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In vitro Antioxidative Activities and Polyphenol Content of *Eugenia polyantha* Wight Grown in Indonesia

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Abstract: Antioxidative activities of three bark extracts (methanol, methanol-water and water) from *Eugenia polyantha* Wight grown in Indonesia were evaluated using various *in vitro* assays; 2, 2-diphenyl-1-picrylhydrazyl radical-scavenging, hydrogen peroxide-scavenging and β-carotene bleaching assays. From the assays, *Eugenia polyantha* bark extracts were found to be potential antioxidative activities. The methanol-water extract showed the highest level of free radical-scavenging activity (Effective dose (ED_{50}) = 0.18 mg mL^{-1}) and protection from β-carotene bleaching (85.7% at 100 μg mL^{-1}). The methanol-water extracts showed the highest total phenolic content (856 mg gallic acid equivalent (GAE)/g and 161 mg catechin equivalent (CE)/g) and total antioxidative capacity (449 mg ascorbic acid equivalent (AAE)/g). Furthermore, it exhibited dose-dependent antioxidative activities. A relationship between total antioxidative capacity and total phenolic content was recognized in the three extracts from *Eugenia polyantha* bark.

Key words: Antioxidative activity, total phenolic content, *Eugenia polyantha* wight, bark extracts

INTRODUCTION

Free radical reactions occur in the human body and food systems. Free radicals, in the form of reactive oxygen and nitrogen species, are an integral part of normal physiology. Over production of these reactive species can occur due to oxidative stress brought about by an imbalance of the bodily antioxidant defense system or free radical formation. These reactive species can react with biomolecules, causing injury and death (Halliwell, 2008).

Free radical and reactive oxygen species are well known inducers of cellular and tissue pathogenesis leading to diseases like cancer and inflammatory disorders, as well as the aging process (Halliwell, 1994; Aviram *et al*., 2000). Oxidative stress induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases, such as cancer (Muramatsu *et al*., 1995), atherosclerosis (Steinberg *et al*., 1989) and gastric ulcer (Das *et al*., 1997). Many antioxidants, occurring naturally in plants, have been identified as scavengers of_ free radicals or active oxygen (Zheng and Wang, 2001). Reactive Oxygen Species (ROS) cover a wide range of chemical components, including superoxide anion, hydrogen radicals, nitric oxide and peroxynitrite and have the potential to initiate degenerative processes in the human body (Wang *et al*., 2002). Antioxidants provide protection to living organisms from damage caused by the uncontrolled production of ROS and concomitant lipid peroxidation, protein damage and DNA strand breaks (Ghosal *et al*., 1996).

The use of plants as a source of remedies for the treatment of diseases dates back to prehistory; however, plants used in traditional medicines are still understudied, particularly in clinical microbiology (Kirby, 1996). In developing countries where medicines are expensive, investigations of antioxidative activities in ethnomedicinal plants are needed. The use of traditional medicines is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs (Perry *et al*., 1999). It is obvious that these phytochemicals will find their way into the realm of drug discovery. Several species belonging to genus Eugenia of the Myrtaceae like *Eugenia jambolana* have been used traditionally for a wide variety of enthnomedical properties such as antidiabetes, antidiarrhea antinematodes and anti-inflammatory. Among them is *Eugenia polyantha* Wight, a deciduous tropical tree with spreading branches and simple leaves which can reach 60 to 90 feet and grows wild in the scrub forests of Indonesia. *Eugenia polyantha* leaves have been widely

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used in Indonesian culinary as a food additive (Dalimartha, 2001). They have also been used for antiulcer, antidiabetes, anti-inflammatory and antidiarhea treatments.

In view of the wide spread ethnomedical uses of *E. polyantha*, this study was conducted to evaluate *in vitro* antioxidative activities of bark extracts from *E. polyantha* using three standard procedures; 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging, hydrogen peroxide-scavenging and β-carotene bleaching assays together with the total polyphenol content. The polyphenol content correlation with the antioxidative activities was investigated as an attempt to clarify the high antioxidative activities of *E. polyantha* bark extracts.

