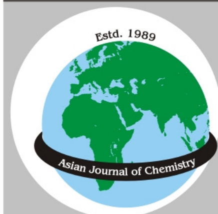

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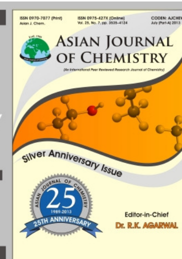

Oil Constituents from the Leaves and Stems of *Drypetes hainanensis*

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The present paper examines the chemical composition and antioxidant capacity of six oil fractions which were prepared by silica gel column chromatography from *D. hananensis* leaves and stems. Totally, 43 compounds have been identified, but 4 compounds were unidentified as well. The oil fractions investigated are characterized by a high content of saturated fatty acid, unsaturated fatty acid and *bis*(2-ethylhexyl)phthalate. In addition, it also contains alcohols, hydrocarbons (saturated group and alkene) and ketone. For the evaluation of the mentioned antioxidant capacities, two different methods were performed: the 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) and ferric reducing/antioxidant power assay (FRAP). The oil fractions have a moderate antioxidant effect when tested by each method, respectively, but significant less effective than ascorbic acid and butylated hydroxytoluene. And the oil fractions are moderate to good in scavenging effect on DPPH radical but low in reducing power. The synergy among unsaturated fatty acid is suggested as possible factor, which influenced the scavenging DPPH power of the oil fractions. The unsaturated fatty acid concentrations influenced its antioxidant power, too. To our best of knowledge, this is the first report so far on the study of the content and antioxidant capacity of oil fractions from *D. hainanensis* leaves and stems.

Key Words: *Drypetes hainanensis*, Oil constituents, GC-MS, Antioxidant.

INTRODUCTION

Drypetes hainanensis Merr., a big tree, is one of the many species of the genus Drypetes (Euphorbiaceae) encountered in China. In Hainan tropical monsoon forests more than 10 species have been identified[1]. These plants are well known in folk medicine of Africa and Hainan; many are used to treat various diseases including tumors, gonorrhea, toothache, dysentery, coryza, sinusitis, boils and swellings in West and Central Africa[2-5]. The results of our previous screening on this species showed that the crude extract possessed potent antioxidant properties[6]. However, little information is available about the constituents of the *D. hananensis*. This background prompted us to explore the phytochemistry of *D. hananensis*. Therefore, our objective was to isolate the chemistry constituents from the leaves and stems of *D. hananensis* by chromatograph with Si gel and the components in the oil substances analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). In addition, antioxidant properties of the constituents, including antioxidant activities and reducing power were determined in the present study and the results are reported here.

EXPERIMENTAL

Collection of plant material: The leaves and stems of *D. hainanensis* (Euphorbiaceae) were harvested from Bawangling rain forest (Southwest Hainan) in July 2011. The botanical identification of the plant was done at the College of Materials and Chemical Engineering, Hainan University, where the voucher specimens were conserved.

Extraction and isolation: The dried and powdered stems of *D. hainanensis* (17.9 kg) were extracted with 95 % EtOH (75 L × 3 L) at room temperature for 7 days. The filtrate was concentrated to dryness under reduced pressure to yield a semisolid residue (879 g) that constituted the crude extract (DH). The crude extract was triturated successively with water (2 L) and then subjected to further extraction, respectively with petroleum ether-soluble fraction (MSO), ethyl acetate-soluble fraction (EtOAc) and *n*-butanol-soluble fraction (NBA) and a residue fraction (H_2O). Part of the ethyl acetate-soluble fraction (300.0 g) was subjected to column chromatography over silica gel 60 (200-300 mesh, 3000 g) and eluted with $CHCl_3$-MeOH (99:1) in increasing polarity to give a total of 330 fractions (2000 mL each). Thin layer chromatography permitted to combine the resulted fractions into 14 series, A-N. Further

column chromatography of series A (7.0525 g, oily substances), on silica gel 60 (200-300 mesh, 200g) using hexane/ $CHCl_3$ (100:0 to 70:30) in increasing proportions yielded oily fraction (150 mL each), Fr. 21-40 (F-1, hexane/$CHCl_2$, 90:10, 204.5 mg), Fr. 41-50 (F-2, 90:10, 99.5 mg), Fr. 51-122 (F-3, 90:10, 212.1 mg), Fr. 123-154 (F-4, 90:10, 86.1mg), Fr. 181-209 (F-5, 80:20, 1331.6 mg), Fr. 210-308 (F-6, 70:30, 2477.1 mg), respectively.

