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Antioxidants of Purple and White Greater Yam (*Dioscorea alata* L.) Varieties from the Philippines

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Aqueous methanolic (50% MeOH) extracts of the tubers (peel and flesh) of nine cultivars of greater yam (*Dioscorea alata*) were determined to have relatively high antioxidant activities among which two cultivars (*Ubing upo*, purple; LA 096, white) had activities as high as those of α-tocopherol and butylhydroxyanisole (BHA). The aqueous methanol extract of the purple variety was fractionated on XAD-2 column chromatography serially with water, 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH and acetone. High activities were found in the 50% and 75% MeOH fractions; these were dried and further subjected to semi- or preparative HPLC chromatography using Develosil column. Two major peaks were isolated with high antioxidant activities, P1 and P2. P1 was established to be a purple anthocyanidin with UV-Vis and ^{1}H NMR spectral data that are very similar to alatanin C (Yoshida et al., *Tetrahedron Lett.* 32: 5579-5580 (1991)). Initial results for P2 indicated its phenolic nature with a glucose moiety and a molecular weight of 306. Both P1 and P2 had antioxidant activities higher than those of butylated hydroxyanisole and α-tocopherol.

Key Words: antioxidants, *Dioscorea alata*, greater yam, alatanin

INTRODUCTION

Antioxidants prevent oxidation and oxidative damage to cells by scavenging oxygen radicals or hydroxy radicals which have been implicated to attack polyunsaturated fatty acids in cell membranes giving rise to lipid peroxidation (Aust and Sringen 1982; Osawa et al.1990; Kaur and Perkins 1991; Ramarathnam et al. 1995). At high levels, this type of oxidation has been linked not only to aging but also to mutagenesis, carcinogenesis, atherosclerosis (Halliwell and Whiteman 2004; Addis and Warner 1991; Ramarathnam et al. 1995) and to diabetes (Mooradian et al. 1994; Wehmeier and Moorradian 1994).

Many flavonoids have been studied and reviewed to have comparable, if not better, antioxidative properties than butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Jovanovic et al.1994). Using a number of generally accepted antioxidative assay systems, Tsuda et al. (1994a&b) showed that free cyanidin (synthetic or hydrolysis products) and one of its glucosides possess antioxidative activity better and comparable to α-tocopherol, respectively. Cyanidin and its simple glucosides are known to be digestive degradation products of the anthocyanin pigments commonly found in foods.

Greater yams (*Dioscorea alata* L.) are commonly known as *ube* in the Philippines and are popularly utilized in sweetened food delicacies because of their attractive violet color and unique taste. Thus, *ube* is used in native delicacies such as *halaya* (yam pudding with milk), *sagobe* (with parboiled chocomilk and glutinous rice balls), *puto* (rice cake), *halo-halo*, *hopia*, and different types of rice cake using glutinous rice such as *suman*, *sapin-sapin*, *bitso* and *bibingka.* It also has become a great favorite as flavor and/

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or filling for ice cream, tarts, bread, cakes etc. Although the color of greater yams varies from very light (almost white) to intense violet, its taste remains the same.

On the other hand, some species of yams including *D. alata* have also been utilized as herbal medicine e.g., in China. We had earlier screened in our laboratory a number of greater yam varieties and found high antioxidant activities in some of them (Mendoza et al.1994). Chen et al. (2004) reported the free radical scavenging ability of compounds from Taiwan yams extracted by a continuous hot pressurized solvent extraction process. Hou et al. (2001) also reported high antioxidant activity of the storage protein (dioscorin) of *Dioscorea batatas* tuber. The mucilage consisting of polysaccharides of the tuber of *D. batatas* was likewise reported to exhibit high antioxidant activity (Hou et al. 2002). Lin et al. (2005) recently reported the antioxidant activities of crude and partially purified mucilages of three different cultivars of yam (*D. alata*). A steroid yam extract called dioscorea was reported to exhibit antioxidant activity which can modify serum lipid levels (Araghiniknam et al. 1996). A feeding experiment with freeze-dried powder or *D. alata* in hyperhomocysteinemia rats showed antioxidative effects including alleviation of lipid peroxidation, oxidative stress and platelet aggregation (Chang et al. 2004).

