Determination, Localization, and Partial Characterization of Natural Antioxidants in Musaea

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Analysis of the antioxidative activity of six banana varieties *(Bungulan, Lakatan, Latundan, Pisang walung, Pundol and Saba)*, abaca (*Musa textilis*) and Heliconia by the thiobarbituric and thiocyanate tests showed that *Lakatan, abaca* and *Pundol* had appreciable activity in the leaf. This was observed in both chloroform and 80% ethanol extracts as well as in both young and mature leaves.

The ethanol extract of the pseudostem of abaca showed the highest antioxidant activity compared to the corm, leaf and bract of two banana varieties and abaca.

High pressure liquid chromatography of the abaca pseudostem ethanol extract showed seven peaks. Antioxidant activity was located in the second peak.

Thin layer chromatography of the active peak gave two bands with Rf Values of 0.12 and 0.65. Partial characterization of these bands using chromatography revealed that the Rf 0.65 constituent was phenolic and the Rf 0.12, nonphenolic. The band of Rf 0.65 corresponded to chlorogenic acid.

Keywords: Musaea, banana, abaca, Heliconia, *Musa textilis*, antioxidant activity.

Banana (Musa spp), the premier fruit crop of the Philippines, has other uses in addition to the consumption of its fruit. The banana leaves have traditionally been used for wrapping food. The flowers (male bud) of certain cultivars such as *Saba* are cooked and eaten as vegetable. The rest of the plant are not all utilized.

Antioxidants naturally occur in biological systems as protection against the toxic effects of normal oxygen metabolism. The presence of antioxidants such as ascorbic acid and tocopherol in the banana fruit has been reported. In a recent study, high antioxidant activity was detected in the leaf wax of *Musa zebrina*, a banana cultivar from Malaysia (2). Other types of antioxidants may therefore be present in various parts of the banana plant, especially those which are just thrown away and which comprise a large volume of agricultural wastes. These antioxidants may also be involved in the protection of the plant against certain pests and diseases.

This paper reports on the (1) screening of six cultivars of banana as well as other members of the Musaea, abaca *(Musa textilis)* and heliconia for antioxidant activity in the leaves; (2) localization of the antioxidant activity in different parts of the plant and (3) isolation and partial identification of the antioxidant active principle.

MATERIALS AND METHODS

Sample Collection and Preparation

Mature and young leaves of banana cultivars *Bungulan, Lakatan, Latundan, Pisang Walung, Pundol* and *Saba*, abaca *(Musa textilis)* and Heliconia were collected from the National Plant Genetic Resources Laboratory of the Institute of Plant Breeding, College of Agriculture, UPLB. These were extracted twice with chloroform, (10 g: 50 mL) and concentrated in vacuo. The residue of each sample was then extracted with 80% ethanol (100 mL), treated with activated carbon (20 mg/mL) filtered and concentrated in vacuo. To localize the antioxidative activity, samples of leaves, pseudostems, corms and bracts of abaca, Pundol and Lakatan were collected and extracted with 80% ethanol (10 g: 50 mL), treated with activated charcoal (20 mg/mL), filtered and concentrated in vacuo.

Assay for Antioxidative Activity

The reaction mixture consisted of the following:

10 uL of methyl linoleate, 5 mL of 0.02% sodium dodecylsulfate (SDS) in 0.05 M Tris HCl/0.15 M KCl buffer pH 7.4; 50 uL of 0.033% H_2O_2; 50 uL of 20 mM $FeCl_2$ and 100 uL of the antioxidant solution. For test samples, 1 mg/mL solution was used. Butylated hydroxyanisole (BHA) (1.8 mg/mL) was used for comparison.

The reaction mixtures were put in screw capped test tubes and incubated in a slanted position in a shaking water bath at 37°C for 16 hr. Percent lipid peroxidation was determined by the thiocyanate (SCN) and thiobarbituric acid (TBA) tests.

