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Bioassay-guided Isolation and Identification of Antioxidative Compounds from the Bark of *Eugenia polyantha*

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Abstract: *Eugenia polyantha* bark extracts were found to have potential antioxidative activities. This study is an effort to investigate the antioxidative compounds in *E. polyantha*. *In vitro* antioxidatve assay were used as guided tools for the isolation of antioxidative compounds. The methanol-water extracts showed the highest level of free radical-scavenging activity (ED_{50}) = 180 μg mL^{-1} and protection from β-carotene bleaching (8.7 μg mL^{-1}). The methanol-water (1:1) extracts exhibited strong DPPH scavenging activity and protection against beta carotene bleaching and was subjected to repeated silica gel column chromatography. The n-butanol, acetone and ethyl acetate solubles exhibited the highest antioxidative activities, derivatization was conducted to the isolated antioxidative compounds prior to identification. Catechin, gallic acid and rutin were isolated from those solubles as active compounds present in the *Eugenia polyantha* bark.

Key words: *Eugenia polyantha*, antioxidative, isolation, bioassay guided

INTRODUCTION

The reduction of molecular product such as superoxide anion, hydroxyl radical and peroxyl radical is known to have potent harm as reactive free radicals (Williams and Jeffrey, 2000). Several degenerative diseases as cancer (Muramatsu *et al*., 1995), atherosclerosis (Steinberg *et al*., 1989) and gastric ulcers (Das *et al*., 1997), are known to be induced by oxygen radicals which occurs as products of oxidative stress. According to Zheng and Wang (2001), many natural occuring antioxidative compounds could act as free radicals scavenger, therefore they have the potential to be used as improving food quality agents, in a way of terminating the chain reaction of free radicals in the biological system.

Polyphenols are ubiquitous plant compounds which are increasingly receiving attention as chemopreventive agents against cancer, cardiovascular disorders and neurodegenerative diseases (Fremont, 2000). The polyphenols are products of the shikimate and acetate malonate pathways and posses a phyenlpropanoid unit as basic carbon skeleton. Phenolic compounds include the simple phenols, phenolic acids, hydrocinnamic acids, stilbenes, flavonoids, biflavonoids and protocyanidinis (Robards and Antolovich, 1997).

The main use polyphenols in plants are their free radicals scavenging capacity (Kroon and Williamson, 2005), while several antioxidative properties such as inhibition of peroxidation and oxygen radical scavenging are the main biological properties of flavonoids and phenolic acids. To improve the efficacy of antioxidative study in plant extracts, it is important to use establish assay and considering each oxidative conditions, system composition and antioxidative mechanism. (Prior *et al*., 2005).

Eugenia polyantha Wight (Myrtaceae), known in Indonesia as 'salam', is a ever green trees with spreading branches and simple leaves that grows wild and also cultivated in Indonesia. The use of *E. polyantha* leaves as Indonesian culinary additives dated to the colonial era. *E. polyantha* has shown potential antioxidative activity towards free radicals and protection from beta carotene bleaching (Lelono *et al*., 2009). In view of the widespread ethnomedical uses of *E. polyantha*, this study was conducted to isolate and identify the active compounds from bark extracts of *E. polyantha* using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging bioassay-guided fractionation. The identification of the active compounds was part of an attempt to clarify the antioxidative activities of *E. polyantha* bark extracts.

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MATERIALS AND METHODS

Plant material: The *E. polyantha* bark was collected and air-dried at The Research Centre for Chemistry, Indonesian Research Institute of Sciences, Serpong, Tangerang, Indonesia in mid July 2009. A voucher specimen was deposited in the Laboratory of Plant Chemistry, Faculty of Agriculture, Ehime University, Japan.

Chemicals: Linoleic acid, 1,1-diphenyl-2-picrylhydrazl (DPPH) and β-carotene were purchased from Wako Chemical Co. Ltd., (Osaka, Japan). The silica gel used for column chromatography was Wakogel C-200 (Wako Pure Chemical Industries Ltd., Osaka, Japan). TLC (Thin layer chromatography) aluminum sheet (0.25 mm) (Silica gel 60 F254, 20×20 cm) and PLC (preparative thin layer chromatography) glass plates (2 mm) (Silica gel 60 F254, 20×20 cm) were obtained from Merck (Darmastadt, Germany). All solvents were of the highest purity or high performance chromatography (HPLC) grade.