Because of the importance of *D. alata* as food and as a nutraceutical, we undertook a study of the antioxidant activities of several cultivars of greater yam of varying colors of purple to white from the Philippines. We hereby report the identification of greater yam cultivars with high antioxidant activities in their tubers (peel and flesh) and the isolation and structural characterization of the antioxidants in the methanolic extract of a selected purple cultivar with high antioxidant activity.

MATERIALS AND METHODS

Materials

Mature (10–12 months after planting) greater yam tubers were obtained from the National Plant Genetic Resources Laboratory of the Institute of Plant Breeding (IPB), College of Agriculture, University of the Philippines Los Baños, College, Laguna, Philippines. Some of the samples were obtained from the town of Los Baños, Laguna, Philippines. Linoleic acid, α-tocopherol and BHA were purchased from Wako Pure Chemical Industries Ltd., Japan. All other chemicals used were of pure analytical grade from various commercial sources.

Preparation of Crude Extracts

Tubers (peel and flesh) of nine cultivars of greater yam (1 kg) with color ranging from white to intense purple were sliced (2 to 5 mm thick), immersed quickly in liquid nitrogen and lyophilized. The lyophilized tubers were ground or powdered with a grinder or with a mortar and pestle. Two hundred g powder (60–100 mesh) were extracted with methanol:water (1:1) at 1:4 (w/v) at least three times at room temperature. The crude extract was filtered through filter paper (Whatman No. 1 or equivalent) and the filtrate was concentrated using a rotary evaporator to dryness. The dried crude extract (about 5 g) was weighed (about 5 g) and stored in the freezer before assay.

Antioxidative Activity Assay

The assay for antioxidative activity using the linoleic acid system (Osawa and Namiki 1981) was followed. Each sample (25 to 50 μg for purified fraction or 200 μg for crude extract) was added to a 10 mL solution mixture prepared as follows: 9.8 ml phosphate buffer with linoleic acid (prepared by mixing 25 mL of 100 mM phosphate buffer, pH 7.4, 3.5 g SDS, 0.35 ml linoleic acid and 225 ml water) mixed with 50 μl freshly prepared H_2O_2 (1.1 μl of 32% H_2O_2 per ml water) and 50 μl of 20 mM $FeCl_2$. The solution was incubated at 37 ^oC for 2 to 4 h and the degree of oxidation was determined according to the thiocyanate method (Mitsuda et al. 1966) which measured the peroxides by taking the absorbance at 500 nm after color development with $FeCl_2$ and ammonium thiocyanate. α-Tocopherol and BHA (100 or 200 μg each; amount indicated for specific experiment) were used as standard samples. A test solution with H_2O_2 –$FeCl_2$ but without antioxidant sample was taken as control (100% relative lipid peroxidation) while that without H_2O_2 -$FeCl_2$ and sample (autoxidation) was taken as blank. Each sample had four replicates. Statistical analysis was performed on the peroxidation values by using Student's t-test at 95% confidence level. Insignificant levels of lipid peroxidation, in comparison to the BHA or tocopherol standards, are indicated.

Batchwise XAD-2 Column Fractionation of Crude Extract

The crude extract from the tuber with the highest antioxidant activity (*Ubing upo*) was fractionated through an XAD-2 column (Nomura Chemicals, Japan) measuring 4.3 cm i.d. x 64 cm i.d. and was eluted using the following solvents in series: water, 25% aqueous methanol, 50% aqueous methanol, 75% aqueous methanol, 100% methanol and acetone. The volume of solvent used was 3x the bed volume of XAD-2 (2.7 l). The fractions were concentrated *in vacuo* for antioxidative assay.

