Phytochemical Screening and Antioxidant Activities of Hexane, Chloroform, and Aqueous Extracts of *Derris reticulata* stem

พฤกษเคมีเบื้องต้นและฤทธิ์ต้านอนุมูลอิสระของต้นชะเอมเหนือที่สกัดด้วยเฮกเซน คลอโรฟอร์มและน้ำ

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ABSTRACT

Recently, the aqueous extract of *Derris reticulata Craib.* (Leguminosae) has been shown to exhibit an anti-diabetic activity in alloxan-induced diabetic rats. It was proposed that the anti-diabetic activity was due to its cytoprotective effect, probably by its antioxidant activity. The main objective of this study was aim to compare antioxidant activity of *D. reticulata* stem extract obtained by sequential extraction method with three different solvents, hexane, chloroform and water. The results from phytochemical analysis showed that the compositions of hexane extract were similar to those of the chloroform extract, but quite different from those of the aqueous extract. It was found that the aqueous extract contained the least total phenolic compounds. In accordance with phenolic contents, the hexane and chloroform extracts exhibited more antioxidant potential than the aqueous extract. The results from TLC revealed that the major constituent of hexane and chloroform extracts was the compound observed on TLC with R_f 0.854. Taken together, the results suggested that this major compound may be responsible for antioxidant activities of hexane and chloroform extracts. Now, isolation and identification of this compound as well as the examination of its antioxidant and anti-diabetic activities are in progress.

บทคัดย่อ

เมื่อไม่นานมานี้มีรายงานพบว่าสารสกัดด้วยน้ำของต้นชะเอมเหนือ (*Derris reticulate*, วงศ์ Leguminosae) มีฤทธิ์ต้านเบาหวานจากการศึกษาในหนูขาวที่ถูกชักนำให้เกิดเบาหวานด้วย alloxan โดยฤทธิ์ดังกล่าวได้รับการเสนอว่าเป็นผลจากฤทธิ์ปกป้องเซลล์ซึ่งอาจจะเนื่องมาจากฤทธิ์ต้านอนุมูลอิสระของสารสกัด การศึกษานี้มีวัตถุประสงค์หลักเพื่อเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากต้นชะเอมเหนือที่ได้การสกัดเป็นลำดับขั้นด้วยตัวทำละลายที่แตกต่างกัน 3 ชนิด คือ เฮกเซน คลอโรฟอร์มและน้ำ ผลจากการตรวจสอบพฤกษเคมีเบื้องต้นแสดงให้เห็นว่าส่วนประกอบของสารสกัดด้วยเฮกเซนและคลอโรฟอร์มมีความคล้ายคลึงกันแต่ค่อนข้างแตกต่างจากสารสกัดด้วยน้ำ และพบว่าสารสกัดด้วยน้ำมีปริมาณสารฟีนอลิกน้อยที่สุด นอกจากนั้นยังพบว่าสารสกัดด้วยเฮกเซนและคลอโรฟอร์มมีฤทธิ์ต้านอนุมูลอิสระมากกว่าสารสกัดด้วยน้ำ ซึ่งผลดังกล่าวนี้สอดคล้องกับปริมาณสารฟีนอลิกที่พบในสารสกัดทั้งสามชนิด ผลจากการทำ TLC เผยให้เห็นว่าสารประกอบหลักในสารสกัดด้วยเฮกเซนและคลอโรฟอร์มน่าจะเป็นสารชนิดเดียวกันซึ่งปรากฏเป็นค่า R_f เท่ากับ 0.854 บนแผ่น TLC ผลการทดลองทั้งหมดที่ได้ชี้แนะว่า สารประกอบหลักที่พบชนิดนี้อาจจะเป็นสารออกฤทธิ์ในการต้านอนุมูลอิสระของสารสกัดเฮกเซนและคลอโรฟอร์ม ซึ่งขณะนี้กำลังอยู่ระหว่างการสกัดสารนี้ให้บริสุทธิ์เพื่อพิสูจน์เอกลักษณ์ รวมทั้งทดสอบฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านเบาหวาน

