SHORT COMMUNICATION

Chemical constituents and antioxidative activity of the extracts from *Dyera costulata* leaves

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Abstract

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Chloroform and n-butanol extracts from the leaves of *Dyera costulata* exhibited EC_{50} values for DPPH radical scavenging activity of 79.8±0.2 and 12.0±0.1 μg/ml, respectively. Chemical investigation of the extracts was conducted by means of column chromatography. β-Amyrin and rhamnazin were isolated from the chloroform extract and quercetin-3-O-α-L-rhamnopyranoside was isolated from the n-butanol extract. The chemical structures of the isolated compounds were identified by comparison of their spectroscopic data with those reported in the literature. The EC_{50} value of quercetin-3-O-α-L-rhamnopyranoside in DPPH radical scavenging activity was 9.37±0.02 μM. Its antioxidative activity is therefore about 8 times higher than that of the well known antioxidant, BHT, which has an EC_{50} value of 80.78±0.01 μM.

Key words : antioxidative, flavone, quercetin, rhamnazin, *Dyera costulata*

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บทคัดย่อ

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สารเคมีและฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากใบตีนเป็ดแดง

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สารสกัดด้วยคลอโรฟอร์มและนอร์มอลบิวธานอลจากใบตีนเป็ดแดง (*Dyera costulata* Hook.f.) มีค่าความเข้มข้นที่สามารถออกฤทธิ์ได้ 50% (EC_{50}) ต่อการต้านอนุมูลอิสระของ DPPH คือ 79.8±0.2 ไมโครกรัม/มล. และ 12.0±0.1 ไมโครกรัม/มล. ตามลำดับ สารบีตา-อมัยริน และแรมนาซิน แยกได้จากสารสกัดด้วยคลอโรฟอร์ม ส่วนสารเควอซิติน-3-โอ-แอลฟา-แอล-แรมโนพัยราโนไซด์ แยกได้จากสารสกัดด้วยนอร์มอลบิวธานอล สูตรโครงสร้างทางเคมีของสารที่แยกได้หาโดยการเปรียบเทียบข้อมูลทางด้านสเปกโตรสโกปีกับสารที่เคยมีรายงานไว้ ค่าความเข้มข้นของเควอซิติน-3-โอ-แอลฟา-แอล-แรมโนพัยราโนไซด์ที่สามารถออกฤทธิ์ได้ 50% ต่อการต้านอนุมูลอิสระของ DPPH คือ 9.37±0.02 ไมโครโมลาร์ ฤทธิ์ในการต้านอนุมูลอิสระของสารตัวนี้แรงกว่า BHT ซึ่งเป็นสารต้านอนุมูลอิสระมาตรฐานประมาณ 8 เท่า โดย BHT มีค่าความเข้มข้นที่สามารถออกฤทธิ์ได้ 50% ต่อการต้านอนุมูลอิสระของ DPPH คือ 80.78±0.01 ไมโครโมลาร์

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Dyera costulata Hook.f. (*D. costulata*) is known in Thai as "Teen Pet Daeng", a large tree having yellow heartwood, in the family Apocynaceae. It occurs in primary evergreen lowlands or on hills and can be found in the South of Thailand, peninsular Malaysia, Sumatra, Borneo, and the intervening islands (Ridley, 1923; Whitmore, 1973). The bark and leaves of *D. costulata* have been used in folk medicine for the treatment of fever, inflammation and pain by traditional doctors in the South of Thailand. Recently, Reanmongkol *et al*. (2002) found that the chloroform extract from the leaves of the tree possesses marked analgesic effect in mice. Antioxidative activity relates to analgesic and anti-inflammatory activities, many medicinal plants or natural products having antioxidative effect also possess analgesic and anti-inflammatory effects (Hodek *et al*., 2002; Felzenszwalb *et al*., 1998; Samuelsen, 2000; Chung *et al*., 2001). The chemical constituents of this plant have been reported only from the leaves. They were the bisindole alkaloids: ochrolifuanines A, E, F and 18-dehydroochrolifuanines A, E, F (Mirand *et al*., 1983). We are interested in the other chemical constituents and antioxidative activity from the leaves of this plant

Materials and Methods

The instruments used in this study were as follows; NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on Varian Unity Inova 500 MHz spectrometer; MS were recorded using a Incos 50 Finnigan Mat and Jeol type JMS-HX 101A spectrometer; IR and UV spectra were recorded using a Jasco type IR-810 infrared spectrometer and a Hewlett-Packard type 8452A diode array spectrophotometer, respectively. TLC was performed on Merck silica gel type 60 GF_{254} plates and the detection of compounds was accomplished either by exposure to UV light at 254 nm or by spraying with vanillin/sulfuric acid reagent. All chemicals were analytical grade and purchased from chemical companies.