MATERIALS AND METHODS

This research was carried out in the Faculty of Agriculture, Ehime University, Japan during 2007-2008 to evaluate the antioxidative activities and total poly phenol content of *E. polyantha* extracts.

Plant material: The *E. polyantha* bark was collected from a 7 years old tree with 5 m height and d.b.h. (diameter at breast height) of 20 cm in mid July 2007 at The Research Centre for Chemistry, Indonesian Research Institute of Sciences, Serpong, Tangerang, Indonesia. The bark was air-dried prior to extraction. A voucher specimen was deposited in the Laboratory of Plant Chemistry, Faculty of Agriculture, Ehime University, Japan for further use.

Chemicals: Linoleic acid, 1,1-diphenyl-2-picrylhydrazl (DPPH), β-carotene and hydrogen peroxide were purchased from Wako Chemical Co. Ltd. (Osaka, Japan). Tween 40, catechin, gallic acid, ascorbic acid, phenol reagents, aluminium trichloride, sodium acetate and rutin were purchased from Sigma-Aldrich Co. Ltd. (Tokyo, Japan). All solvents were of the highest purity or high performance chromatography (HPLC) grade.

Extraction: The bark was cut into small pieces and air-dried prior to extraction. The dried bark (200 g) was successively extracted for 8 h at 80°C with methanol, methanol-water (50%) and water, respectively. The extraction was repeated twice. Each solution was concentrated with a rotary evaporator under reduced pressure. The methanol extract was a dark brown sticky semisolid (50.21 g). The water-methanol extract was a light brown non sticky solid (47.78 g), while the water extract was a light brown solid (68.34 g).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay: The free radical-scavenging activity of each extract was determined as described by Braca *et al.* (2001). Plant extracts were added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was measured under constant mixing at room temperature after 30 min and percent inhibitory activity was calculated from $[(A_0\text{-}A_1)/A_0]\times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard.

Hydrogen peroxide-scavenging assay: The hydrogen peroxide-scavenging ability of each extract was determined according to the method described by Rosen and Rauckman (1984). A solution of hydrogen peroxide (2 mmol L^{-1}) was prepared in a phosphate buffer (pH 7.4). The hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity of 81 M^{-1} cm^{-1}. Each extract (100 μg mL^{-1}) was added to the hydrogen peroxide solution (0.6 mL). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank containing phosphate buffer without hydrogen peroxide. For each sample, a separate blank was used for background subtraction. The percent inhibitory activity was calculated from $[(A_0\text{-}A_1)/A_0]\times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard.

Beta-carotene bleaching assay: β-carotene (10 mg) was dissolved in 10 mL of chloroform. The carotene-chloroform solution (0.2 mL) was pipetted into a flask containing 20 mg of linoleic acid and 200 mg of Tween 40. Chloroform was removed using a rotary evaporator under reduced pressure at 40°C for 5 min and 50 mL of distilled water was added slowly to the residue with vigorous agitation in order to form an emulsion. A 4.8 mL aliquot of the emulsion was added to a tube containing 0.2 mL of the sample solution and the absorbance at 470 nm was immediately measured against a blank that was an emulsion without β-carotene. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored spectrophotometrically by measuring the absorbance at 470 nm for 60 min. Control samples contained 0.2 mL of ethanol instead of the sample extracts. Ascorbic acid was used as the reference. The antioxidative activity was expressed as percent inhibition with reference to the control after 60 min of incubation using the following formula:

$$AA = 100(DR_C\text{-}DR_S)/DR_C$$

where, AA is the antioxidative activity; DR_C is the degradation rate of the control $\{DR_C = [\ln(a/b)/60]\}$; DR_S

is the degradation rate in the presence of the sample $\{DR_S = [\ln(a/b)/60]\}$; where a is the absorbance at time 0; and b is the absorbance after 60 min.

