 20651	*B. cereus* ATCC 11778	*E. coli* ATCC 25922	*S.* Typhi DMST 22844	*V. cholera* O1 DMST 9700	*S. flexneri* DMST 4423	*S. dysenteriae* DMST 15110
HC	10 x 10	15 x 15	-	-	-	-	-	-
PD	-	10 x 10	-	-	-	-	-	-
PE	-	10 x 10	-	-	-	-	-	-
PS	-	15 x 15	-	-	-	-	-	10 x 10
UR	10 x 10	13 x 13	-	-	-	-	-	-
CS	36 x 36	37 x 37	29 x 29	18 x 18	18 x 18	30 x 30	27 x 27	23 x 23
CP	-	-	-	-	-	-	-	-
SCh	19 x 19	24 x 24	-	-	-	15 x 15	14 x 14	11 x 11
SV	20 x 20	25 x 25	11 x 11	-	12 x 12	12 x 12	14 x 14	11 x 11
SCe	-	-	-	-	-	-	-	-
CH	-	-	-	-	-	-	-	-
GP	13 x 13	16 x 16	-	-	-	11 x 11	-	12 x 12
MM	-	15 x 15	10 x 10	-	-	-	-	-
NC	-	-	-	-	-	-	-	-
DE	13 x 13	17 x 17	-	-	-	-	-	13 x 13
OI	20 x 20	24 x 24	15 x 15	11 x 11	11 x 11	17 x 17	15 x 15	13 x 13
Tetracyclin	33 x 33	11 x 11	38 x 38	-	-	32 x 32	-	-

The four ethyl acetate extracts of CS, SCh, SV and OI were selected to test for their antimicrobial activities based on the antimicrobial activity results of their water extracts. The results revealed that the ethyl acetate extract of CS still maintained activity toward all test pathogenic microorganisms. The activities against *E. coli* ATCC 25922 of OI, and *S. Typhi* DMST 22842, *V. cholerae* O1 DMST 9700 and *S. flexneri* DMST 4423 of SV ethyl acetate extracts were lost whereas the activity against *E. coli* ATCC 25922 and *B. cereus* ATCC 11778 of SCh acetate extract was presented when compared to their water extracts. Among these active ethyl acetate extracts, CS exhibited the most active activity with low MIC and MBC values than others. However, this activity was not as strong as activity of its water extract. Similar results were found in the ethyl acetate extracts of OI, SCh and SV (Table 6).

3. DISCUSSION

Extraction of phytochemicals in plant materials is influenced by many factors and the extracts from the extraction are always mixture of different classes of compounds that soluble in the solvent system used [18]. Water is a polar extracting solvent and because of its polarity and ability to form hydrogen bonds, it can be an excellent solvent for dissolving many types of hydrophilic molecules such as sugar, glycoside and polyhydroxylated compounds whilst the ethyl acetate was used for partitioning of less polar organic soluble parts from the extracted water solution. Previous studies reported red dye water soluble flavonoids which can be mostly used in food, beverage, cosmetic and garment [19, 20] from the heartwood of CS and the traditional extraction of these flavonoids is to boil the wood in water [18].

Several studies reveal that the TPC has a strong relationship with the antioxidant activity [21]. Potent antioxidant ability tested by the DPPH, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods of brazilin, a major phenolic compound, in CS extract has been reported [20, 22, 23] while the antioxidant and antibacterial assessments of SCh, SV and OI are limited. Promden et al. [24] reported strong antioxidant capacity according to the DPPH and oxygen radical absorbance capacity (ORSC) assays of the methanol extracts from the stem of SV and OI while the antioxidant

property measured by DPPH, FRAP, ABTS, *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) assays of SCh was reported by Ghadage et al. [25]. Phenolics and flavonoids found in the wood of CS and the stem bark, leaf and twig of OI from previous phytochemical studies are believed to play an important role on antioxidant property of their extracts [18, 32-34].

Table 6. MIC/MBC values of the antimicrobial activity of the water and ethyl acetate extracts.