Thiocyanate test. To 100 uL of the reaction mixture, the following were added: 50 uL butylated hydroxytoluene (BHT, 1% in ethanol); 4.7 mL of 75% ethanol; 100 uL of 30% ammonium thiocyanate; and 100 uL of 20 mM $FeCl_2$ in 3.5% HCl. After 3 min absorbance was read at 500 nm (3).

Thiobarbituric acid test. To 1 mL aliquot of the reaction mixture the following were added: 50 uL of BHT (1% in ethanol), 0.5 mL of 20% trichloroacetic acid and 1 mL of 0.63% TBA. The resulting mixture was heated in a boiling water bath for 15 min, cooled and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm (4).

Fractionation of Active Components

Crude 80% ethanol extract of the abaca pseudostem was fractionated using a Waters Associate HPLC equipped with a Model 680 gradient controller connected to a Shimadzu CRIB Chromatopac Data Processor, with a Develosil ODS-5 [8.0 mm (i.d) x 150 mm) semi-preparative column (Nomura Chemical Co. Ltd) and a UV detector at 280 nm. The Sepak-filtered sample was eluted isocratically with acetonitrile: 0.1% TFA (70:30 v/v) at a flow rate of 1 mL/min. Fractions comprising the peaks were pooled and concentrated in vacuo and assayed for antioxidative activity.

Thin Layer Chromatography (TLC)

The active peak was subjected to TLC (Merck $60F^{254}$ 0.22 mm thickness) using chloroform-methanol-water (64:50:10) as the solvent system. Bands were detected under UV (254 nm) in a Camag UV cabinet and by spraying the plate with Berlin Blue Reagent (5). To prepare Berlin blue reagent, equal volume of aqueous 1% solutions of ferric chloride and potassium ferricyanide were mixed together, making an orange-brown solution with no blue color, and sprayed. Phenols give an immediate blue color (6).

Standard phenolic acids, namely, chlorogenic acid, p-coumaric acid, ferulic acid and quercetin were run on the same plate with the sample.

RESULTS AND DISCUSSION

Antioxidative Activities of Leaf Extracts

Crude chloroform extracts. Chloroform leaf extracts from mature and young leaves showed strong antioxidative activity as determined by the thiocyanate (SCN) and thiobarbituric acid (TBA) tests (Table 1).

In the SCN test, *Lakatan*, abaca and *Pundol* consistently showed strong antioxidative activity in both young and mature leaves. *Lakatan* extracts were able to inhibit lipid peroxidation by as much as 85% and 87% for the young and mature leaf extracts respectively [to be designated from hereon as 85% (87%)]. On the other hand, abaca and *Pundol* extracts inhibited lipid peroxidation by 92%(78%) and 86% (88%) respectively. Leaf extracts of Heliconia showed a higher degree of antioxidative activity in the young leaves (84%) than in the mature leaves (69%).

Using the TBA method, formation of TBA reactive substances was greatly reduced by *Lakatan*, abaca and *Pundol* leaf extracts. Their activities in terms of % inhibition are 93% (86%), 93% (89%) and 86% (87%) respectively. Young leaf extracts of Latundan and Heliconia also showed strong antioxidative activities in the TBA test.

Table 1. Antioxidative activities of leaf extracts of different cultivars of banana, abaca and heliconia.

Sample		% Lipid Peroxidation			
		Chloroform extracts		80% Ethanol extracts	
		SCN	TBA	SCN	TBA
Control		100	100	100	100
		100	100	100	100
BHA		8	2	5	10
		4	2	2	5
Bungulan	Y	35	35	58	63
	M	38	30	36	68
Lakatan	Y	15	7	37	38
	M	13	14	60	82
Latundan	Y	33	10	22	27
	M	45	40	102	90
Pisang walung	Y	28	33	43	40
	M	23	27	41	73
Pundol	Y	14	14	35	38
	M	12	13	24	70
Saba	Y	32	37	55	42
	M	25	25	18	43
Abaca	Y	12	7	28	33
	M	8	11	22	49
Heliconia	Y	16	5	33	34
	M	31	20	25	73

M, mature and Y, young leaves; SCN, thiocyanate and TBA, thiobarbituric acid methods.