The amounts of phenolic compounds, flavonoids and flavonols: The total phenolic content of plant extracts was determined using Folin-Ciocalteu reagent (Yu *et al*., 2002). Aliquots (100 μL) of the plant extracts were mixed with the Folin-Ciocalteu reagent (500 μL) and 20% sodium carbonate (1.5 mL). Each mixture was shaken thoroughly and made up to 10 mL using distilled water. The mixture was allowed to stand for 2 h. Then, absorbance at 765 nm was measured. The phenolic content was determined using a calibration curve obtained from various concentrations of gallic acid and catechin. Values are expressed in Gallic Acid Equivalents (GAE) and Catechin Equivalents (CE).

The flavonoid content was determined with a calibration curve using rutin as a reference compound by the method of Maksimovic *et al.* (2005). One milliliter of plant extract in methanol (10 mg mL^{-1}) was mixed with 1 mL of aluminium trichloride in ethanol and made up to 25 mL. The absorption at 415 nm was read after 40 min. Blanks were prepared from 1 mL of plant extract and a drop of acetic acid and then diluted to 25 mL with ethanol. The absorption of a standard rutin solution in ethanol was measured under the same conditions. The amount of flavonoid in plant extracts in Rutin Equivalents (RE) was calculated by:

$$X = (A.m_0)/(A_0.m)$$

where, X is the flavonoid content, mg g^{-1} plant extract in RE, A is the absorbance of the plant extract solution, A_0 is the absorbance of the standard rutin solution, m is the weight of the plant extract and m_0 is the weight of rutin in the solution.

The amounts of flavonols were determined by the method of Yermakov *et al.* (1987). The rutin calibration curve was obtained from a mixture of 2 mL of various concentrations of rutin in ethanol solution, 2 mL of aluminium trichloride (20 mg mL^{-1}) and 6 mL of sodium acetate (50 mg mL^{-1}). The absorbance at 440 nm was recorded after 2.5 h. The same procedure was used for 2 mL of plant extract (10 mg mL^{-1}) instead of the rutin solution. The flavonols content was calculated using a calibration curve obtained from various concentration of rutin and expressed as rutin equivalents.

Statistical analysis: All data are given as the Mean+SD of three measurements. Statistical analyses were performed using SPSS 15 Software for Windows. Data were subjected to an Analysis of Variance (Anova) followed by the Duncan test. Correlations between antioxidative activities, total phenolic capacity and total antioxidative capacity were tested using the Pearson correlation. $p<0.05$ was considered significant.

RESULTS

DPPH radical-scavenging activity: The three successive bark extracts from *E. polyantha* showed strong DPPH free radical-scavenging activity (Fig. 1). The scavenging effect of the extracts was as follows; methanol-water extract (ED_{50} = 0.181±0.04) mg mL^{-1}> methanol extract (ED_{50} = 0.335±0.16) mg mL^{-1}> water extract (ED_{50} = 0.354±0.11) mg mL^{-1}. *Eugenia polyantha* extracts exhibited a dose-dependent effect on DPPH free radical-scavenging activity. All extracts showed similar activity at 0.001 mg mL^{-1} and significantly increased activity at 0.1 mg mL^{-1}. The methanol-water extract exhibited greater free radical-scavenging activity than pyrogallol or ascorbic acid as a positive control at 1 mg mL^{-1}.

Hydrogen peroxide radical-scavenging activity: The values of hydrogen peroxide radical-scavenging activity of three extracts from *E. polyantha* were compared with that of ascorbic acid as a standard. The water extract from *E. polyantha* had the highest level of hydrogen peroxide radical-scavenging activity compared to other extracts (Fig. 2). In hydrogen peroxide radical-scavenging ability at a concentration of 100 μg mL^{-1}, the extracts ranked as follows; methanol extract (28±0.54), methanol-water extract (15±4.22), water extract (74±0.53) and ascorbic acid (81±0.67). The water extract showed more hydrogen peroxide radical-scavenging activity than the methanol and methanol-water extracts at 100 μg mL^{-1}.