Apparatus: Melting points were measured on a Yanaco micro melting point apparatus (Yanaco Co., Ltd., Kyoto, Japan) and were uncorrected. Ultraviolet (UV) spectra were measured with a Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). HPLC was conducted with a Shimadzu system consisting of a LC-10ADvp pump equipped with a CTO 10 Avp column oven, SPD 10Avp UV-visible spectrophotometric detector and Frac 10A fraction collector. Peak analysis and assignment were conducted using Class-LC10/M10 and a Chiral CD-Ph (5 μm 4.6×250 mm) column. Mass spectra were recorded at an electron energy of 70 eV with a direct inlet on a Shimadzu GC-MS QP 5050A (Shimadzu Corp., Kyoto, Japan); ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-EX400 (JEOL, Ltd., Tokyo, Japan) at 400 MHZ and 100 MHZ, respectively. Chemical shifts are expressed as ä in ppm and TMS was used as the internal standard. The coupling constant (J) was recorded in Hz.

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 $[(A_0-A_1)/A_0]\times100$, 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 Tween40. The 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:

$$AA = 100\left(\frac{DRC\text{-}DRS}{DRC}\right)$$

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 $\{DR_S = [\ln(a/b)/60]\}$; is the absorbance at time 0; and b is the absorbance after 60 min.

Statistical analysis: All data are given 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.

Extraction and isolation: The bark and stem (1000 g) of *E. polyantha* were air dried, cut into small pieces and extracted twice with methanol-water (50:50) at 60°C for 8 h. The methanolic-water solution was concentrated with a rotary evaporator under reduced pressure to give a methanol-water extract (325.23 g). Activity-guided isolation was conducted by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging. Fractions showing potential activity were further purified by column chromatography. For the isolation of antioxidative compounds, the methanol-water extract of *E. polyantha* bark (100 g) was suspended in water and then partitioned with n-hexane, chloroform, ethyl acetate, n-butanol, acetone and methanol to give the respective [n-hexane (5.32 g), chloroform (10.21 g), ethyl acetate (7.05 g), n-butanol (35.28 g), acetone (6.51 g) and methanol (20.11 g)]

solubles. The fractions collected were first checked by TLC and spots were detected under a UV lamp (254 nm), or by spraying with bromocresol green, with diazotized sulphanilic acid and with concentrated sulphuric acid after heating the TLC plate. Repeated bioassay-guided fractionation using silica gel column chromatography, Sephadex LH-20 and preparative TLC, of the active fractions lead to the isolation of three active compounds.

Acetylation of purified compounds: Acetylation of compounds 2 and 3 was carried out according to the method of Markham (1982). About 10 mg of compound, dissolved in 0.5 mL of pyridine, was added to 0.5 mL of acetic anhydride in a stoppered conical flask and kept overnight at room temperature. Ice-cold water (10 mL) was then poured in to the reaction mixture with constant stirring and left for 1 h. The insoluble acetylated compound was separated by filtration, using Whatman filter paper No.1. Recrystallization of each product was conducted to purify the acetate derivatives.

Compound 1: Yellow powder, m.p. 174-177°C, $[\alpha]_D$ (MeOH) = + 18.6°. EIMS m z^{-1} (rel.int) 290 (M^+) (21), 152 (35), 139 (100), 123 (31), 69 (15). ^{1}H NMR (400 MHZ, acetone-d_6) δ: 2.5 (dd, J =16.1, 8.2 Hz, H-4β), 2.9 (dd, J = 16.1, 5.3 Hz, H-4α), 3.3 (s, H-3), 4.5 (d, J = 7.5 Hz, H-2), 5.9 (d, J = 2.2 Hz, H-6), 6.0 (d, J = 2.2 Hz, H-8), 6.7 (m, J = 8.2 Hz, H-6'), 6.8 (m, J = 8.1 Hz, H-5'), 6.9 (d, J = 1.5 Hz, H-2'). ^{13}C NMR (100 MHZ, acetone-d_6) δ: 50.2 (C-4), 68.8 (C-3), 83.2 (C-2), 95.9 (C-8), 96.6 (C-6), 101.1 (C-4a), 115.7 (C-2'), 116.1 (C-5'), 120.5 (C-6'), 132.7 (C-1'), 146.0 (C-3'), 146.1 (C-4'), 157.4 (C-8a), 157.7 (C-5), 158.2 (C-7).