Note: Antioxidative activity is expressed in terms of % inhibition of lipid peroxidation (100%-% lipid peroxidation).

Crude 80% ethanol leaf extracts. Crude 80% ethanol extracts from mature and young leaves also had marked antioxidative activities as monitored by the SCN and TBA tests (Table I).

Peroxide formation (SCN test) was observed to be lower in systems with extracts from abaca [72% (78%)], *Pundol* [65% (76%)] and Heliconia (67% 75%)]. For *Lakatan* and *Latundan,* only the young leaf extracts showed strong antioxidative activity, 63% and 78%, respectively. Mature *Saba* leaf extract was able to inhibit lipid peroxidation to the extent of 82%.

On the other hand, the TBA test revealed that leaf extracts from abaca, *Pundol* and *Saba* had significant antioxidative activities, 67% (51%), 62% (30%) and 58% (57%) respectively. Appreciable activities were also detected in young leaves of *Lakatan* (62% inhibition), Heliconia (66% inhibition) and *Latundan* (73% inhibition).

For both the assays of chloroform and 80% ethanol extracts in which 0.1 mg of the extract was used, none of them exhibited stronger antioxidative activity than 0.18 mg of butylated hydroxyanisole (BHA). The chloroform extracts however, showed quite a comparable antioxidative activity to BHA.

In all the assays, no relation between maturity and antioxidative activity was observed. Rather the differences in activity were only of varietal nature.

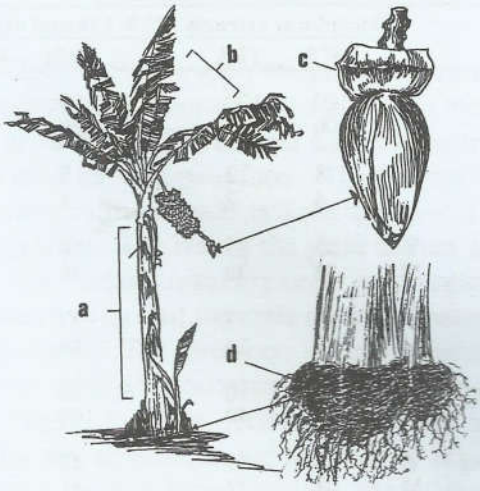

Fig. 1. Parts of the banana plant used for the localization of antioxidative activity: a) pseudostem, b) leaves, c) bract, d) corm.

Localization of Antioxidative Activity

Of the eight plant samples screened, three which showed significant antioxidative activity were selected for localization of antioxidative activity. These were abaca, Pundol and Lakatan. The parts selected for localization were the leaf, pseudostem, corm and bract (Fig. 1).

The highest antioxidative activity was detected in the crude alcohol extract of abaca pseudostem (81% inhibition in the TBA test and 78% inhibition in the SCN test, Fig 2). Notably, all the extracts from abaca exhibited strong antioxidative activities compared to the other extracts. In general, antioxidative activity was found to be highest in the leaf, followed by the corm, pseudostem and bracts.

Isolation and Identification/Characterization of Antioxidative Components

High-pressure liquid chromatography (HPLC). The HPLC chromatogram of the 80% ethanol extract of abaca pseudostem separated on a semi-preparative ODS-5 (8 mm (i.d) x 150 mm) column is shown in Fig 3. At a flow rate of 1 mL/min, seven peaks were resolved with the following retention times: 0.92 min, 1.13 min, 2.30 min, 3.36 min, 4.06 min, 4.66 min and 5.60 min.