Key Words: *Derris reticulata*, Antioxidant, Phytochemical screening
คำสำคัญ: ชะเอมเหนือ สารต้านอนุมูลอิสระ พฤกษเคมีเบื้องต้น

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Introduction

Nowadays, antioxidants are topics of interest in many research areas including nutrition, health and cosmetics due to their ability to interrupt free radical process. Free radicals are unstable molecules with unpaired electrons in their outer orbits. Free radicals are reactive molecules generated by cellular processes and environmental stress such as immune system, electron transfer, cellular respiration, chemical substances and UV radiation. Free radical can damage DNAs, proteins and lipids leading to cellular dysfunction, injury and death. Excess free radicals cause inflammation, cancer, premature aging disorder, atherosclerosis and diabetes (Kannan and Jain, 2000). Free radicals mostly found in cellular systems are reactive oxygen species and reactive nitrogen species for example hydrogen peroxide, superoxide radical, hydroxyl radical and nitric oxide. Normally, free radical mentioned above can be removed from the body by antioxidant pathways, either enzymatic or non-enzymatic systems, such as superoxide dismutase, catalase, glutathione peroxidase, vitamin C and thiol antioxidant. However, the excess free radicals can also be destroyed by external antioxidants such as supplements containing vitamin C, flavonoid and carotenoid, which can be found in plants, vegetables and fruits (Valko et al., 2007).

It has long been widely accepted that plants are one of the major sources of antioxidants. These external antioxidants have been proven for their efficiency in interrupting free radical processes with fewer side effects than synthetic chemicals. Therefore, the natural products from medicinal plants are more interesting for reducing the risk of several ailments such as diabetes, cancer, ischemia injury and aging (Blasa et al., 2010). One of our research projects has been focused on antioxidant effects of *Derris reticulata* Craib. (Leguminosae). This climbing plant is known in Thai as Cha-em-nuea. It has long been traditionally used as expectorant to relieve cough. Moreover, anti-inflammatory activity in arthritis and anti-herpes simplex virus type I have been reported (Laupattarakasem et al., 2003; Wisetsutthichai et al., 2005). Recently, the aqueous extract of *D. reticulata* has been shown in alloxan-induced diabetic rats. by our group (Kumkrai et al., 2014). It was proposed that the anti-diabetic activity of *D. reticulata* was partly due to its cytoprotective effect, probably by an antioxidant activity. However, the aqueous extract used in our previous study contained many compounds; it was difficult to identify the active ingredients that are responsible for antioxidant and anti-diabetic activities. In the present study, we sequentially extracted *D. reticulata* stem with three solvents differ in polarity (hexane, chloroform and water), screened for phytochemical compounds and finally compared their antioxidant potentials. The results from this study would provide useful information for the future investigation to identify the active ingredients responsible for anti-diabetic activity of *D. reticulata*.

Objective of the study

The aim of this study was to compare antioxidant activities among the hexane, chloroform, and aqueous extracts from *D. reticulata* stem. Phytochemicals and phenolic contents of the extracts were investigated. Thin layer chromatography (TLC) was also conducted to examine major compounds of the extracts.

Methodology

Plant collection and extraction

The stems of *D. reticulata* were obtained from Prachinburi province, Thailand (January - April, 2011). Plant verification has been confirmed by a plant taxonomist, Dr. Paul J. Grote. Voucher specimens (Pharm-Chu-006) were preserved at School of Pharmacology, Suranaree University of Technology (SUT). The stems were cut into small pieces and dried at 50 ˚C in hot air oven. The extracts were obtained by sequential extraction method with hexane and chloroform using Soxhlet extractor at 80˚C. Then the residues were subjected to water decoction at 100 ˚C.