Compound 2: White amorphous powder, m.p. 239-241°C, EIMS m z^{-1} (rel.int). 170(M^+) (100), 153 (92), 135 (13), 125 (20), 79 (10). ^{1}H NMR (400 MHZ, CD_3OD) : δ 7.1 (2H, s, H-2, 6). ^{13}C NMR (100 MHZ, CD_3OD) δ : 110.3 (C-2, 6), 122.1 (C-1), 139.5 (C-4), 146.4 (C-3, 5), 170.5 (C-7).

Compound 2 methyl ester: White powder, m.p. 68-69°C. EIMS m z^{-1} (rel.int) 226 (M^+) (100), 211 (43), 195 (24), 183 (9), 155 (17), 125 (6). ^{1}H NMR (400 MHZ, $CDCl_3$) δ : 3.9 (3H, s, methoxy carbonyl), 3.9 (9H, s, methoxy C-3,4,5), 7.0 (2H, s, H-2, 6). ^{13}C NMR (100 MHZ, $CDCl_3$) δ : 52.2 (methoxy C-4), 56.2 (methoxy C-3, 5), 60.9 (methoxy carbonyl C-4), 106.8 -δ 107.4 (C-2, 6), 125.1 (C-1), 142.2 (C-4), 152.9-153.0 (C-3, 5), 166.7 (C-7).

Compound 2 triacetate: White powder, m.p. 171-172°C. EIMS m z^{-1} (rel.int) 296 (M^+) (9), 279 (2), 254 (65), 237 (8), 212 (91), 194 (51), 170 (100), 152 (77). ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 2.3 (3H, s, acetyl C-5), 2.3 (3H, s, acetyl C-3), 2.3 (3H, s, acetyl C-4), 7.9 (2H, s, H-2, 6). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 20.2 (acetyl-methyl C-4), 20.5 (acetyl-methyl C-3,5), 122.8 (C-2, 6), 127.4 (C-1), 139.3 (C-4), 143.5 (C-3,5), 166.4 (acetyl-carbonyl C-4), 167.6 (acetyl-carbonyl C-3, 5), 169.7 (methoxy carbonyl C-7).

Compound 3: Yellow amorphous powder, m.p. 192-195°C. EIMS m z^{-1} (rel. int) 610 (M^+) (9), 307 (42), 154 (99), 137 (100), 89 (34). The ^{1}H and ^{13}C NMR signals of the isolated compound, coincided with those of the rutin standard.

Compound 3 deca-acetate: White powder, m.p. 158-162 °C. FAB-Mass m z^{-1} (rel.int) 1031 $(M+H)^+$ (46), 971 (7), 561 (25), 525 (10). The ^{1}H and ^{13}C NMR signals of the compound 3 deca-acetate, coincided with those of the rutin deca-acetate standard.

Hydrolysis of compound 3: Yellow powder, m.p. 315°C, EIMS m z^{-1} (rel.int) 302 (M^+) (100), 301 (18), 273 (10), 153 (30), 137 (11). ^{1}H NMR (400 MHZ, CD_3OD) δ: 6.2 (1H, d, J = 2.0 Hz, H-6), 6.4 (1H, d, J = 2.0 Hz, H-8), 6.9 (1H, dd, J = 8.4 Hz, H-5'), 7.6 (1H, d, J = 8.4, 2.1 Hz, H-6'), 7.7 (1H, d, J = 2.1Hz, H-2'). ^{13}C NMR (CD_3OD) δ: 94.4 (C-8), 99.2 (C-6), 104.5 (C-10), 116.0 (C-2'), 116.2 (C-5'), 121.7 (C-6'), 124.2 (C-1'), 137.2 (C-3), 146.2 (C-3'), 148.0 (C-2), 148.8 (C-4'), 158.2 (C-9), 162.5 (C-5), 165.6 (C-7), 177.3 (C-4).