Plant material

The leaves of *D. costulata* were collected in August 2000 in Songkhla Province, Thailand. The plants were identified by Associate Professor Dr. Sanan Subhadhirasakul and voucher specimens of plant materials (Specimen No. SS 43080801) have been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of

Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

Preparation of extracts

The dried, coarsely-powdered leaves of *D. costulata* (13.5 kg) were macerated with methanol 40 l for five days and filtered; the filtrate was then evaporated to give a syrupy mass. The marc was re-macerated with methanol (40 l) four times, filtered and evaporated as before. The evaporates were combined to give the methanol extract. The extract was mixed with 2% sulfuric acid aqueous solution (5×1000 ml) and filtered. The acidified filtrate was washed with n-hexane (2×900 ml), then made basic (pH 9) with 25% NH_4OH and extracted with $CHCl_3$ (6×750 ml). The combined $CHCl_3$ extract was washed with water (2×1000 ml), dried with anhydrous Na_2SO_4 and evaporated under reduced pressure to give the $CHCl_3$ extract (13.9 g). The basic aqueous solution was further extracted with n-butanol (3×750 ml). The combined n-butanol extract solution was evaporated under reduced pressure to give the n-butanol extract (93.5 g).

Isolation of pure compounds

A portion of the $CHCl_3$ extract (10.0 g) was separated using silica gel column chromatography. The column was eluted with mixtures of ethanol and chloroform by increasing the percentage of ethanol, and finally with pure ethanol. Fractions of 75 ml were collected. Fractions 12-17 from the column were combined and further separated by SiO_2 column chromatography using MeOH:EtOAc: n-hexane (0.2:2.5:7.0) as eluting solvent. Fractions of 50 ml were collected. Compound **1** (14.8 mg) was isolated as crystals from fraction 4. Fractions 14-15 were combined, and showed one major spot on TLC. They were further purified by SiO_2 column chromatography using $CHCl_3$ as eluting solvent to give an amorphous compound **2** (5.6 mg).

A portion of the n-butanol extract (10.0 g) was chromatographed on SiO_2 column chromatography using MeOH:$CHCl_3$:n-hexane (1:3:1) as eluting solvent. Fractions of 75 ml were collected. Fractions 20-40 from the column were combined (1.225 g) and further separated by SiO_2 column chromatography using 30% MeOH/$CHCl_3$ as eluting solvent. Compound **3** (429.7 mg) was obtained from fractions 12-30 as prismatic crystals.

DPPH radical scavenging assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method is one of those used for testing the antioxidative activity (Yamasaki *et al*., 1994). The scavenging activity of samples corresponds to the degree of quenching of the DPPH as described by Hatano *et al*. (1989). Samples for testing were prepared by dissolving in absolute ethanol. The concentrations of the tested samples were 50, 100, 200 and 400 µg/ml. Each concentration was tested in triplicate. A portion of sample solution was mixed with the same volume (500 µl) of 6×10^{-5} M DPPH in absolute ethanol in a vial and allowed to stand at room temperature for 30 minutes. The absorbance (A) was then measured at 520 nm. Butylated hydroxy toluene (BHT), which is a well known antioxidant, was used as a positive control. The results were expressed as percentage inhibition, % inhibition = $[(A_{control} - A_{sample})/A_{control}]\times100$. EC_{50} (effective concentration of sample at 50% inhibition) was obtained by linear regression analysis of the dose-response curve, plotted between % inhibition and concentration (µg/ml).

Results and Discussion

Compound **1** was obtained as white needles, mp (uncorr.) = 190-191ºC. It was identified as β-amyrin from the following observations (Figure 1). It showed one characteristic of a triterpene by giving a purple spot with vanillin/sulfuric acid reagent. The mass spectrum showed a molecular ion peak at *m/z* 426. The IR spectrum exhibited hydroxyl absorption (3300 cm^{-1}). The ^1H NMR of this compound revealed signals for eight singlet methyl groups at δ 0.79, 0.82, 0.86 (2×CH_3), 0.94, 0.97, 1.00, and 1.14 ppm. The vinylic proton signal at δ 5.18 (1H, dd, J = 3.8, 3.6 Hz) could be assigned to H-12 (Figure 1). A single proton observed at δ 3.22 (ddd, J = 10.7, 6.1, 4.8 Hz) could be assigned to the position C-3 (Figure 1). These ^1H NMR

