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The evaluation of antioxidant and free radical scavenging activities of *Eugenia polyantha* leaves extracts

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Abstract : *Eugenia polyantha* Wight (*E. polyantha*) has been widely used as culinary additives especially in Indonesia. This plant also used as medicinal herb to treat several illnesses like diarrhea, skin infection and diabetes. Three antioxidant assays were used to evaluate the antioxidant capacity of its four different extracts, petroleum ether (PE), chloroform (CE), methanol (ME) and water (WE). 1,1-diphenyl-2-dipicrylhydrazyl free radical scavenging activity (DPPH), 2,2-azino-bis(3-ethylbenzothiozoline-6-sulphonic acid) cation radical scavenging (ABTS) and ferric-reducing antioxidant power (FRAP) assays were used to determine the antioxidant potential of these extracts. The result showed that all four extracts have the capability to scavenge DPPH free radical where the value of IC_{50} was also calculated from the result. ME was the strongest scavenger with IC_{50} value 0.01746 mg/mL, followed by WE (0.01896 mg/mL), PE (0.02308mg/mL) and CE (0.02932mg/mL). ABTS assay proved that ME has the highest antioxidant capacity with TEAC value is 6.18331 TEAC (mmol), followed by CE (1.91150 TEAC (mmol), WE (1.48987 TEAC (mmol) and PE (0.32187 TEAC (mmol). Lastly, result from FRAP test showed that ME has the greatest reducing power followed by WE, CE and PE.
Keywords : *Eugenia polyantha* Wight, antioxidant activity, free radical scavenging, DPPH, ABTS, FRAP.

1.Introduction

Free radicals are known to have high activities to trigger a chain reaction in the cell[1,2]. According to Niki[3], free radicals disturb redox balance causing oxidative stress which leads to oxidative damage of biological molecules. Consequently, one will be exposed to degenerative diseases which affect cardiovascular and cerebrovascular systems or worst, cancer[4-6]. Antioxidants are referred to as compounds which are able to inhibit the oxidation of different biomolecules and helps in repairing the damages caused to the body tissues due to oxidation processes[7]. They act against free radical or any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate[8,9]. It is important to find an effective antioxidant from natural origin including herbs from safety point of view because these natural substances have been eaten safely for long time[10] Examples of antioxidants from herbs

include flavonoids, phenolic acid, carotenoids and polyphenols[11].Studies on natural antioxidants especially in plants and fruits consumed have demonstrated that they may be an alternative in curbing the deleterious effects of free radicals. Over 1000 plants had been recognized to possess antioxidant activity including *Eugenia polyanta* Wight[12].

Eugenia polyantha Wight or *Syzygium polyanthum* is one of the most widely used medicinal plant in Indonesia[13]. This plant is commonly known as Indonesian Bay-leaf which is known by locals as 'daun salam'. It is rarely grown in the West but mostly available in South-East Asia especially Indonesia, Malaysia and Burma. Besides its medicinal properties to treat diarrhea, skin infection and diabetes the leaves of *E. polyantha* is usually added as flavoring in Indonesian and Malayan cuisine[13-14]. Previous study reported the antimicrobial and antioxidant activity as well as the cytotoxicity and phytochemical analysis of extracts of the plant. Flavanoids, eugenol, carbohydrate, tannin, alkaloid, steroid and triterpenoid are believed to be active in this plant[15]. Thus, this study focus on evaluating the antioxidant capacity of 4 different extracts of leaves of *E. polyantha* by using 3 different assays ie DPPH radical scavenging activity, ABTS cation radical assay and ferric-reducing antioxidant power (FRAP).

1.0 Methodology

1.1 Chemicals and reagents

1,1-diphenyl-2-dipicrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt 98% (ABTS), potassium peroxodisulfate, 99% (USA), phosphate buffered saline (PBS) were obtained from Sigma-Aldrich. There were 2 types of methanol used, HPLC grade and analytical grade. 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) (Calbiochem). Potassium hexacyanoferrate (lll) was purchased from R&M Chemical (Essex, UK). Trichlorocetic acid (TCA) and Ferric chloride were supplied from Sigma-Aldrich. Butylated hydroxytoluene (BHT) and quercetin were used as standards.