Sample code	MIC/MBC value (mg/mL)							
	S. aureus MSSA 2933	*S. aureus* MSRA 20651	*B. cereus* ATCC 11778	*E. coli* ATCC 25922	*S.* Typhi DMST 22844	*V. cholera* O1 DMST 9700	*S. flexneri* DMST 4423	*S. dysenteriae* DMST 15110
Water extract								
HC	1.56/3.12	1.56/1.56	-	-	-	-	-	-
PD	-	1.56/1.56	-	-	-	-	-	-
PE	-	1.56/1.56	-	-	-	-	-	-
PS	-	3.12/3.12	-	-	-	-	-	25.0/12.5
UR	1.56/3.12	1.56/1.56	-	-	-	-	-	-
CS	0.78/0.78	0.78/1.56	0.39/0.39	3.12/3.12	0.78/1.56	0.78/0.78	0.78/0.78	0.78/0.78
CP	-	-	-	-	-	-	-	-
SCh	1.56/1.56	0.78/1.56	-	-	-	6.25/6.25	6.25/6.25	3.12/3.12
SV	1.56/1.56	1.56/1.56	3.12/3.12	-	1.56/6.25	1.56/3.12	6.25/6.25	3.12/3.12
SCe	-	-	-	-	-	-	-	-
CH	-	-	-	-	-	-	-	-
GP	6.25/6.25	6.25/6.25	-	-	-	-	-	6.25/3.12
MM	-	1.56/3.12	-	-	-	-	-	-
NC	-	-	-	-	-	-	-	-
DE	1.56/1.56	1.56/1.56	-	-	-	-	-	6.25/3.12
OI	0.78/1.56	1.56/1.56	0.78/0.78	6.25/6.25	3.12/3.12	3.12/3.12	6.25/3.12	1.56/1.56
Ethyl acetate extract								
CS	0.78/6.25	1.56/6.25	3.12/3.12	3.12/6.25	6.25/6.25	1.56/1.56	0.78/3.12	0.78/0.78
SCh	12.5/50.0	12.5/25.0	12.5/12.5	50.0/50.0	-	50.0/50.0	25.0/25.0	6.25/3.12
SV	12.5/50.0	12.5/50.0	50.0/50.0	-	-	-	-	6.25/6.25
OI	3.12/6.25	3.12/12.5	1.56/1.56	-	25.0/25.0	6.25/6.25	6.25/12.5	0.78/1.56

Previous research had indicated that polyphenols and flavonoids are potent xanthine oxidase inhibitors and superoxide scavengers [29]. This suggestion supports the inhibition of xanthine oxidase activity of CS and OI extracts since they possessed high TPC and good radical scavenging capacity. In addition, Nguyen et al. [30] reported potent xanthine oxidase inhibitory property of the compounds isolated from the heartwood of CS as compared with allopurinol. There is no information of xanthine oxidase inhibitory property for OI has been documented. Although CH ethyl acetate extract had weak DPPH radical scavenging activity and low TPC it showed the strongest xanthine oxidase inhibition. This result can be explained by the presence of carbazole alkaloids and coumarins in the root of CH [31-34]. The xanthine oxidase inhibitory activity of carbazoles and coumarins [35-37] are known and has been reviewed. However, this is the first time report on the xanthine oxidase inhibitory activity of the root extract of CH species.

In this study, the CS and OI extracts had good antioxidant activity with high TPC content and the radical scavenging capacity. This was in agreement with the antibacterial activity when the extracts were tested against the pathogenic bacteria. It is likely that the TPC of the CS and OI extracts may be playing an important role in lysing cell wall and cytoplasmic membrane of the bacterial pathogens thereby causing leakage of bacterial cell contents [35]. Interestingly, Gram-positive bacteria are more sensitive toward phytochemical bacterial agents than Gram-negative bacteria [36]. This is due to the Gram-negative microorganisms have an outer membrane lipopolysaccharide coating on a cell wall. Therefore, hydrophilic type of molecules like polyphenols could not be easily passed into their cells. In addition, the extract may be disrupting of peptidoglycan biosynthesis of Gram-positive bacteria, and exerted to damage cell wall and cell membranes, which resulted in cell death [37]. The antibacterial activity result of CS of our present study is supported by information reported in literatures [22, 38-41] whereas the antibacterial property of constituents of OI or its extracts has never been reported yet except for the anti HIV-1 activity of the OI methanol extract [26]. These findings suggest that the CS and IO extracts could be used as a potential source of antibacterial.

4. CONCLUSION

The results of our present study indicated that among the tested extracts, the water and ethyl acetate extracts from the stem bark of CS and OI exhibited potent antioxidant property and antibacterial activity against Gram-positive and Gram-negative bacteria. The extracts of these two plants can be used as natural antioxidants, natural food preservative and related microbial infection treatment. These two extracts which are proven to be rich in natural antioxidant may potentially help in health promotion and prevent bacterial infection which may cause serious problems especially for breastfeeding women. The ethyl acetate extract from the root of CH possessed an excellent xanthine oxidase inhibitory activity followed by the ethyl acetate extracts of CS and OI respectively. Therefore, these three plants could be considerate to use as alternative rheumatism diseases plant sources.