HPLC of XAD-2 Fractions with High Activity

The XAD-2 fractions with antioxidant activities relatively higher than or comparable with the reference standards were subjected to analytical high performance liquid chromatography (HPLC) on Develosil ODS-5 column (4.6 mm i.d. x 250 mm) and pre-column (4.6 mm i.d. x 10 mm) (Nomura Chemical Co., Ltd.) using a JASCO (Japan Spectroscopic Co., Ltd.) photodiode-array detector (Model 910). A linear gradient elution using water-methanol was used.

Isolation of Antioxidative Compounds from XAD-2 Fraction with Highest Activity

Preparative and semi-preparative HPLC runs were done on the XAD-2 fraction with highest activity using either JASCO HPLC with UV/visible detector Model UV-970) or a Shimadzu LC6-AD HPLC (Shimadzu Works Co., Ltd.) with a JASCO Uvidec-100-III detector. The columns used were Develosil ODS-5 20 mm x 250 mm and 8 mm x 250 mm for preparative and semi-preparative separations. Linear gradient elution from 0.1%TFA (aqueous) to 100% MeOH and gradient elution from 0.1% TFA in 10% acetonitrile to methanol were used as solvent systems to purify the compounds. The purity of the isolated compounds was checked using the photodiode array detector (DAD) system.

Spectroscopic Analysis of Isolated Compounds

The UV spectra of the isolates dissolved in methanol were taken using a JASCO spectrophotometer. The FAB-mass spectra were recorded at the Nagoya University Instrument Center, School of Agricultural Sciences. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker ARX-400 NMR instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C) in CD_3OD and/or D_2O (with 10%TFA) containing tetramethylsilane (TMS) as internal standard.

RESULTS AND DISCUSSION

Screening of Antioxidative Activity of Greater Yam Cultivars

All of the white and purple greater yam cultivars exhibited considerable antioxidant activity (Figure 1). The greater purple yam variety *Ubing upo* had the highest activity followed by the white variety LA-096. Their antioxidant activities were similar to those of the control of α-tocopherol and BHA. *Ubing upo* was eventually chosen for the isolation of the antioxidant principle based on its availability and high antioxidant activity. In an earlier report from our laboratory, seven cultivars of greater yam

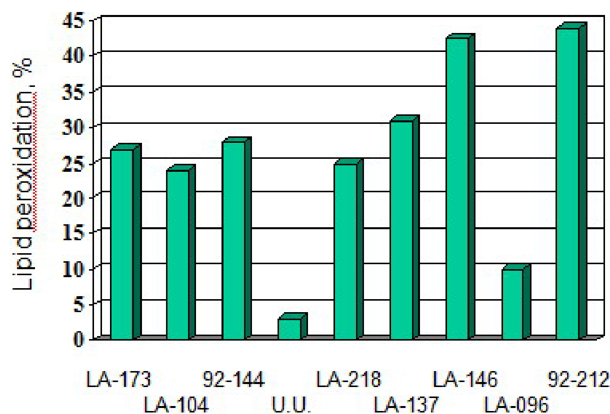

Figure 1. Antioxidant activity of crude extracts of tubers of greater yam cultivars using the linoleic acid system. The color of the tubers ranged from purple (LA-173, LA-104, 92-144 and Ubing Upo (U.U.) to white (LA-218, LA-137, LA-146, LA-096 and 92-212). BHA and α-tocopherol (200 μg each) exhibited 10% and 12% lipid peroxidation, respectively. A control containing no added samples or standard represents 100% lipid peroxidation.

were shown to have high antioxidant activities including five which had higher antioxidant activity than that of α-tocopherol (Mendoza et al. 1994). The antioxidant activities reported in this present paper and the earlier report are from the peel and flesh of the tubers of *D. alata*, whereas the antioxidant activity in greater yam from Taiwan is from the mucilages of the tubers of *D. alata* (Lin et al. 2005) and *D. batatas* (Hou et al. 2002).

Fractionation of Antioxidant Compounds on XAD-2 Column and their HPLC-DAD Analysis

The crude methanolic extract of the purple-colored Ubing-upo greater yam variety was fractionated on XAD column. Table 1 shows that the purple-colored 50% and

Table 1. Antioxidative activities of Ubing upo crude extract, XAD-2 fractions and standards, and their corresponding yields from 1 kg of greater yam.