GC and GC-MS analysis: The oil substances fractions were analyzed on a Hewlett- Packark, HP6890 equipped with a mass spectrometer, USA fused silica capillary column (30 m × 0.25 mm. i.d., 0.25 µm film thickness), using helium as carrier gas at 1.0 mL/min. Samples were injected 1.0 µL at the following condition. Initial column temperature of 120 ºC for 1 min, elevated to 250 ºC at a rate of 6 ºC/min, kept at the final temperature for 5 min. Injector temperature was 250 ºC. The carrier gas, helium, was adjusted to a linear velocity of 1 mL/ min. Ion source temperature was 230 ºC. The ionization energy was 70 eV with a scan time of 1 s and mass range of 10-500 AMU.

Identification of components: The identification of the constituents was assigned on the basis of comparison of retention data and mass spectra with data banks. NIST 98 (National Institute of Standards and Technologies 98) Mass Spectra Library was also used as a reference.

Antioxidant activities

DPPH radical scavenging assay: In order to measure antioxidant activity, DPPH free radical scavenging assay was used. The radical scavenging activity of extracts was determined following the methods of Changwei *et al.*[7] and Al-Zubairi *et al.*[8] with slight modification.

Briefly, the oily fractions stock solutions were prepared at 20 mg/mL in dimethyl sulfoxide (DMSO) and different concentrations (0.5-10 mg/mL) of each extract were prepared. The sample solution (100 µL) with various concentrations was added to 100 µL of 0.1 mM DPPH in 95 % ethanol in microtiter 96-well plates. Control contained DPPH solution and solvent without test sample. Blank contained test sample, 95 % ethanol solution without DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature, the absorbance was measured at 517 nm using xMarK (BIO-RAD, USA). Ascorbic acid and BHT were used as positive control. The measurement was performed in triplicate. Activity of scavenging (%) was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{A_0 - (A - A_b)}{A_0}\right] \times 100$$

where, A_0 was the absorbance of the control, A was the absorbance of test sample and A_b was the absorbance of blank. The correlation between each concentration and its percentage of scavenging was plotted and the EC_{50} was calculated by interpolation. The activity was expressed as EC_{50} (the effective concentration of each fraction that scavenges 50 % of DPPH radicals).

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous-blue-coloured form in the presence of antioxidants. It is a relatively simple method frequently used in the assessment of antioxidant activity of various fruits, vegetables and some biological samples[9]. Briefly, The FRAP assay was done according to Benzie *et al.* with some modifications[10]. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ solution. The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3{\cdot}6H_2O$ solution and then warmed at 37 ºC before using. The fractions were appropriately diluted with ethanol and immediately used in the antioxidant assay. A 20 µL sample which was prepared at 2 mg/mL, was mixed with 150 µL of FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm using xMarK (BIO-RAD) after incubation at 37 ºC for 0.5 h. The results could be expressed in micromole of Fe^{2+} equivalent. The standard curve was linear between 25 and 1500 µM Fe^{2+}. Results are expressed in µM Fe^{2+}/g fresh mass. All the measurements were taken in triplicate and the mean value ± standard error (SE) are reported.

Statistical and analysis: The data were statistically analyzed by one-way analysis of variance followed by Duncan's multiple range tests using SAS 8.0 Software. The $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Ethyl acetate-soluble fraction sample A was further separated in to 5 fractions by flash silica chromatography, using hexane/$CHCl_3$ (90:10) with increasing amounts of $CHCl_3$ (up to 30 %) as eluent and all the fractions were investigated by GC-MS, which means that all components reported in Table-1 were identified by both techniques. In total, 43 compounds have been identified, 4 compounds were unidentified as well. The *D. hainanensis* oils investigated are characterized by a high content of saturated fatty acid, unsaturated fatty acid and *bis*(2-ethylhexyl)phthalate (DEHP). In addition, it also contains alcohols, hydrocarbons (saturated group and alkene) and ketone.