5. MATERIALS AND METHODS

5.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, Germany), Folin-Ciocalteu reagent (Carlo Erba Reagents, France), sodium carbonate (Kemaus, Australia), tetracycline (Sigma, USA), Mueller Hinton Agar (Difco, USA), Mueller Hinton Broth (Difco, USA), dimethyl sulfoxide (Riedel-de Haën, Germany and RCI Labscan, Thailand), xanthine and allopurinol (Wako Pure Chemical Industries, Japan), xanthine oxidase, buttermilk (Calbiochem, EDM millipore, USA) and sodium dihydrogenphoasphate monohydrate (Merck, Germany) were used for the experiments.

5.2. Plant material and sample preparation

Sixteen plant species were selected based on the traditional use as galactogogue plants in northeast Thailand (Table 7). The plant samples were collected in 2018 from Maha Sarakham province except for *Salacia verrucosa* (SV) which was collected from Amnat Charoen province by K. Wongpakam and identified by S. Sedlak, Walai Rukhavej Botanical Research Institute, Mahasarakham University (MSU) where the voucher specimens (Wongpakam 19-XX) are deposited. Three dried plant samples were received from the medicinal folkers in Maha Sarakham province (Table 7). Fresh samples were washed, cut into small pieces and air dried. Dried samples were kept in zipped plastic bag and stored at room temperature until use.

5.3. Plant extraction

Each plant sample (50 g) was refluxed in water (500 mL, 24 h). After filtration, the aqueous layer was collected and a portion of 100 mL of it was subjected to freeze dry to obtain the water extract. Another portion of ~400 mL of the aqueous layer was further partitioned twice with 150 mL of ethyl acetate and the collected ethyl acetate layer was subjected to evaporate under reduced pressure to get the ethyl acetate extract from water. The extraction yield (%) of each plant was calculated based on the equation 1 given below.

$$\text{Extraction yield (\%)} = \left[\frac{\text{(g of water extract + g of ethyl acetate extract)}}{\text{50 g of dried plant material}}\right] \times 100 \qquad \text{(Eq. 1)}$$

5.4. Evaluation of antioxidant activity

The antioxidant activity of the water and ethyl acetate extracts was determined for their DPPH radical scavenging activities and TPC.

5.4.1. DPPH radical scavenging activity

The radical scavenging activity was tested toward a stable DPPH radical called DPPH method. The DPPH radical gives purple color with maximum absorption at wavelength (λ) of 515-517 nm in alcoholic solvent. When the DPPH radical reacts with antioxidants it turns to a reduced form DPPH-H which is a hydrazine derivative, resulting in decolorization of its purple color to yellow or colorless. The DPPH radical scavenging activity was determined by the modified method described by Seephonkai et al. [42]. Briefly, extract solution was prepared in 50% methanol for the water extract and in methanol for the ethyl acetate extract. A well shake mixture of 1 mL of the extract solution at different concentrations (5 – 150 µg/mL) and 2 mL of 0.2 mM DPPH in methanol was kept in the dark at room temperature for 30 min. Later, the absorbance of the mixture (Abs of sample) was measured at λ 517 nm by UV-Vis Spectrophotometer (Thermo Scientific

GENESYS 20). The absorbance at the additional 50% methanol or methanol instead of the extract solution was expressed as Abs of control. The %RSA of each concentration was calculated based on equation 2 described below. A graph plotted between the %RSA and tested concentrations led to calculation of IC_{50} of the samples. Ascorbic acid was used as a standard antioxidant.

$$\%RSA = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} * 100 \qquad \text{(Eq. 2)}$$

Table 7. Information of sixteen galactogogue Thai medicinal plants materials.