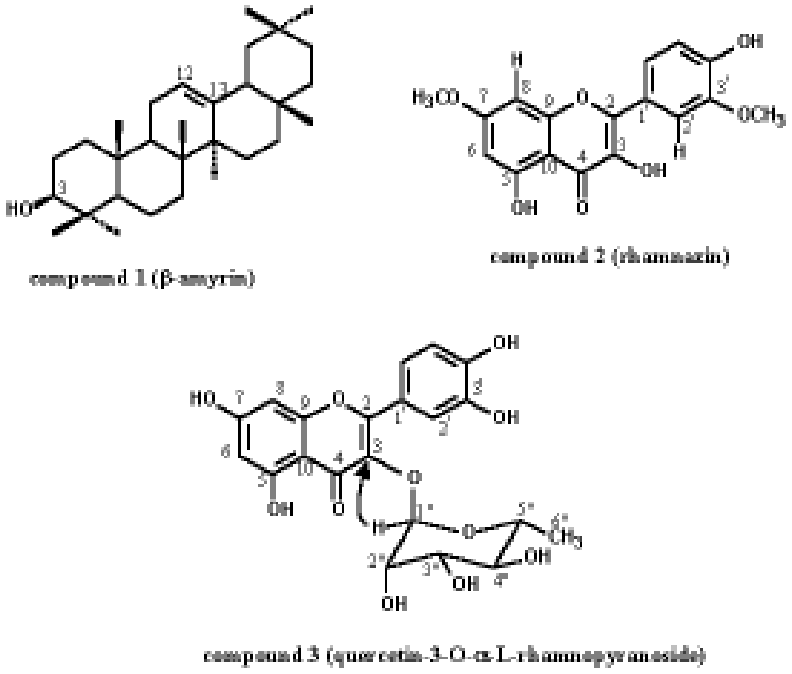

Figure 1. Chemical structures of compounds 1, 2, 3, showing the significant HMBC correlations of compound 3.

signals were regarded as being due to a pentacyclic triterpene (Mahato and Sen, 1997). The ^{13}C NMR spectrum of 1 showed a total of 30 carbon atoms, among them two olefinic carbon signals (δ 145.58 and 121.96 ppm) and one oxygen-attached carbon signal (δ 79.03 ppm) were observed. The remaining carbons showed signals having chemical shifts between 15 and 55 ppm. These ^{13}C NMR data were identical with those reported in a literature for β-amyrin (Seo *et al.*, 1975) (Table 1). The TLC patterns of compound **1** and authentic β-amyrin were also found to be identical.

Compound **2** was obtained as yellow amorphous powder. It was identified as 3,4',5-trihydroxy-3',7-dimethoxyflavone or 3',7-dimethoxyquercetin or rhamnazin (Agrawal, 1989). It gave a quasi-molecular ion peak $(M+H)^+$ at m/z 331 in the positive ion FAB-MS spectrum. The complete analysis of the ^{13}C and ^{1}H NMR spectral data of compound **2** was assigned with information provided from COSY, HMQC and HMBC correlations. Its ^{1}H NMR spectrum exhibited two methoxyl signals at δ 3.91 and 4.02 ppm. A singlet signal at δ 11.75 ppm was assigned to the hydroxyl proton at the position C-5. Presumably because of intramolecular hydrogen bonding, the chemical shift of the hydroxyl proton appeared at low field. Doublet signals at δ 6.40 and 6.52 (J = 2.3 Hz) were assigned to H-6 and H-8 on the A-ring, respectively. The signal at δ 7.83 (d, J = 2.1 Hz) was assigned to H-2', whereas the signals at δ 7.08 (d, J = 8.5 Hz) and 7.79 (dd, J = 2.1, 8.5 Hz) were assigned to H-5' and H-6', respectively. The chemical shift of all carbons was assigned based on the HMQC and HMBC spectra as shown in Table 2.

Compound **3** was obtained as yellow prismatic crystals. The structure of **3** was identified as quercetin-3-O-α-L-rhamnopyranoside (Figure 1) from the following observations. It gave a quasi-molecular ion peak $(M+H)^+$ at m/z 449 in the positive ion FAB-MS spectrum. The IR spectrum exhibited the presence of carbonyl group at 1660 cm^{-1} and hydroxyl groups at 3350 cm^{-1}. The UV

Table 1. ^{13}C NMR spectral data (δ ppm) in $CDCl_3$ for β-amyrin (Seo *et al*., 1975) and compound 1 .

Position	β-Amyrin	1	Position	β-Amyrin	1
1	38.5	38.35	16	26.2	25.85
2	27.0	26.63	17	32.5	32.23
3	78.9	79.03	18	47.2	47.04
4	38.7	38.55	19	46.8	46.64
5	55.1	55.03	20	31.1	30.82
6	18.3	18.03	21	34.8	34.48
7	32.6	32.38	22	37.2	36.90
8	39.7	39.56	23	28.1	27.80
9	47.6	47.45	24	15.5	15.14
10	37.0	36.71	25	15.5	15.23
11	23.4	23.21	26	16.8	16.45
12	121.7	121.96	27	26.0	25.69
13	145.0	145.58	28	27.3	26.94
14	41.7	41.50	29	33.2	33.08
15	28.3	28.11	30	23.6	23.38

Table 2. ^{13}C NMR spectra data (δ ppm) for compound 2, quercetin-3-O-α-L-rhamnopyranoside (3a) and compound 3.