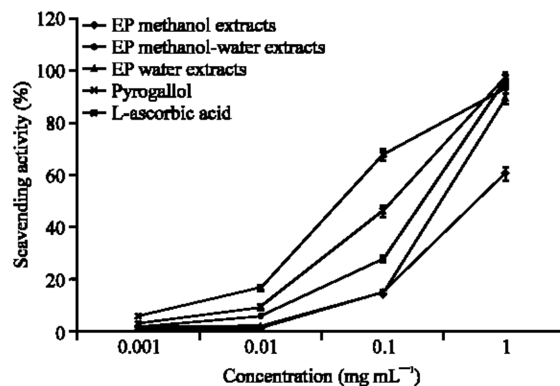

Fig. 1: Scavenging activity of *E. polyantha* extracts against the 1,1-diphenyl -2-picrylhydrazyl radical. Values are shown as the Mean±SD of three parallel measurements (n = 3)

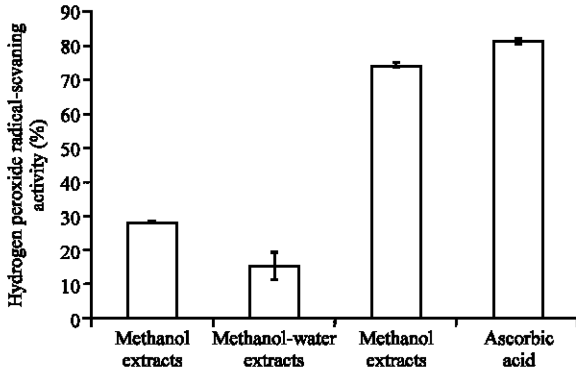

Fig. 2: Scavenging activity of *E. polyantha* extracts (100 µg mL^{-1}) against the hydrogen peroxide radical. Values are shown as the Mean±SD of three parallel measurements (n = 3)

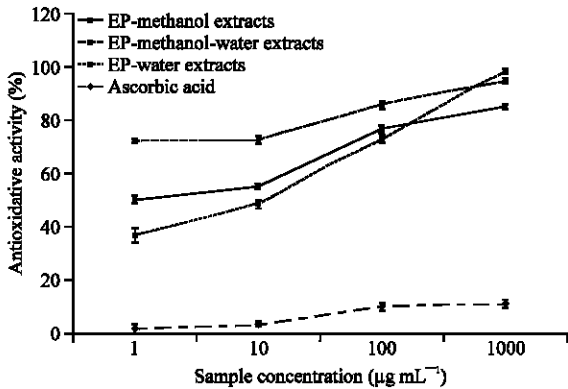

Fig. 3: Result of β-carotene bleaching assay of *E. polyantha* extracts. Values are shown as the Mean±SD of three parallel measurements (n = 3)

Beta-carotene bleaching assay: In the β-carotene bleaching assay, the three extracts from *E. polyantha* were compared with a well known natural antioxidant, ascorbic acid. In antioxidative activities, the extracts ranked in the following order: Methanol-water extract (85.72%±0.57) > methanol extract (76.43%± 0.91) > water extract (74.33%±0.53). From Fig. 3, it is clear that all the *E. polyantha* extracts are capable of inhibiting β-carotene bleaching and the activity depends on the amount of extract. The water extract showed the highest level of activity at 1 mg mL^{-1} and also demonstrated a significant increase in activity compared to the other extracts and positive control. In comparison of the β-carotene bleaching activity, ascorbic acid showed the lowest antioxidative activity.