Plant species (Code)	Family	Used part	Voucher no.
Hubera cerasoides (Roxb.) Chaowasku (HC)	Annonaceae	Stem bark	Wongpakam 19-08
Polyalthia debilis (Pierre) Finet & Gagnep. (PD)	Annonaceae	Stem bark	Wongpakam 19-09
Polyalthia evecta (Pierre) Finet & Gagnep. var. *evecta* (PE)	Annonaceae	Stem bark and root	Wongpakam 19-11
*Polyalthia suberosa** (Roxb.) Thwaites (PS)	Annonaceae	Stem bark	Wongpakam 19-14
Uvaria rufa Blume (UR)	Annonaceae	Stem bark	Wongpakam 19-07
*Caesalpinia sappan*** L. (CS)	Fabaceae	Stem bark	Wongpakam 19-01
Celastrus paniculatus Willd. (CP)	Celastraceae	Stem bark	Wongpakam 19-10
Salacia chinensis L. (SCh)	Celastraceae	Stem bark	Wongpakam 19-04
Salacia verrucosa Wight (SV)	Celastraceae	Stem bark	Wongpakam 19-03
Siphonodon celastrineus Griff. (SCe)	Celastraceae	Stem bark	Wongpakam 19-13
*Clausena harmandiana** (Pierre) Pierre ex Guillaumin (CH)	Rutaceae	Root	Wongpakam 19-15
*Glycosmis pentaphylla** (Retz.) DC. (GP)	Rutaceae	Stem bark	Wongpakam 19-05
Micromelum minutum (Forst.f.) Wight & Arn. (MM)	Rutaceae	Stem bark	Wongpakam 19-16
Naringi crenulata (Roxb.) Nicolson (NC)	Rutaceae	Stem bark	Wongpakam 19-12
Diospyros ehretioides Wall. ex G.Don (DE)	Ebenaceae	Stem bark	Wongpakam 19-06
Ochna integerrima (Lour.) Merr. (OI)	Ochnaceae	Stem bark	Wongpakam 19-02

* Samples were received as dried plant materials from Thai folk healers in Maha Sarakham province.
** *Caesalpinia sappan* L. was recently renamed to *Biancaea sappan* (L.) Tod. based on taxonomic revision and International Plant Names Index (IPNI) produced by the Royal Botanic Gardens, Kew.

5.4.2. Total phenolic content (TPC)

The TPC was determined using Folin-Ciocalteu (FC) method. The FC reagent is phosphomolybdic-phosphotungstic acid complex and the complex itself produces yellow color. When the phenolic compounds react with the FC reagent under a basic condition, the phosphomolybdotungstic acid complex is reduced by the phenolate anion resulting to molybdenum blue complex giving blue (or green) color with maximum absorption at λ of 760-765 nm. The TPC of the extracts was measured according to modified method reported by Seephonkai et al. [42]. Briefly, the extract solution of 1 mL at the concentration of 50 µg/mL was taken to mix with 2 mL of FC reagent (10-flod dilution in water) and 3 mL of 7.5% (w/v) sodium carbonate solution. The mixture was shook and left to stand for 30 min in the dark at room temperature. Supernatant from centrifugation of the mixture was taken to measure for its absorbance at λ 761 nm. The TPC was calculated based on standard linear regression of gallic acid (concentrations of 5 – 10 µg/mL) and expressed as mg GAE/g of extract as shown below (Equation 3).

$$\text{TPC} = \frac{X\ (\text{mg GAE/L}) * \text{Dilution factor} * V}{\text{Sample weight (mg)}} \qquad \text{)Eq. 3(}$$

X: Quantity of phenolics in extract solution calculated from standard linear regression of gallic acid
V: Total volume of the extract solution

5.5. Evaluation of xanthine oxidase inhibitory activity

5.5.1. Xanthine oxidase activity assay

The xanthine oxidase enzyme was freshly prepared by dissolving the stock enzyme into 100 mM sodium dihydrogenphosphate, pH 7.5 with a ratio of 1:19 by volume. The xanthine oxidase assay was carried out by adding the enzyme, 5 µL, into a solution of xanthine substrate 100 µM in 100 mM sodium dihydrogenphosphate, pH 7.5. The enzyme activity was measured as ΔAbs_{295}/min using UV-spectrophotometer (Jasco V-530). The enzyme activity in Unit of mUnit/mL was determined using the Beer-

Lambert law equation; the extinction coefficient (ε) of uric acid at 295 nm used was 12.65 $mM^{-1}cm^{-1}$ (1 Unit = 1 µmol of uric acid increased/min).

5.5.2. Sample preparation

Sample solution (3 mg in 1000 µL DMSO) was diluted with 100 mM sodium dihydrogenphosphate, pH 7.5 as 1 mg/mL before using in the xanthine oxidase inhibitory assay. In the assay solution, the final sample concentration used was 100 µM, and %DMSO was 1.5%.