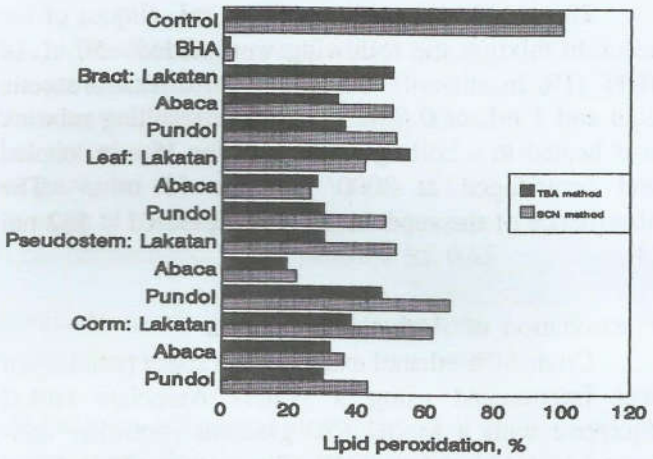

Fig. 2. Antioxidative activities of 80% ethanol extracts of four different parts of two banana varieties and an abaca *(M. textilis)* accession.

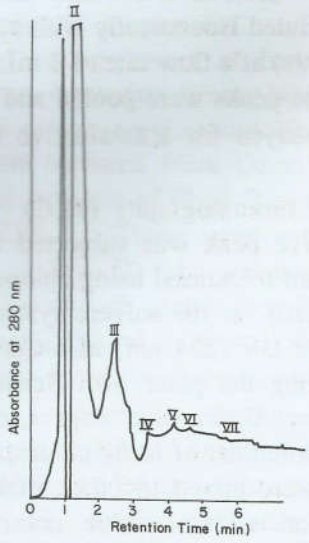

Fig 3. HPLC chromatogram of the crude 80% ethanol extract of abaca pseudostem. Conditions: column, Develosil ODS-5 (8mm (i.d.) x 150 mm); eluent, CH_3CN: 0.1% TFA (70:30 v/v); flow rate, 1 ml / min; detector, UV 280 nm.

Assaying the components of these peaks, prepared as 1 mg/mL solutions, by the TBA and SCN tests gave the results shown in Fig 4. Peak 2 had the strongest antioxidative activity, 72% in the SCN test and 83% in the TBA test. These components even exhibited stronger antioxidative activity than 0.18 mg of BHA (68% inhibition - SCN test and 60% inhibition - TBA test). Components of peaks 3 and 6 also showed appreciable antioxidative activity particularly in the SCN test. Coming out of the column at 2.30 min and 4.66 min respectively, their antioxidative components can be totally different but equally as effective as the antioxidants of peak 2.

Thin layer chromatography (TLC). Three solvent systems were tested, namely ethyl acetate-formic acid-acetic acid-water (EFAW), n-butanol-acetic acid-water (BAW) and chloroform-methanol-water (CMW) to separate peak 2 components. Only the latter was able to successfully separate the antioxidative components.

Developed in chloroform-methanol-water (64:50:10), the plates were subjected to two detection techniques: without chemical treatment and with chemical treatment.

Under UV (254 nm), the chemically untreated plate yielded two fluorescent bands with Rf values of 0.12 and 0.65 (Fig 5b). The band at Rf 0.65 gave a positive reaction to ferric chloride-potassium ferricyanate spray (Fig 5a) indicating that the activity was caused by a phenolic compound. The negative reaction of the band at Rf 0.12, however, does not indicate that it is not antioxidative in nature. It can possibly be a non-phenolic synergistic antioxidant.