Position	2^x	3^{ayz}	3^y	Position	2^x	3^{ayz}	3^y
2	145.59	156.4	156.58	1"	-	101.9	101.99
3	135.61	134.4	134.39	2"	-	70.4	70.52
4	175.14	177.7	177.89	3"	-	70.6	70.69
5	160.81	161.2	161.44	4"	-	71.5	71.36
6	97.91	98.6	98.81	5"	-	70.1	70.19
7	165.75	164.3	164.32	6"	-	17.3	17.60
8	92.22	93.5	93.97	7-OCH_3	55.85	-	-
9	156.79	157.0	157.40	3'-OCH_3	56.10	-	-
10	103.89	104.2	104.23				
1'	122.90	121.0	121.23				
2'	110.26	115.4	115.59				
3'	146.42	145.1	145.34				
4'	147.73	148.3	148.56				
5'	114.60	115.8	115.81				
6'	121.78	121.0	121.23				

^{x} In $CDCl_3$, ^{y} In DMSO-d_6, ^{z} Markham *et al*., 1978.

absorption maxima (log ε) bands at 216.0 nm (4.65), 256.0 nm (4.65) and 350.0 nm (4.53) suggested a flavonol analogue (Agrawal, 1989). The complete analysis of ^{13}C and ^1H NMR spectral data of compound **3** was assigned with information provided from COSY, HMQC and HMBC correlations. The ^1H NMR (CD_3OD) spectrum of **3** showed characteristics of a quercetin moiety (Abe *et al*., 1995). Doublet signals at δ 6.20 and 6.37 (J = 2.2 Hz) were assigned to H-6 and H-8 on the A-ring, res-

pectively. Signal at 7.34 (d, J = 2.2 Hz) was referred to H-2', whereas, signals at δ 6.91 (d, J = 8.4 Hz) and 7.32 (dd, J = 2.2, 8.4 Hz) were assigned to H-5' and H-6', respectively. An anomeric proton of the sugar was observed at δ 5.35 (d, J = 1.7 Hz) and originated from that of α-rhamnose (Bashir *et al*., 1991). Signals of other sugar protons appeared at δ 4.22 (dd, J = 1.7, 3.3 Hz, H-2"), 3.75 (dd, J = 3.3, 9.4 Hz, H-3"), 3.31 (m, H-4"), 3.42 (m, H-5") and 0.94 (3H, d, J = 6.3 Hz, H-6"). The signal at δ 0.94 was assignable to a methyl group of rhamnose. In the HMBC spectrum (Figure 1), significant cross peak was observed between an anomeric proton signal of rhamnose (δ 5.35 ppm) and a C-3 signal (δ 136.78 ppm). The ^{13}C NMR (DMSO-d_6) spectral data of **3** (Table 2) were identical with those reported in the literature (Markham *et al*., 1978).

The crude chloroform extract and the crude n-butanol extract from the leaves of *Dyera costulata* exhibited EC_{50} values for DPPH radical scavenging activity of 79.8±0.2 and 12.0±0.1 μg/ml, respectively. Compound **3**, isolated from n-butanol extract showed an EC_{50} of 9.37±0.02 μM. Its antioxidative activity is therefore about 8 times higher than that of the well known antioxidant, BHT, which has an EC_{50} value of 80.78±0.01 μM. Compounds **2** and **3** are classified as flavonols, which are the most widely occurring group of flavonoids. This is the first report of finding these compounds in *D. costulata*. Quercetin possesses many types of biological activity, such as anti-allergic, anti-inflammatory, anticancer, antiosteoporotic, antispasmodic, and antihepatotoxic (Carlo, 1999), as well as having antioxidative activity (Choi, 2002). It is clear that the high radical-scavenging property of compound **3** is from its aglycone, quercetin. Flavonoids have a variety of biological effects in mammalian cell systems, *in vitro* as well as *in vivo*. They have been shown to exert antimicrobial, antiviral, antiulcerogenic, cytotoxic, antineoplastic, mutagenic, anti-inflammatory, antioxidant, antihepatotoxic, antihypertensive, hypolipidemic and antiplatelet activities (Guardia *et al*., 2001). Therefore, the presence of flavonoids in this plant is in accord with its traditional uses as an antinociceptive, antipyretic and anti-inflammatory drug.

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