5.5.3. Xanthine oxidase inhibitory assay

The Xanthine oxidase inhibitory assay was modified from Gawlik-Dziki et al. [43] and Shintani et al. [44]. In the assay condition, a solution of xanthine oxidase 0.57 mUnit/mL and 50 µg/mL of sample extract solution (inhibitor) in 100 mM sodium dihydrogenphosphate, pH 7.5, 1000 µL, was prepared in 1.5 mL cuvette. The mixture solution was incubated at the room temperature (25 °C) for 10 min. Then, 100 µM final concentration of sample solution was added to initiate the assay reaction. ΔAbs_{295}/min was measured using the UV-visible spectrophotometer. A 1.5% DMSO in 100 mM sodium dihydrogenphosphate buffer, pH 7.5, was used as the negative control. The %xanthine oxidase inhibition of the sample was calculated using equation 4 as shown below. A standard control was allopurinol.

$$\%Xanthine\ oxidase\ inhibition = \left(\frac{\left(\frac{\Delta Abs_{295}}{min}\right)_{control} - \left(\frac{\Delta Abs_{295}}{min}\right)_{sample}}{\left(\frac{\Delta Abs_{295}}{min}\right)_{control}} \right) \times 100 \qquad \text{(Eq. 4)}$$

5.6. Evaluation of Antibacterial activity

All of the water extracts were screened for the antibacterial activity using Agar well diffusion method. The active water extracts were determined for their MIC and MBC values against target bacteria using microdilution method. After that, only the ethyl acetate extracts which were partitioned from active water extracts were subjected to evaluate the antibacterial property.

5.6.1. Microbial strains and cultivation

Eight pathogenic bacteria; three Gram-positive bacteria, methicillin-susceptible *Staphylococcus aureus* (MSSA) DMST 2933, methicillin-resistant *Staphylococcus aureus* (MRSA) DMST 20651 and *Bacillus cereus* ATCC 11778, and five Gram-negative bacteria, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhi DMST 22842, *Vibrio cholerae* O1 DMST 9700, *Shigella flexneri* DMST 4423 and *Shigella dysenteriae* DMST 15110, were selected to test the antibacterial activity of the plant extracts. These bacterial strains were sourced from Medical Microorganisms Department of Medical Sciences Thailand. The selected strains were cultured on Mueller Hinton Agar (MHA) at 37 °C for 16 – 18 h. Then, single colony of bacterial pathogens was inoculated into Mueller Hinton Broth (MHB) at 37 °C for 4 h with shaking at 250 rpm. After that, bacterial culture was adjusted to 4 – 5 × 10^6 CFU/mL and stored at 4 °C before use.

5.6.2. Sample preparation

The tested sample was dissolved in 10% methanol or 10% dimethyl sulfoxide (DMSO) for primary screening of the inhibition zone diameters. Later 10% methanol was used to dissolve sample to make a concentration of 100 mg/mL and then diluted to 50, 25, 12.7, 6.25, 3.12, 1.56 and 0.78 mg/mL by two-fold dilution method for the determination of MIC/MBC values. Tetracycline was used as a positive control.

5.6.3. Agar well diffusion method

The antibacterial activity of the extracts was determined by the agar well diffusion method [45, 46]. A sterile cotton swab was dipped into the standardized bacterial suspension and then swabbed over the entire surface of the MHA plates before being cut by using a 7 mm sterile cork borer. The 50 mg/mL of the extracts were added to each well (0.1 mL per well). The plates were then incubated at 37 °C for 16 – 18 h. The zone of inhibition surrounding in each well was measured. The negative controls used were 10% (v/v) methanol and 10% DMSO. Tetracycline (250 µg/mL) were used as positive control.

5.6.4. Determination of MIC and MBC of active extract

The MIC and MBC values were determined using the microdilution method [45, 46]. Single colony of the pathogenic bacteria was inoculated into MHB medium and was then incubated at 37 °C with shaking (250 rpm) for 3 h. The bacterial suspension was standardized to 4 – 5 × 10^6 CFU/mL. Later, 10 µL of the standardized inoculum was added to the wells of a 96-well polystyrene tray. Then, 90 µL of each diluted extracts (50, 25, 12.5, 6.25, 3.125 and 1.56 mg/mL) in MHB were separately added. A control growth well and an uninoculated control well were included in each plate. Tetracycline (250 µg/mL) was used as the reference standard. The MIC was defined as the lowest concentration of extract that remained clear after incubation, relative to the control well. Then, the suspension in each well was streaked on MHA for determining the MBC value. The MBC was defined as the lowest concentration of extract capable of killing the test bacteria.

5.7. Statistical analysis

The DPPH radical scavenging and xanthine oxidase inhibitory activities, and TPC assays were carried out in triplicate. The results were reported as mean ± standard deviation (SD). Data were analyzed for statistical significant (p <0.05) by post-hoc test (IBM SPSS statistics software, version 22, USA). The p values less than 0.05 were considered statistically significant.

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Appendix A. Supplementary Material

Supplementary material related to this article can be accessed at https://dx.doi.org/10.29228/jrp.42.

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