Total phenolic, flavonoid and flavonol contents: The total phenolic content each extract of *E. polyantha* was determined using the Folic-Ciocelteau assay and expressed as gallic acid equivalents (GAE mg g^{-1}) and catechin equivalents (CE mg g^{-1}), respectively as shown in Table 1. The methanol-water extract had the greatest total phenolic content (856±28.2 mg GAE g^{-1} and 161±18.3 mg CE g^{-1}) and total antioxidative capacity expressed as ascorbic acid equivalents (449±23.5 mg AAE g^{-1}). A correlation between total phenolic content and total antioxidative capacity was observed (Fig. 4). The total phenolic content (GAE and CE) and total antioxidative capacity (TOAC) of the methanol-water extract exhibited a positive correlation with DPPH free radical-scavenging activity and beta carotene bleaching activity. The DPPH free radical-scavenging activity and beta carotene bleaching activity

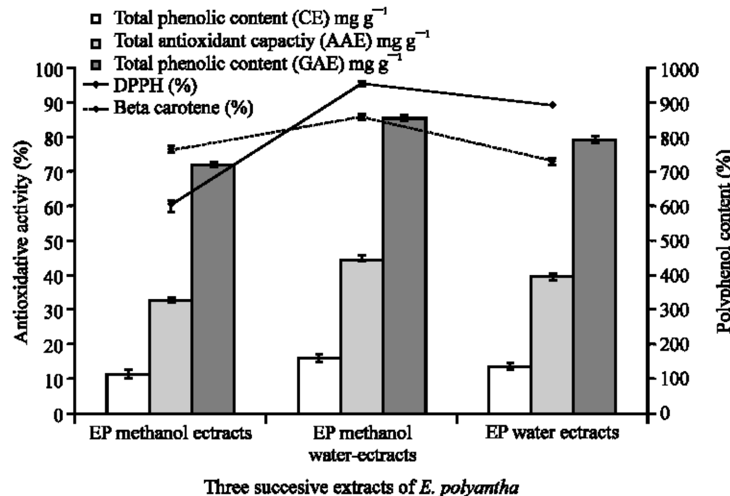

Fig. 4: Correlation of antioxidative activities of the three *E. polyantha* bark extracts with total phenolic content and total antioxidative capacity. Values are shown as the Mean±SD of three parallel measurements (n = 3)

Table 1: Total phenolic content of *E. polyantha* bark extracts

Eugenia polyantha bark extract	Total polyphenol content (mg g^{-1} extracts)				
	Total phenol (Gallic acid equivalents)	Total phenol (Catechin equivalents)	Total flavonoid (Rutin equivalents)	Total flavonol (Rutin equivalents)	Total antioxidative capacity (Ascorbic acid equivalents)
Methanol extract	718±26.4	113±12.2	60.6±15.2	19.4±4.5	329±21.1
Methanol water-extract	856±28.2	161±18.3	26.52±7.1	18.9±6.1	449±23.5
Water extract	793±34.9	141±12.8	22.12±4.8	16.7±3.5	398±26.5

Values are shown as the mean+SD of three parallel measurements (n = 3)

were directly influenced by the total phenolic content and the total antioxidative capacity of each *E. polyantha* extract.

DISCUSSION

Extracts of the bark of *Eugenia polyantha* exhibited strong antioxidative activities including DPPH free radicals-scavenging activity, hydrogen peroxide-scavenging activity and protection from β-carotene bleaching. The DPPH radical-scavenging capacity of the extracts could be explained by the presence of phenolic components (Siddhuraju *et al*., 2002). The effect on DPPH radical scavenging was thought due to their hydrogen donating ability and radical scavenging activity (Oke *et al*., 2009). As the concentration of phenolic compounds or the degree of hydroxylation of the phenolic compounds increases, so too will DPPH radical-scavenging activity (Sanchez-Moreno *et al*., 1999). Hydrogen peroxide itself is not very reactive, yet it is can be toxic to cells because of hydroxyl radical production (Kumaran and Karunaaran, 2007). Dietary polyphenols have also been shown to protect mammalian and bacterial cells from toxicity due to hydrogen peroxide, especially compounds with an orthodihydroxy phenolic structure (catechol moiety) such as quercitin, catechin, gallic acid ester and caffeic acid ester (Nakayama, 1994; Nakayama *et al*., 1993). The phenolic compounds of *E. polyantha* may be involved in the free radical-scavenging activity and removal of hydrogen peroxide. Since the phenolic compounds present in the extracts are good electron donors, they may accelerate the conversion of peroxides into water (Ruch *et al*., 1989).