1.2 Sample preparation and solvent extraction

Five kg *E. polyantha* leaves were collected from Titi Kuning, Medan, Indonesia and identified by The Schoolof Biological Sciences, University of Sumatera Utara, Medan, Indonesia (No.13/MEDA/2012). The leaves were dried and the dry mass obtained was 3.05 kg. Next, the raw and dry-heated *E. polyantha* leaves were ground to fine powder. For serial extraction, 500g of powdered *E. polyantha* leaves was extracted using 2 L petroleum ether. The extraction process was done for 72 hrs with occasional shaking at 37◦C. 15.35g (3.07%) of sticky dark brown-colored petroleum extract (PE) was obtained after the extract was concentrated. The residue was then dried and extracted with 1.6L chloroform. After 72 hrs of extraction, the extract was concentrated and dried, yielding 24.8g (4.96%) of chloroform extract (CE). Similarly, the residue from CE was dried and re-extracted with methanol, 1.3L also for 72 hrs. The methanol extract was concentrated and freeze-dried and 24.8g (4.96%) of dark green coarse powder was obtained. For the preparation of *E. polyantha* water extract, maceration for 24 hrs was done on the residue from methanol extraction where the water solvent was changed daily for consecutively 3days. The final extract resulted from water extraction was 15.25g (3.05%) pale brown powder. These extracts were kept at temperature -20°C before antioxidant tests were carried out.

1.3 Antioxidant capacity assays

2.3.1 1,1-diphenyl-2-dipicrylhydrazyl (DPPH•) free radical scavenging assay

The DPPH scavenging activity assay used was modified from Blois, Sanchez-Moreno and Kim e*t al*. [16-18]. The DPPH stock solution was obtained by dissolving 4 mg DPPH in 100 mL MeOH at room temperature. Serial two fold dilutions of each extracts were performed to obtain 8 different concentrations of extracts and standard (quercetin). Then each well in the 96-well plate was filled with 100µL of extract or standard before DPPH was added. Finally, each well contained 100µL of extracts or standard and 200µL DPPH. Each reaction was performed in triplicates. Blank for this experiment was 100µL extract or standard mixed with 200µL MeOH. The extract was allowed to react with DPPH by incubating the plate at room temperature for 30mins. The absorbance of the extract was measured using spectrometric microplate reader (Perkin-Elmer Lambda 45, Massachusetts, USA) at 517nm. The percentage of radical scavenging activity was calculated using the following equation.

$$\% \text{ RSA} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100\%$$

2.3.2 $ABTS^{\bullet+}$ cation radical scavenging assay

$ABTS^{\bullet+}$ cation radical scavenging method used was slightly modified from Re *et al*[19] and Yusoff *et al*[20]. ABTS stock solution was prepared by dissolving ABTS powder and potassium peroxodisulfate (1:6 w/w) in 10mL deionized water. The solution was then incubated at room temperature for 16 hours in the dark. This step was to generate ABTS radical. As this solution only had 2 days of shelf-life, it was freshly prepared before performing each experiment. 30mL phosphate-buffered saline was then mixed with a few drops of ABTS solution and the absorbance was tested at 734 nm to obtain a stable reading of 0.70±0.02. Once a stable mixture was obtained, the reaction was left to occur in the dark. It was important to avoid any interference in absorbance measured. Each extract was prepared in 3 different concentrations and the test was performed in triplicates. 10µL sample extract was then added with 2mL of stabilized ABTS solution. The absorbance was measured using HITACHI U-2800 spectrophotometer at 734 nm after 6 mins.

2.3.3 Ferric-reducing antioxidant power (FRAP) assay

The assay used was based on Oyaizu's [21]. The ability of ME, WE, CE or PEE to reduce Fe^{3+} in ferric chloride which is yellow in color to green-colored ferrous Fe^{2+} was determined by reacting 1mL extract and standards in 2.5 mL PBS solution, pH 7.4 and 2.5mL 1% potassium hexacyanoferrate (lll). Triplicates of each 5 different concentration of samples and standards were established. The solution was then incubated for 20 mins in 50°C water bath before 2.5 mL of tricholoacetic acid, TCA (10%) was added. Each solution was centrifuged at 3000 rpm for 10 mins (Eppendorf 5403, Engelsdorf, Germany)after which the 2.5mL of the upper layer (supernatant) was mixed well in a test tube with 2.5mL distilled water and 0.5mL ferric chloride. The absorbance of extracts and standards were then measured at 700nm using spectrophotometer (HITACHI, U-2800)