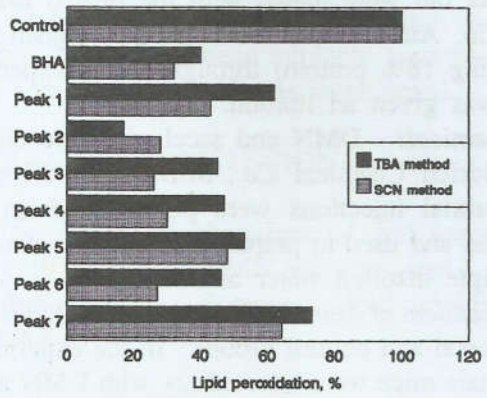

Fig. 4. Antioxidative activities of the HPLC fractions of the 80% ethanol extracts of abaca pseudostems.

Standard phenolic compounds, namely, p-coumaric acid, chlorogenic acid, ferulic acid and quercetin developed in the same plate as the sample (Fig 5b) had the following Rf values: 0.87, 0.66, 0.88 and 0.91. The closeness of the Rf values of chlorogenic acid and the band at Rf 0.65 and their similar reaction to ferric chloride - potassium ferricyanate (dark blue spots) indicate that chlorogenic acid is a major component responsible for the antioxidative properties of the 80% ethanol extract from abaca pseudostem.

In studies on sweet potato, Dalisay (7) reported that chlorogenic acid is a resistance factor of sweet potato against the rot fungus *Diplodia tubericola.* This may also be true in abaca.

The foregoing observations indicate the presence of antioxidative defense systems in members of the Musaea family. These antioxidants may contribute to some physiological mechanisms such as defense mechanisms against diseases and insect pests in the banana plant. This study also provides a basis for the use of banana as source of natural antioxidants for possible uses in food and medicine.

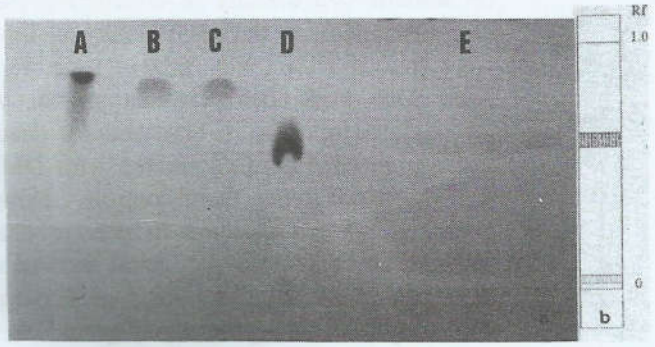

Fig. 5. TLC pattern of (a) HPLC active fraction and standard phenolic acids. Plate Merck silica plate, $60F_{254}$; eluent, $CHCl_3$: MeOH : H_20 (64:50:10 v/v); detector, Berlin Blue Reagent and (b) HPLC active fraction viewed under UV light (254 nm).

LITERATURE CITED

1. Simmonds, N.W. 1959. Bananas. New York: Longmanns, Green, Co. Ltd.
2. Sekiya, K. 1985. Natural antioxidants in plant leaf waxes. B.S. Thesis, Dept of Food Science and Technology, Faculty of Agric, Nagoya University.
3. Osawa, T. and M. Namiki. 1985. Natural antioxidants isolated from *Eucalyptus* leaf waxes. J. Agric. Food Chem 33:777-780.
4. Ottolenghi, A. 1959. Interaction of ascorbic acid and mitochondrial lipides. Arch Biochem. Biophys. 79, 355-363.
5. Wagner, H., S. Bladt and E.M. Zgaishi. 1984. Plant Drug Analysis. A Thin Layer Chromatography Atlas, Germany: Springer-Verlag.
6. Pratt, D.E. and E.E. Miller. 1984. A flavonoid antioxidant in Spanish peanuts *(Arachis hypogaea).* J. Am Oil Chemists Society 61:1064-7.
7. Dalisay, R.F., G.G. Divinagracia and E.M.T. Mendoza. 1986. Biochemical basis of resistance to Java black rot caused by *Diplodia tubericola* (EII. Ev.) Taubenh) in sweet potato roots *(Ipomea batatas* (L.) Lam.). Philipp Phytopathology 22:15-28.