The major compounds in fraction 1 were DEHP (69.31 %), isopropyl palmitate (8.39 %), hexadecanoic acid, methyl ester (7.02 %), octadecanoic acid (6.13 %) and octadecanoic acid methyl ester (4.85 %). In fraction 2, the major compounds were hexadecanoic acid, methyl ester (26.00 %), isopropyl palmitate (22.91 %), DEHP (18.22 %), octadecanoic acid methyl ester (12.78 %) and octadecanoic acid (9.95 %). In fraction 3, the major compounds were 8-octadecenoic acid, methyl ester (24.75 %), DEHP (10.81 %), phytol (10.25 %), 2-pentadecanone, 6,10,14-trimethyl (9.77 %), hexadecanoic acid, methyl ester (8.54 %) and 8,11-octadecenoic acid, methyl ester (8.48 %). In fraction 4, the major compounds were DEHP (19.19 %), 11-octadecenoic acid, methyl ester (18.81 %), 9,12,15-octadecatrienoic acid, met (17.29 %), 2-pentadecanone, 6,10,14-trimethyl (10.72 %) and heptadecane, 2,6,10,15-tetramethyl (7.02 %). In fraction 5, the major compound was DEHP (89.21 %). In fraction 6, the major compound was DEHP (95.78 %). In addition, in fraction 3, it contains eicosane, 1-nonadecene, 9-hexacosene, octacosane, cyclotetradecane, 1,7,11-trimethyl, 1-hexacosene, Z-14-nonacosane, docosane and tricosane. DEHP was high in the

TABLE-1
CHEMICAL COMPOSITION OF OILY SUBSTANCES ISOLATED FROM DH-EtOAc

No.	Identified components	F-1 Pa (%)	F-2 Pa (%)	F-3 Pa (%)	F-4 Pa (%)	F-5 Pa (%)	F-6 Pa (%)
1	Methyl tetradecanoate	–	1.49	–	–	–	–
2	Tridecanoic acid, 12-methyl-, meth	–	–	0.37	–	–	–
3	Eicosane	–	–	1.13	3.58	–	–
4	Isopropyl Myristate	0.34	1.58	0.33	–	–	–
5	Nonanoic acid, 9-oxo-, methyl este	–	–	1.60	1.11	–	–
6	Pentadecanoic acid, methyl ester	–	0.36	–	–	–	–
7	2-Pentadecanone, 6,10,14-trimethyl	–	–	9.77	10.72	–	–
8	1-Butanol, 3-methyl-, benzoate	0.46	–	–	–	–	–
9	Hexadecanoic acid, methyl ester	7.02	26.00	8.54	5.83	–	–
10	Docosane	–	–	–	2.44	–	–
11	9-Hexadecenoic acid, methyl ester,	–	–	4.24	–	–	–
12	Isopropyl Palmitate	8.39	22.91	4.54	2.42	–	–
13	Hexadecanoic acid, ethyl ester	–	–	2.09	–	–	–
14	Isophytol	–	–	0.89	–	–	–
15	Heptadecanoic acid, methyl ester	0.65	2.85	0.49	–	–	–
16	1-Nonadecene	–	–	0.43	–	–	–
17	Ethyl 9-hexadecenoate	–	–	1.10	–	–	–
18	Octadecanoic acid, methyl ester	4.85	12.78	–	–	–	–
19	11-Octadecenoic acid, methyl ester	–	–	–	18.81	–	–
20	9-Octadecenoic acid (Z)-, methyl	–	–	–	–	–	0.50
21	Octadecanoic acid	6.13	9.95	–	–	–	–
22	8-Octadecenoic acid, methyl ester	–	–	24.75	–	–	–
23	9,12-Octadecadienoic acid (Z,Z)-,	–	–	–	5.96	–	–
24	8,11-Octadecadienoic acid, methyl	–	–	8.48	–	–	–
25	Heptadecane, 2,6,10,15-tetramethyl	–	–	–	7.02	–	–
26	Phytol	–	–	10.25	–	–	–
27	9,12,15-Octadecatrienoic acid, met	–	–	2.94	17.29	–	–
28	Cyclopropaneoctanoic acid, 2-octyl	–	1.46	–	–	–	–
29	Eicosanoic acid, methyl ester	0.62	0.70	–	–	–	–
30	Octadecanoic acid, 2-methylpropyl	0.52	–	–	–	–	–
31	Ethanol, 2-(octadecyloxy)-	–	–	0.89	–	–	–
32	dl-2-Ethylhexyl chloroformate	0.79	–	–	–	–	–
33	Docosa-2,6,10,14,18-pentane-22-al,	–	–	–	1.90	–	–
34	9-Hexacosene	–	–	0.64	–	–	–
35	Docosanoic acid, methyl ester	0.91	0.93	–	–	–	–
36	Octacosane	–	–	1.25	–	–	–
37	Tetracosanoic acid, methyl ester	–	0.75		–	–	–
38	Cyclotetradecane, 1,7,11-trimethyl	–	–	1.21	–	–	–
39	Tricosane	–	–	–	2.28	–	–
40	1-Hexacosene	–	–	0.87	–	–	–
41	*Bis*(2-ethylhexyl) phthalate	69.31	18.22	10.81	19.19	89.21	95.78
42	Z-14-Nonacosane	–	–	2.40	–	–	–
43	2,6,10-Dodecatrien-1-ol, 3,7,11-tr	–	–	–	1.46	–	–
44	X_1	–	–	–	–	5.19	–
45	X_2	–	–	–	–	2.19	–
46	X_3	–	–	–	–	1.14	0.77
47	X_4	–	–	–	–	2.27	2.94
Total	–	99.99	99.98	100.01	100.01	100.00	99.99