Sample[a]	Relative lipid peroxidation (%)	Yield (mg)
Crude extract	61 ± 3.1	5000
Water fraction	Not assayed	
25% MeOH	58 ± 12.5	85.1
50% MeOH	26 ± 1.6	156.4
75% MeOH	23 ± 3.1	99.4
100% MeOH	34 ± 0.1	114.3
Acetone	38 ± 4.7	119.3
BHA	22 ± 3.1	na
α-tocopherol	38 ± 3.1	na

na, not applicable
[a]100 μg of the crude extract, other fractions and standards (BHA and α-tocopherol) used in analysis of antioxidant activity.
Reported values are means ± SD (n=4). A control containing no added samples or standard represents 100% lipid peroxidation.

75% methanol fractions had the highest levels (25% and 23%) of antioxidant activities, which were not statistically significant from each other. They exhibited antioxidant activity (22%) similar to that of BHA at 100 µg. The crude extract showed a moderate antioxidant activity (61%) but lower than that of α-tocopherol (38%).

Both the 50% and 75% fractions gave almost identical HPLC chromatogram patterns (not shown) except for the presence of an early eluting group in the former. Both fractions contained the greatest amounts (based on peak areas) of substances absorbing at the 250–280 nm and 520–540 nm regions. These fractions had noticeably large peaks with spectral patterns indicative of phenolics and flavonoids in particular. These observations plus the fact that these two fractions were the most intensely purple colored fractions indicate that these contain a lot of phenolic compounds, particularly anthocyanin. The two fractions were therefore subjected to further purification of the antioxidant compounds.

Isolation, Purification and Structural Characterization of Antioxidant Compounds

Application of the 50% XAD-2 fraction on preparative HPLC using stepwise elution (Figure 2) from 0.1% TFA (aq), 10 min, to 7% acetonitrile in 0.1% TFA (aq), 20 min, to 100% MeOH, 45 min, resulted in the separation of four groups of compounds. Group III consisted of purple fractions. Group IV consisted of colorless fractions (Figure 2) that came out later in the elution and one peak (designated P2) was found to exhibit a UV spectrum typical of phenolics (Figure 2 inset). It was symmetrical, indicative of its purity, and had high antioxidant activity (Table 2).

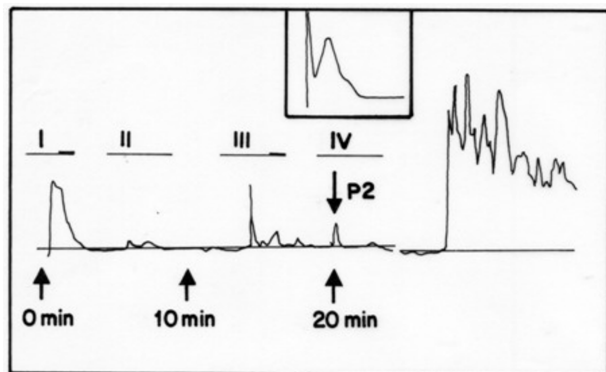

Figure 2. Preparative HPLC of 50% XAD-2 fraction. Conditions: column-ODS 5 (Develosil 20 x 250 mm); detection at 220 nm; stepwise elution from 0.1% TFA(aqueous), 10 min, to 7% acetonitrile in 0.1% TFA (aq), 20 min, to 100% MeOH, 45 min; flow rate 5 mL/min; injection volume, 800 µl. Inset is the UV spectrum of P1.

Table 2. Antioxidative activities of purified compounds from greater yam

Sample	Lipid peroxidation, %
Control	100
P1 (25 µg)	42±20*
P1 (50 µg)	13± 8
P2 (25 µg)	17±10
P2 (50 µg)	5±4**
BHA (100 µg)	2±4
α-tocopherol (100 µg)	46±18

Reported values are means ± SD (n=4). A control containing no added samples or standard represents 100% lipid peroxidation. (Note: (*) indicates no significant difference from 100 µg α-tocopherol, and (**) from 100 µg BHA)

Under different eluting conditions (linear gradient from 0.1% TFA(aq) to 100% MeOH), six anthocyanin peaks separated (Figure 3). The third peak was selected, designated P1 and was further characterized.