In spite that polar compound like ascorbic acid are well known antioxidants, the β-carotene bleaching test did not show antioxidative properties. This interesting phenomenon has been described as the polar paradox (Kulisic *et al*., 2004). Polar antioxidants remaining in the aqueous phase of the emulsion are more diluted in the lipid phase and are thus less effective in protecting linoleic acid. β-carotene undergoes rapid decolorization in the absence of an antioxidant. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing linoleate free radicals and other radicals formed in the system (Siramon and Ohtani, 2007). Furthermore, β-carotene is used as a coloring agent in beverages and its discoloration would markedly reduce the quality of those products (Sakanaka *et al*., 2005).

Plant phenolics constituents are one of the major groups of compounds acting as antioxidants. The key role of phenolic compounds as scavengers of free radicals has been emphasized in several reports (Tepe and Sokmen, 2007; Moller *et al*., 1999; Madsen *et al*., 1996). The antioxidative activity of phenolic compounds is mainly due to their redox properties, which play an important role in adsorption and neutralization of free radicals, quenching of singlet and triplet oxygen, or decomposition of peroxides (Osawa, 1994). Phenolic compounds are classified as simple phenols, a single aromatic ring bearing at least one hydroxyl group and polyphenols with at least two phenol subunits such as flavonoids or three or more phenol subunits, called tannins (Sultana *et al*., 2007). Flavonoids as one of the most diverse and widespread groups of natural compounds, are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical-scavenging properties.

The three methods of extraction gave significantly different levels of antioxidative activity. They also gave a significantly different total phenolic content (GAE and CE) and total antioxidative capacity (TOAC) ($p<0.05$), but not total flavonoid or flavonol content. The total phenolic content (GAE and CE) and total antioxidant capacity (TOAC) of the methanol-water extract from *E. polyantha* bark exhibited a positive correlation with DPPH free radical-scavenging activity ($r = 0.9417$; $r = 0.9236$ and $r = 0.951$, respectively) and beta carotene bleaching protection ($r = 0.6411$, $r = 0.6031$ and $r = 0.6249$, respectively). These results are consistant with those of Holasova *et al*. (2002), who found that total phenol content increased with antioxidative activity. Gheldof and Engeseth (2002) reported a linear correlation between phenolic content and antioxidative activity. Phenolic compounds are commonly found in both edible and inedible plants and have been reported to have multiple biological effects, including antioxidative activity. Crude extracts of fruits, herbs, vegetables, cereals, nuts and other plant materials rich in phenolic compounds are

increasingly attracting interest in the food industry. The importance of the antioxidants in plant materials to the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers (Loliger, 1991). Tanaka *et al.* (1988) reported that polyphenol compounds had inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g was ingested daily from a diet rich in fruits and vegetables.

The extracts of *E. polyantha* bark showed strong DPPH radical-scavenging activity, hydrogen peroxide-scavenging activity and β-carotene bleaching activity. These activities demonstrated a positive correlation with the total antioxidative capacity and total phenolic content of the extracts. Therefore, the antioxidative activities of *E. polyantha* bark extracts were shown to be related to the phenolic components.

CONCLUSION

The three extracts of *E. polyantha* bark had significant *in vitro* antioxidative activities. The extracts had different levels of activity in all the system tested, ranking in the order methanol-water extract > water extract > methanol extract. Total antioxidative capacity and total phenolic content correlated with the antioxidative activities, i.e., DPPH and beta carotene bleaching assay. Further isolation and identification of the individual phenolic compounds responsible for antioxidative activities is needed to understand their mechanisms of action.

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