Pentaacetate of the hydrolytic product of Compound 3: White powder, m.p. 190-192°C). FAB-Mass m z^{-1} (rel.int) 513 $(M+H)^+$ (1), 470 (75), 428 (89), 386 (96), 344 (100), 302 (99), 273 (51), 245 (10), 137 (8), 69 (2). ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 2.3 (12H, s, acetyl methyl), 2.4 (3H, s, acetyl methyl C-3), 6.9 (1H, d, H-5'), 7.3 (1H, s, H-2'), 7.4 (2H, dd, H-6', H-8), 7.7 (H, d, H-6). ^{13}C NMR ($CDCl_3$) δ: 20.5 (acetyl methyl C-7), 20.6 (acetyl methyl C-3', C4'), 21.0 (acetyl methyl C-3), 21.2 (acetyl methyl C-5), 108. 9 (C-8), 113.9 (C-6), 114.7, 123.8 (C-2'), 123.9 (C-5'), 126.4 (C-6'), 127.8 (C-1'), 134.1 (C-3), 142.2 (C-4'), 144.4 (C-3'), 150.4 (C-5), 153.8 (C-7), 154.3, 156.8 (C-2), 169.3 (acetyl carbonyl C-3), 167.7 (acetyl carbonyl C-7), 167.8 (acetyl carbonyl C-5), 167.8 (acetyl carbonyl C-3'), 167.9 (acetyl carbonyl C-4'), 170.1 (C-4, carbonyl).

RESULTS

Screening in vitro for free radical-scavenging activity of *Eugenia polyantha* bark extracts: The *E. polyantha* methanol, methanol-water and water extracts were screened for antioxidative activity in vitro. The extracts demonstrated potential free radical-scavenging activity of 60.4%+1.8, 95.6%+2.1 and 89.2%+1.3 and 76.43%+0.91,

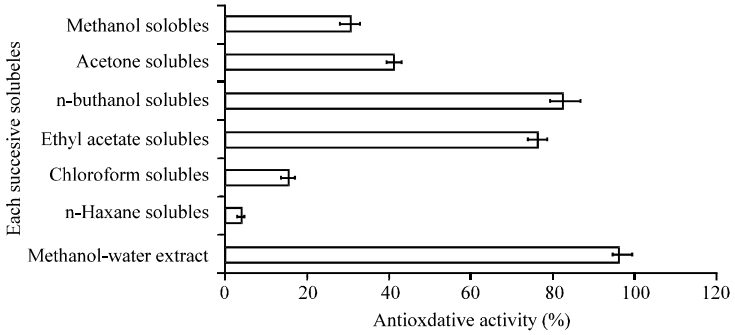

Fig. 1: *In vitro* free radical-scavenging activity of the respective solubles *from Eugenia polyantha* bark extracts

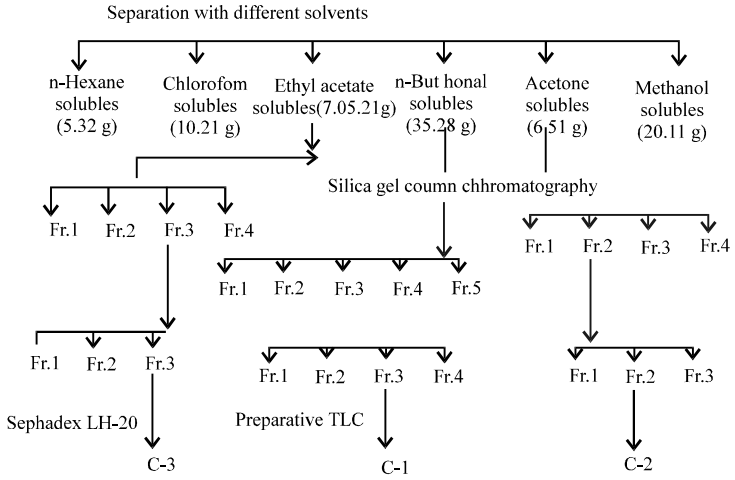

Fig. 2: Scheme for the isolation of antioxidants from the bark of *Eugania polyantha*

Table 1: IC_{50} Value *of Eugenia polyantha* bark extracts

	IC_{50} Value *of Eugenia polyantha* bark extracts (μg mL^{-1})		
In vitro antioxidative assay	Methanol extract	Methanol-Water extract	Water extract
DPPH-scavenging activity	330.21±16.11	180.33±4.17	350.82±12.01
Beta carotene bleaching protection	30.27±2.030	8.76±2.05	50.22±9.040

Values are the Mean±SD for three parallel assessments (n = 3)

85.72%+0.6 and 74.33%+0.5, respectively, in the beta carotene bleaching protection assay. IC_{50} values are shown in Table 1, which showed that methanol water extracts demonstrated the highest protection toward beta carotene bleaching assay (8,76 μg mL^{-1}) and DPPH radical free scavenging (180.33 μg mL^{-1}). The methanol-water extract was selected for further evaluation.