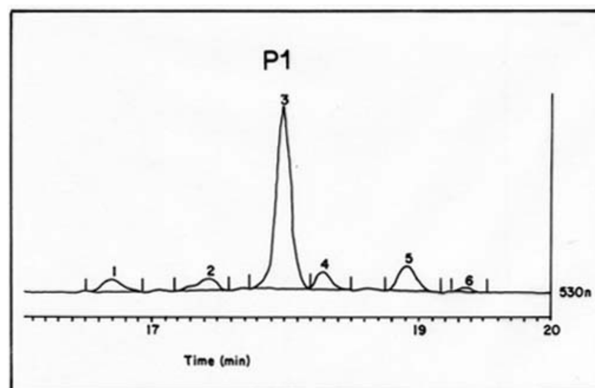

Figure 3. HPLC chromatogram of the 50% MeOH XAD-2 fraction. Six anthocyanin peaks are shown in detail. Conditions: column, ODS 5 (4.6 x 250 mm); linear gradient from 0.1% TFA (aq) to 100% MeOH for 30 min then washed with methanol.

The characterization and identification of compound P2 with high antioxidant activity is the subject of a separate paper.

The purple red compound P1 was found to exhibit the following properties: UV λmax in methanol (nm) 287, 332, and 535 (max). These are similar to the UV λmax of alatanin C of 286, 334 and 539 (Yoshida et al, 1991). Alatanin C is one of three structurally similar anthocyanidins isolated and identified in *D. alata* (Figure 4) (Yoshida et al. 1991). All three alatanins (A, B, and C) possess a common cyanidin nucleus as anthocyanidin. Alatanin A has two sinapic acids and five glucoses, alatanin B with two sinapic acids and four glucoses and alatanin C, with one sinapic acid and two glucose moieties.

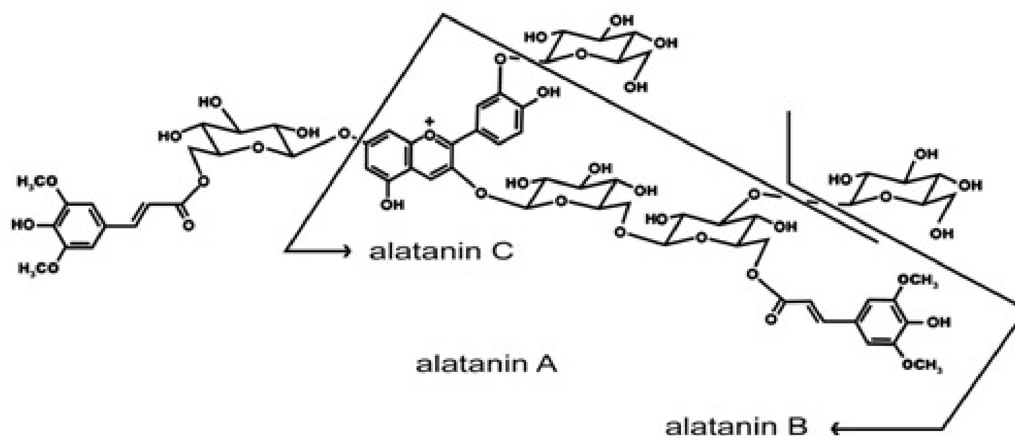

Figure 4. Structure of alatanins A, B, and C [20]. Reprinted from Yoshida K, Kondo T, Kameda K, Kawakishi Lubag AJM, Mendoza EMT, Goto T. Structures of alatanins and C isolated from purple yam *Dioscorea alata*. Tetrahedron Lett. 32: 5575-5578 (1991), with permission fom Elsevier.