Phytochemical screening

The phytochemical screening was carried out for identification of components of the extracts. They were screened for alkaloids, flavonoids, saponins, tannins, triterpenoids, cardiac glycosides, and polyphenol by using standard procedures (Harborne, 1998; Saxena and Saxena, 2012).

Determination of total phenolic content

The total phenolic content was determined by Folin-Ciocalteu method and performed according to (Prior et al., 2005) with some modifications. In brief, 100 µl of 2.5 µg/ml extract was mixed with 2 ml of 2% Na_2CO_3 and incubated for 2 min at room temperature. After incubation, 100 µl of Folin-Ciocalteu reagent (diluted in methanol 1:1 v/v) was added in the reaction mixture and incubated in the dark at room temperature for 30 min. The absorbance at 750 nm was measured using spectrophotometer. The total phenolic content of extract was determined from a standard curve of Gallic acid and results are expressed as mg gallic acid equivalents (GAE) per gram extract.

Antioxidant activity determinations

ABTS radical scavenging activity assay

ABTS radical scavenging activity assay was performed according to the method previously described (Re et al., 1999) with some modifications. The $ABTS^{\cdot+}$ radical cation stock solution was prepared by mixing 5 ml of 14 mM ABTS with 5 ml of 4.9 mM potassium persulphate ($K_2S_2O_8$) and incubated in the dark at room temperature for 16 h. Before use, the $ABTS^{\cdot+}$ radical cation stock solution was diluted with ethanol to attain absorbance of 0.7 ± 0.02 at 734 nm. After that, 50 µl of various concentrations of the extract (0-5,000 µg/ml) was mixed with 1.5 ml of diluted $ABTS^{\cdot+}$ stock solution. Then, the mixture was incubated in the dark at room temperature for 6 min. After incubation, the absorbance was measured at 734 nm using spectrophotometer. Butylated hydroxytoluene (BHT) was used as standard. Results were expressed as IC_{50} (the concentration required for 50% scavenge for free radical).

Ferric reducing antioxidant power assay

FRAP assay was performed according to the method described by (Benzie and Strain, 1996) with some modifications. The FRAP reagent was prepared by mixing 10 mM TPTZ solution (2,4,6-tripyridyl-s-triazine), 20 mM $FeCl_3$, and 300 mM acetate buffer (pH 3.6), in ratio 1:1:10 (v/v/v). Then, FRAP reagent was incubated at 37 ^{o}C until used. A 50 µl of 5 µg/ml extract was mixed with 1.5 ml of the FRAP reagent. The reaction mixture was incubated at room temperature for 4 min and measured the absorbance at 593 nm. The reducing potential of extract was determined from a standard curve of $FeSO_4$ and the FRAP value was expressed at µmol Fe^{2+}/mg dried extract.

Thin layer chromatography (TLC)

TLC was carried out for screening the number of components in extracts solution as follow. The aqueous extract was dissolved in distilled water whereas the hexane and chloroform extracts were dissolved in ethanol. Then, 10 µl (5 µg) of the extract solution was applied on 4×8 cm of TLC plates. After drying, TLC plates was transferred to TLC developing tank containing mobile phase [Dichloromethane: Methanol (95:5) and Chloroform: Methanol: Water (10:30:1)]. Compositions of each extract were first observed under UV lamp and then stained with 10% sulfuric acid for image analysis.

Statistical analysis

Data are expressed as mean ± SD. Comparisons among different groups were performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test. *P*-values less than 0.05 were considered as significantly different.

Results

% Yield and phytochemical compositions

The results in Table 1 showed that the aqueous extract of *D. reticulata* gave the highest yield (10.56%) compared to the chloroform and hexane extracts with 2.68% and 2.02% yields, respectively.

As shown in Table 1, phytochemical screening analysis demonstrated the presence of alkaloids, triterpenoids, flavonoids, tannins and polyphenols for the hexane and chloroform extracts, whereas saponin, triterpenoids, tannins and polyphenols were presented in the aqueous extract.