X_1, X_2, X_3, X_4: unidentified. – = undetectable. Pa: Peak area. F-1, F-2, F-3, F-4, F-5 and F-6: Fractions of the oil substances eluted from a silica gel column.

oil and found in fractions 1-6 (Table-1). It was postulated that the elution of this compound was not completed by one single eluant, partially due to its high content and specific polarity. Along with the increased polarity of the eluants, the eluted fractions consisted of more polar compounds. After fractionation using the silica gel column chromatography, minor components present in the oil could be enriched in the eluted fractions and therefore, were easily recognized and identified.

DEHP is a primary plasticizer, which is one of the most widely used synthetic organic compounds and has a large production in the world. DEHP and plastic components are not combined with covalent chemical bonds. Therefore, DEHP can easily release to environment as well as pollutes air, water and soil. Research has proved that human being and animals cumulate DEHP through several ways such as diet, touching to plastic products and breathing from air and DEHP has a potential toxicity[11]. Xuqiang Wang[12] concluded that the DEHP cumulation ability of plants was nothing to do with the composition and content of fatty acid. It may be related to nonadecane, methyl palmitate, 1-octadecene, eicosane and 1-eicosenethe in wax components, which were contained in the oil from ethyl acetate-soluble fraction sample A, abundantly.

For the reason that, the oil from leaves and stems of *D. hainanensis* contains a large number of DEHP. The results indicated that *D. Hainanensis* conducted DEHP contamination of repair. Phytoremediation plays an important role of bioremediation, using plants to absorb DEHP and to degradation with low-input, effective governance and the characteristics of the construction of ecological environment compared with traditional physical, chemical remediation methods. Hence, *D. hainanensis* may be promoted and cultivated value.

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol. Owing to rapid hydrogen acceptable ability of DPPH, it reacts with antioxidants and gets converted into 1,1-diphenyl-2-picrylhydrazine and hence shows decrease in absorbance. Fig. 1 shows the results of DPPH radical scavenging activity of all oil fractions at various concentrations. With increasing oil concentrations, the scavenging effect on DPPH radical increased. At 10 mg/mL, the scavenging effect of the F-1, F-2, F-3 and F-4 (91.7, 92.5, 61.6 and 77.0 %, respectively) are higher than that of F-5 (8.9 %) and F-6 (7.8 %). However, the scavenging effects of BHT and ascorbic acid were much more effective at an extremely low concentration and were 49.8 and 79.3 % at 0.04 mg/mL. The effective concentration (EC_{50}, meaning the concentration that scavenges 50 % of the initial DPPH radical) values for F-1, F-2, F-3, F-4, ascorbic acid and BHT were 4.57, 4.28, 3.48, 1.40, 0.0107 and 0.0471 mg/mL, respectively. These results revealed that the antioxidant activities were in the descending order of ascorbic acid > BHT > F-4 > F-3 > F-2 >F-1.

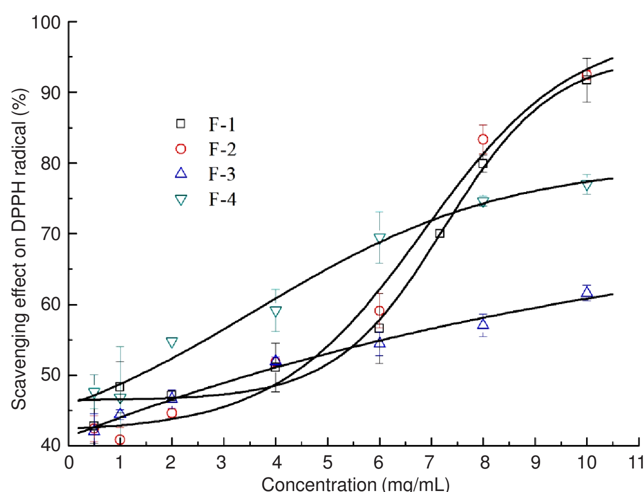

Fig. 1. Scavenging effects on DPPH radical. F-1, F-2, F-3 and F-4, fractions of the oil substances eluted from a silica gel column. Data were expressed as means with standard errors (n = 3)