The ^{1}H NMR (in D4-methanol) spectrum and peak assignments are summarized in Table 3. The assignments are supported by a homonuclear (^{1}H-^{1}H) correlation spectroscopy (COSY) (not shown). Table 3 shows that only very small differences in the ^{1}H NMR signals can be observed between the P1 spectrum and that of alatanin C isolated by Yoshida et al. (1991). An additional singlet at around 2.7 ppm was observed for P1 which may have come from a –CH3 group within P1 or a solvent signal. Slight shifts in other signals may be due to the relatively higher concentration of methanol and water impurities (due mainly to the relatively small amount of P1 purified) as seen in the spectrum of P1. The ^{13}C NMR spectrum of P1 (not shown) supported the proton assignments. The results show that P1 is similar to alatanin C based on NMR and UV/visible spectra. The possibility though, of one or more methylation cannot entirely be discounted as possible signals of such character can be observed in the ^{1}H NMR of P1 (around 3.5 ppm). HPLC analyses of the anthocyanins of the *D. alata* var Ubing upo extract were also noted to exhibit a pattern similar to that obtained by Yoshida et al. (1991).

Table 3. ^{1}H-NMR signal assignments of P1 compared with alatanin C (both in 10% TFAd-CD_3OD at 30 °C).

H-position	P1 ppm (400 MHz)	Alatanin C ppm (500 MHz)[a]	Difference ppm
4	8.68	8.55	0.13
6	6.64	6.62	0.02
8	6.52	6.48	0.04
2'	7.92	7.84	0.08
5'	7.02	7.01	0.01
6'	8.23	8.18	0.05
1	5.28	5.23	0.05
2	3.66	3.68	-0.02
3	3.78	3.78	0
4	3.13	3.26	-0.13
5	4.28	4.28	0
6a	4.07	4.09	-0.02
6b	3.93	3.98	0.05
S1- α	6.10	6.06	0.04
S1-β	7.32	7.27	0.05
S1-2',6’	6.19	6.21	-0.02
-OCH3	3.47#	3.45#	0.02

The antioxidative activity of the isolated anthocyanin P1 which may be alatanin C at the level of 50 μg was similar to that of 100 μg of BHA. P1 at the level of 25 μg had a similar antioxidant activity to 100 μg of α-tocopherol (Table 2). This indicates that P1 is stronger than α-tocopherol and BHA. Chen et al. (2004) reported the extraction of free radical scavenging compounds from *D. alata.* They also observed that the flavonoids such as naringenin, quercetin, kaempherol, pinocembrin, isorhamnetin, caffeic acid, phenethyl ester, galangin, chrysin and acacetin, and four typical phenolic acids were not present in the extracts of the *D. alata* and therefore would not account for the yam’s antioxidant activity.

The strong antioxidative activity of greater yam's anthocyanin or possibly alatanin C is similar to the observations by others on the antioxidative activity of other anthocyanin pigments. Tsuda et al. (1994b) showed the strong antioxidative activity of the anthocyanin pigments cyanidin and cyanidin 3-*O*-β-D-glucoside which are higher and as good as α-tocopherol, respectively. The pigments of the seed coat of red and black bean (*Phaseolus vulgaris*) exhibited strong antioxidant activities (Tsuda et al. 1994b) under varying pH conditions. Cyanidin from red bean showed strong antioxidant activity in the linoleic acid system at pH 7.0 while pelargonidin 3-*O*-β-D-glucoside from red bean and delphinidin 3-*O*-β-D-glucoside from black bean had no antioxidative activity at pH 7.0 and strong antioxidative activity at pH 3.0 and pH 5.0.

Our study showed that cultivars of greater yam (*D. alata*), with colors ranging from white to intense purple, had high antioxidant activities similar or higher than the control BHA and α-tocopherol. Two compounds with high antioxidative activity were isolated from a purple cultivar. P1 is an anthocyanin which NMR spectral data show to be most probably alatanin C and P2 is a colorless unidentified phenolic compound with a sugar moiety.

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