Isolation and characterization of antioxidative compounds: The *E. polyantha* methanol-water bark extract was suspended in water and then partitioned with n-hexane, chloroform, ethyl acetate, n-butanol, acetone and methanol, to give the respective solubles. Each of the solubles was subjected to *in vitro* free radical-scavenging assay. Three fractions; n-butanol (81%), ethyl acetate (77%) and acetone (40%); demonstrated potential activity towards DPPH, as shown in Fig. 1. The most active fraction was fractionated further to isolate the active compounds. Repeated separation using silica gel column chromatography and Sephadex LH-20 afforded three antioxidative compounds; one of the active compounds (Compound 1) was isolated from n-butanol and purified by silica gel column chromatography. Another (compound 2) was isolated and purified from the acetone solubles. The third compound (compound 3) was isolated from the ethyl acetate solubles and subjected to recrystallization from acetone. The extraction process for the isolation of the compounds is shown in Fig. 2. The identification catechin, gallic acid and rutin as the active compounds as described in Fig. 3, were conducted using NMR and coincided with the authentic standard.

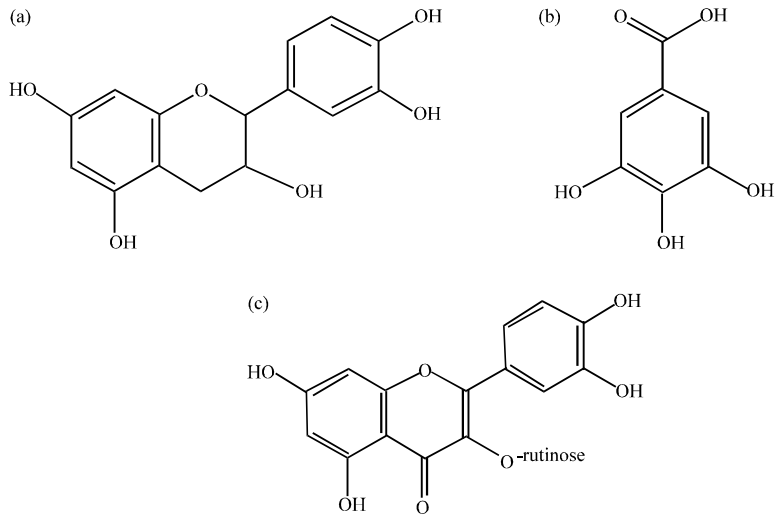

Fig. 3(a-c): Antioxidants compounds isolated from dry bark of *Eugenia polyantha* (a) Catechin, (b) Gallic acid and (c) Rutin

Table 2: DPPH radical scavenging activity and beta carotene bleaching protection of isolated compounds from *E. polyantha* bark

	DPPH -scavenging (%)	Beta carotene bleaching protection (%)
Compound 1	49.89±1.44	26.25±0.89
Compound 2	94.68±1.12	12.53±1.01
Compound 3	34.36±1.71	17.24±0.98

Values are the Mean±SD for three parallel assessments (n = 3)

***In vitro* antioxidative activity:** DPPH has been used to evaluate the antioxidative activity of natural products in organic solvents. The method is based on the reduction of a DPPH solution to DPPH-H in the presence of a hydrogen-donating antioxidant (Sentandreu *et al*., 2008). The methanol:water (1:1) extract of *E. polyantha* bark and stem and the isolated compounds showed potential antiradical activity. Compound 2 showed the highest DPPH radical scavenging activity (94.68%), while Compounds 1 showed the highest activity of beta carotene bleaching protection (26,55%), as shown in Table 2.