Total phenolic compounds and antioxidant activity

Total phenolic contents and antioxidant activities of the extracts are shown in Table 2. Total phenolic contents of the hexane and chloroform extracts were similar (52.29±1.33 and 53.89±0.68 mg GAE/g extract, respectively), but significantly higher than that of the aqueous extract (33.21±0.31 mg GAE/g extract). For antioxidant activity by ABTS method, the hexane extract exhibited the highest potential. The IC_{50} of hexane and chloroform extracts were 441.55±13.56 and 632.69±23.02 µg/ml, respectively, whereas that of the aqueous extract was 1252.68±110.00 µg/ml. However, in this study, the

Table 1 Yields and phytochemical compositions of the chloroform, hexane, and aqueous extracts from *D. reticulata* stem

Phytochemical Screening Tests	Extract		
	Hexane	Chloroform	Aqueous
Alkaloids			
Mayer's test	+	+	-
Wagner's test	+	+	-
Saponins			
Froth test	-	-	+
Triterpenoids			
Salkowski's test	+	+	+
Flavanoids			
Shinoda test	+	+	-
$FeCl_3$ test	+	+	-
Tannins			
$FeCl_3$ test	+	+	+
Lead acetate test	+	+	+
Cardiac glycosides			
Keller Killiani	-	-	-
Polyphenol			
Ferric-chloride-Ferricyanide test	+	+	+
% Yield (dry weight)	**2.02**	**2.68**	**10.56**

reducing powers measured by FRAP assay were not statistically different among all three extracts (*p*-value = 0.064).

Compositions of the extracts detected by TLC

TLC finger prints shown in Figure 1 revealed that the chloroform and hexane extracts contained some similar constituents, and the major compound in both extracts was the compound with R_f= 0.854. In addition, more compounds were observed in the chloroform extract than in the hexane extract. In the case of aqueous extract, the compounds were not separated well in both types of mobile phase used. However, it is clear that the amount of the major compound mention earlier (R_f= 0.854) was much less found in the aqueous extract.

Table 2 Total phenolic content and antioxidant activities of *D. reticulata* extracts

Extract	Total phenolic compound (mg GAE/g extract)	ABTS assay (IC_{50} µg/ml)	FRAP assay (µmol Fe^{2+}/mg dried extract)
Hexane	52.29±1.33[a]	441.55±13.56[a, b]	0.047±0.036
Chloroform	53.89±0.68[a]	632.69±23.02[a]	0.121±0.028
Aqueous	33.21±0.31	1252.68±110.00	0.091±0.026
BHT	-	108.12±8.41	-

Values are expressed as mean ± SD (n = 3).

[a] p< 0.05 statistically significant difference compared to aqueous extract.

[b] p< 0.05 statistically significant difference compared to chloroform extract.

Discussion and conclusion

From our previous report, the aqueous extract of *D. reticulata* stem contained 16.7 yield % (w/w) of dried plant. In the present study, the extracts were obtained from sequential extraction method which was different from the previous report. Due to the polarity of solvent, the hexane extract contained compounds which are more hydrophobic than the chloroform and aqueous extracts, respectively. The results from phytochemical analysis revealed that all extracts from *D. reticulata* stem did not contain cardiac glycosides, whereas triterpenoids, tannins and polyphenol were detected in all three extracts. Saponin appeared only in the aqueous extract. Flavanoids and alkaloids seemed to be completely extracted by hexane and chloroform