Fatty acids are recognized as essential nutrients in both human and animal diets and are implicated to possess numerous health benefits. Their use in the pharmaceutical industry has also been well documented. Saturated "bad" fatty acids are known to contribute to cardiovascular disease, whereas the unsaturated "good" fatty acids are reported to help cellular function and promote a healthy heart. Henry *et al.*[13] reported the antioxidant activities of 29 commercially available C-8-C-24 saturated and unsaturated fatty acids. Most of the unsaturated fatty acids tested showed good antioxidant activities. The present data indicated an increase in antioxidative property with increasing content of unsaturated fatty acid[14]. *D. hainanensis* oil fractions extracts showed moderate scavenging radical activity, which probably contained substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules. The unsaturated fatty acids could be responsible for the results, which were related with the content of the unsaturated fatty acids. F-4 included the highest unsaturated fatty acids, such as 9,12,15-octadecatrienoic acid, met (17.29 %), 11-octadecenoic acid, methyl ester (18.81 %) and 9,12-octadecadienoic acid (Z,Z)- (5.96 %). Therefore, F-4 had the strongest effect on free radical in all oil fractions. However, F-5 and F-6 were abundant with DEHP (89.2, 95.8 %, respectively) which didn't have antioxidant activity. At 10 mg/mL, the scavenging effect of the F-5 and F-6 were just 8.9 and 7.8 %.

The FRAP assay results are presented in Fig. 2 as μM Fe^{2+}/g dry weight of oil fractions. The reducing power of the oil fractions increased with increased content of unsaturated fatty acids. However, The reducing powers of ascorbic acid (18030.0 ± 66.1 μM Fe^{2+}/g) and BHT (10196.7 ± 118.1 μM Fe^{2+}/g) were significantly ($p < 0.05$) higher than that of all oil fractions. The reducing powers of ascorbic acid and BHT ranged from 400-fold to 6000-fold than the oil fractions, respectively. These results indicate that all oil fractions have low reducing power.

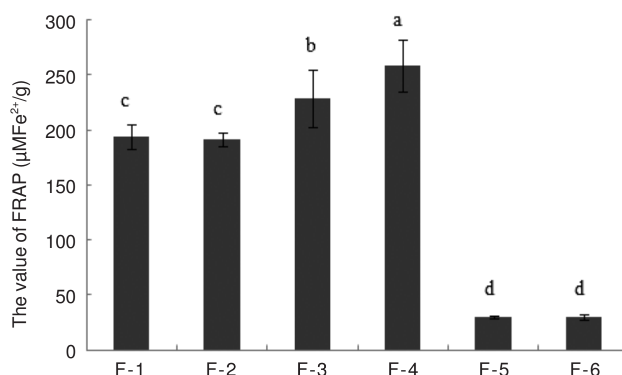

Fig. 2. Antioxidant capacities (μM Fe^{2+}/g) of different oil fractions from *D. hainanensis* leaves and stems according to FRAP assay. Data were expressed as means with standard deviations (n = 3). Different letters in the figure indicate significant difference ($p < 0.05$)

The FRAP assay can react with Fe^{2+} and SH-group containing antioxidants and DPPH method use organic radicals, so it is expected that using these two methods accurately reflects all of the antioxidants in a sample. However, it must be noted that there was some diversity between the FRAP and DPPH results. Unsaturated fatty acids may be due to its proton donating capability as shown in DPPH radical scavenging results. Acting as an electron donor that can react with free radicals, it converts them to more stable products and terminates radical chain reactions. Hence, the oil fractions had shown modest antioxidant power with DPPH method, but low reducing power with FRAP assay simultaneity.

Conclusion

To the best of our knowledge, this is the first report so far on the study of the content and antioxidant capacity of oil fractions from *D. hainanensis* leaves and stems. As this plant

is distributed in a large quantity in Hainan tropical regions and its oil demonstrated moderate antioxidant activity and reducing power because of unsaturated fatty acids. In addition, our results indicated that *D. hainanensis* can cumulate a large number of DEHP. Our work suggested that the *D. hainanensis* may be promoted and cultivated value to conduct DEHP contamination of repair and could be utilized as an effective and safe antioxidant source. And the results obtained in the present study are in agreement to a certain degree with the traditional uses of the plants estimated. The obtained results could form a good basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds.

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