DISCUSSIONS

Bioassay-guided fractionation of the n-butanol soluble afforded compound 1, which was isolated as a yellow amorphous solid and tested positive for diazotized sulphanilic acid, vanillin-HCl and $FeCl_3$, indicating the presence of flavonoids and phenolics, respectively. Compound 1 has an optical rotation to+18.6° in methanol under room temperature, indicating a positive polarization orientation. In the ^{1H} NMR spectrum, four doublets at δ 5.9 (J = 2.2 Hz), 6.0 (J = 2.2 Hz), 4.5 (J = 7.5 Hz) and 6.9 (J = 1.5 Hz) were assigned to the H-6, H-8, H-2 and H-2' protons of the catechin moeity. The two double doublets at δ 2.5 (J =16.1, 8.2 Hz, H-4β) and 2.9 (J = 16.1, 5.3 Hz, H-4α) were assigned to the CH_2 protons. The multiplets at δ 6.74 and 6.77 were assigned to the H-6' and H-5' protons. The EI-MS spectrum of the compound showed a M^+ peak at 290. These result revealed compound 1 to be a catechin. To distinguish the compound from its epimer, epictaechin, HPLC was conducted with a chiral column. Compound 1 showed an Rt of 4.41 min, while the catechin standard showed an Rt of 4.41 min and epicatechin showed an Rt of 3.04 min. The co-injection of catechin and epicatechin into the system together with the isolated compound 1 is shown in Fig. 4. On the basis of the data, compound 1 was identified as a catechin.

Repeated fractionation of the acetone solubles obtained from *E. polyantha* methanol water solubles, afforded compound 2, which was isolated as a white amorphous solid and tested positive for diazotized sulphanilic acid and bromocresol green solution, indicating the presence of phenolic and carboxylic groups, respectively. Compound 2 had the ability to reduce Tollens reagent and Fehling solution, confirming the presence of the carboxyl group. A lone singlet proton signal at δ 7.05 (2H, s, galloyl-H) at H-2 and H-6, in the ^{1}H NMR spectra and five carbon signals in the low field [δ 110.3 (C-2, 6), δ 122.1 (C-1), δ 139.5 (C-4), δ 146.4 (C-3, 5), δ 170.5 (C-7)] in the ^{13}C NMR spectra, indicated the compound to be gallic acid. The molecular ion peak at 170 the EI-MS spectrum and absence of a mixed melting depression in comparison with the standard, confirmed that the compound is gallic acid. The structure of compound 2 was further confirmed by its methyl and acetate derivatives, gallic acid methyl ester and gallic acid triacetate, respectively.