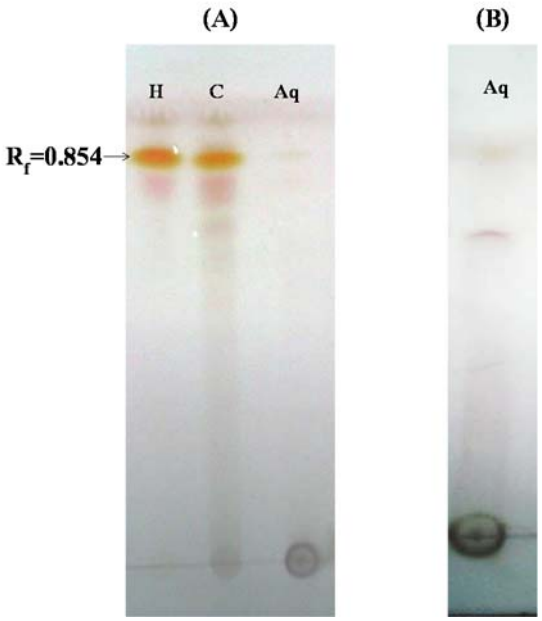

Figure 1 TLC finger print of three *D. reticulate* extracts. (A) TLC plate in Dichloromethane: Methanol (95:5) and (B) TLC plate in Chloroform: Methanol: Water (10:30:1). H, C and Aq denote the type of extracts (hexane, chloroform and aqueous extracts, respectively).

because these types of compound could not be detected in the aqueous extract by the phytochemical screening method used.

D. reticulata extract has been shown to possess antioxidant and anti-diabetic activities. It has been reported that the antioxidant activity of plant

materials was well correlated with the content of their phenolic compounds (Xin-Hua et al., 2001). In this study, it was found that the aqueous extract contained less total phenolic compounds than the other two extracts. We further examined antioxidant activity of the extracts and found that in accordance with the phenolic content, the hexane and chloroform extracts exhibited significantly more antioxidant potential than the aqueous extract.

As shown in Figure 1, the major compound of hexane and chloroform extracts obtained from the present study was the compound shown in TLC with R_f 0.854. The flavonoid lupinifolin has been reported to be a major compound in *D. reticulate* (Chivapat et al., 2009). Taken together, the compound detected at R_f 0.854 in this study could be lupinifolin and may be responsible for antioxidant activities of both hexane and chloroform extracts. Now the isolation and identification of this compound as well as the examination of its anti-diabetic and antioxidant activities are in progress.

References

Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996; 239: 70-6.

Blasa M, Gennari L, Angelino D, Ninfali P. Fruit and vegetable antioxidants in health. In: Preedy RRWR, editor. Bioactive foods in promoting health. San Diego : Academic Press; 2010. p. 37-58.

Chivapat S, Chavalittumrong P, Attawish A, Soonthornchareonnon N. Toxicity study of lupinifolin from stem of *Derris reticulata* Craib. J Thai Tradit Altern Med 2009; 7: 146-55.

Harborne JB. Phytochemical methods a guide to modern techniques of plant analysis. 3rd ed. London: Chapman & Hall; 1998.

Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology 2000; 7: 153-63.

Kumkrai P, Kamonwannasit S, Chudapongse N. Cytoprotective and anti-diabetic effects of *Derris reticulata* aqueous extract. J Physiol Biochem 2014; 70: 675-84.

Laupattarakasem P, Houghton PJ, Hoult JR, Itharat S. An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis. J Ethnopharmacol 2003; 85: 207-15.

Prior RL, Wu X, Schaich K. Standardized method for the determination of antioxidant capacity and phenolics in food and dietary supplements. J Agric Food Chem 2005; 53: 4290-302.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999; 26: 1231-7.

Saxena M, Saxena J. Phytochemical screening of *Acorus calmus* and *Lantana camara*. IRJP 2012; 3: 324-6.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39: 44-84.

Wisetsutthichai S, Techamahamaneerat S, Junyaprasert V, Soonthornchareonnon N. Gel preparation development from *Derris reticulata* Craib. extractas an anti-herpes simplex drug. Thai Journal of Phytopharmacy 2005; 12: 11-22.

Xin-Hua W, Chang-Qing L, Xing-Bo G, Lin-Chun F. A comparative study of *Phyllanthus amarus* compound and interferon in the treatment of chronic viral hepatitis B. Southeast Asian J Trop Med Public Health 2001; 32: 140-2.