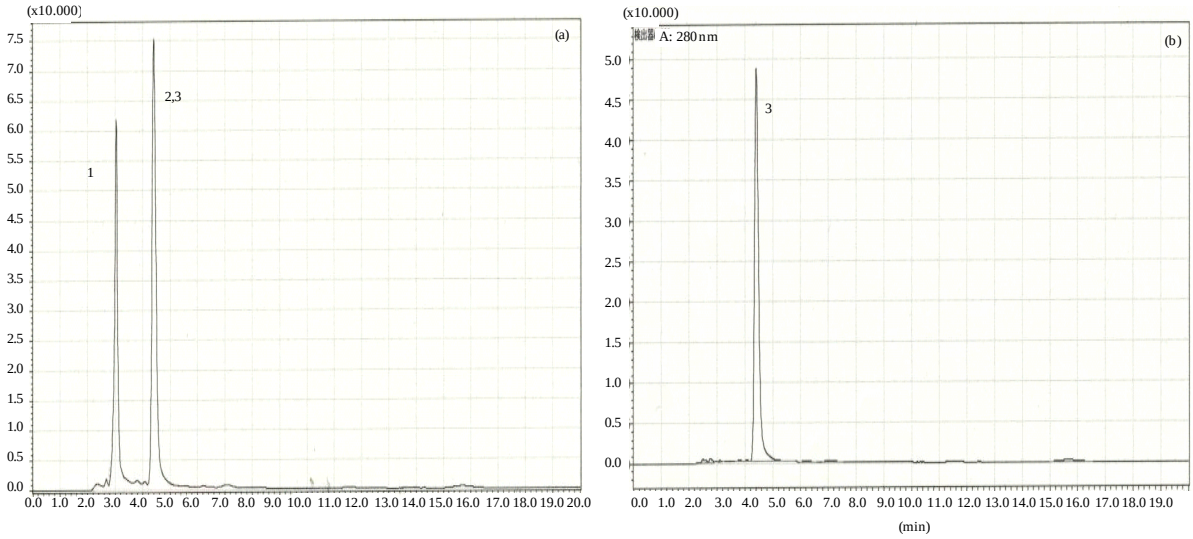

Fig. 4(a-b): HPLC chiral colum chromatogram of (1) Epicatchin, (2) Catechin and (3) Compound 1, (1) Epicatechin Rt 3.038 min, (2) Catechin Rt 4.410 min and (3) Compound 1 Rt 4.407 min

Further separation of the ethyl acetate fraction using Sephadex LH-20, afforded compound 3, which tested positive with vanillin-HCl and $FeCl_3$ for flavonoids and phenolics, respectively. The M^+ peak was observed at 610 in the EI-MS spectrum. The ^1H and ^{13}C NMR spectra of compound 3 coincided with those of the standard. The physical and spectroscopic data indicated that compound 3 is rutin. The structure of the compound was confirmed by its hydrolysis and the acetate derivatives. The ^1H NMR spectra of the product of compound 3's hydrolysis showed the presence of two meta coupled doublet (J = 2.0 Hz) protons on the A-ring at δ 6.37 and 6.17 ppm, which were assigned to H-6 and H-8, respectively. Two sets of doublet and one set of double doublets at δ 7.72 (1H, d, J = 2.1 Hz), δ 6.87 (1H, d, J = 8.4 Hz) and δ 7.62 (1H, dd, J = 8.4, 2.1 Hz), were observed which were characteristic of H-2', H-6' and H-5', respectively. The C ring was a flavanone moiety. The ^{13}C NMR spectra displayed fourteen carbon signals at δ 89.4-165.6, and one carbonyl carbon signal at δ 177.3 (C-4). Mass spectrometry indicated that the m z^{-1} of $[M]^+$ was 302. The melting point was recorded at 315°C and the NMR and MS data confirmed that the aglycone was quercetin. The sugars produced by the hydrolysis of compound 3 were detected on the TLC plate and coincided with the glucose and rhamnose standards in terms of Rf values. On the basis of the these results, compound 3 was identified as rutin.

The antioxidative activity of phenolic compounds correlates to their chemical structure. The activity of natural antioxidants, such as phenolic acids, is due to the presence and position of free hydroxyls as hydrogen-donating hydroxyl groups on the aromatic ring in relation to the carbonyl functional group and also affected by such factors as the glycosylation of aglycones and other hydrogen-donating groups (Rice-Evans *et al.*, 1996). Monohydroxy benzoic acid with the-OH moiety at the *ortho*-or *para*-position relative to the carbonyl showed low activity (Balasundram *et al.*, 2006). The antioxidative activity of phenolic acids increases with the degree of hydroxylation, therefore the high antiradical scavenging activity of compound 2 can be attributed to the presence of three available hydroxyl groups.

The structure-activity relationship of flavonoids is generally more complicated than that of the hydroxybenzoic moiety due to the relative complexity of the molecules. Hydroxylation in the B ring confers stability to the aroxyl radical through electron delocalization (Saskia *et al*., 1996), which increases the radical-scavenging activity. A double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C, enhances the radical-scavenging capacity of flavonoids (Pietta, 2000). The combination of a 3-OH in ring C will also enhance the active radical-scavenging capacity of flavonoids. Substitution of 3-OH resulted in altering the torsion angel and cooplanarity which then reducing the antioxidative activity. The higher activity of compound 1 compared to compound 3, is consistent with this notion, whereas the substitution of 3-OH in ring C with rutinose resulted in lower activity, indicating the aglycone form of the compounds to have more activity than the glycoside form Seeram and Nair (2002).

CONCLUSION

Catechin, gallic acid and rutin were identified as the major phenolic compounds responsible for the antioxidative activity of the *Eugenia polyantha* bark and stem extracts, based on in vitro DPPH radical-scavenging and beta carotene bleaching assays. The antioxidative activity could be used to extend the use of *Eugenia polyantha* bark, as a source of natural antioxidants.

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