2.0 Results and Discussion

3.1 Free radical scavenging activity on 1,1-diphenyl-2-dipicrylhydrazyl (DPPH•)

The DPPH assay used was based on the scavenging or trapping of 1,1-diphenyl-2-dipicrylhydrazyl radical. The percentage scavenging of DPPH radical was calculated using the equation stated previously and a graph of radical scavenging activity was plotted for each extracts and standards. The higher the scavenging activity indicated higher antioxidant capacity. The value of IC_{50} of the extracts was also determined. It was the concentration required to cause 50% free radical inhibition *in-vitro*. Table 1 shows that ME had the highest free radical scavenging activity among the other three extracts where the value of IC_{50} was the lowest with a value of 0.01746 mg/mL. The weakest radical scavenger was CE with highest IC_{50}, 0.02932mg/mL. However, all extracts showed lower antioxidant capacity than quercetin as standard. The IC_{50} of quercetin was 0.00803 mg/mL. The value of IC_{50} of strong antioxidant will be approximately equal or much lower than value recorded for quercetin.

Table 1. The IC_{50} value of 4 extracts from *E. polyantha* on DPPH free radicals.

Samples	IC_{50} (mg/mL)x ± SD
E. polyantha PE	0.02308±0.001
E. polyantha CE	0.02932±0.005
E. polyantha ME	0.01746±0.001
E. polyantha WE	0.01896±0.001
Quercetin	0.00803±0.001

Data are presented as mean±standard deviation (CE=chloroform extract, PE=petroleum ether extract, WE=water extract, ME=methanol extract).

The disappearance of violet color of DPPH to pale yellow will be the indicator of the end point of the scavenging activity of extracts (Figure not shown). DPPH• radical had been reduced where it accepted proton from antioxidant which acted as proton donor. The resulted decolorization is stoichiometric with respect to

number of electrons captured[22]. More electron captured by antioxidant lightened the intensity of violet color of DPPH where lower absorbance was observed. *E. polyantha* extracts especially ME and WE showed a significant color change to pale yellow although the concentration was low when reacted with DPPH. However, CE and PE were observed to change to paler yellow color but at higher concentration. This was due to their weak DPPH scavenging capacity. Antioxidant capacity of extracts from highest to lowest was ME> WE> PE> CE. All extracts performed a quite significant radical scavenging activity. The extract concentrationn is the factor that affects the intensity of this reaction.

3.2 Antioxidant capacity by $ABTS^{\bullet+}$ assay

This antioxidant assay was based on the reaction of antioxidant with $ABTS^{\bullet+}$ radicals generated. Similar to DPPH assay, the mechanism involved the transfer of proton from the antioxidant substrate to the radicals. The antioxidant capacity of extracts was compared with the antioxidant capacity of Trolox, an analogue of vitamin E. In this test, sample extract which can decolorize ABTS from dark blue-green to colorless and give lowest absorbance at 734nm was considered to have the highest antioxidant capacity. But, the value of Trolox equivalent antioxidant capacity (TEAC) must be determined so that the antioxidant capacity can be significantly compared with Trolox standard. The TEAC value was the ratio between the slope of linear plot for scavenging of ABTS radical cation by the extracts compared to the slope of ABTS radical cation scavenging by Trolox[23]. The result showed (Table 2) that ME contained the highest value of TEAC in millimol extract with 6.18331 TEAC (mmol). On the other hand, *E. polyantha* CE has the lowest antioxidant capacity with TEAC value, 0.32187 TEAC (mmol). The sequence of antioxidant capacity of extracts in decreasing order was ME>CE>WE> PE.

Table 2. The total antioxidant capacity of 4 extracts from *E. polyantha* expressed as TEAC (mmol).

Sample extracts	TEAC (mmol)
PE	0.32187±0.006
CE	1.91150±0.015
ME	6.18331±0.046
WE	1.48987±0.005

Data are presented as mean±standard deviation (PE=petroleum ether extract; CE=chloroform extract, ME=methanol extract, WE=water extract)

3.3 Ferric-reducing antioxidant power (FRAP) assay

This assay was performed to determine the reducing power of antioxidant. FRAP assay was based on the measurement of an endpoint like DPPH assay[24]. The result shows, as concentration of sample extract increased, the absorbance also increased. The absorbance of extracts was directly proportional to their reducing power. The graph shows (Figure 1-2) the comparison between the four extracts of *E. polyantha* with two selected standards, BHT and quercetin. WE and ME seemed to have higher reducing power compared to CE and PE. At low concentration (range from 0.0935mg/mL to 1.5mg/mL), both ME and WE showed high absorbance while CE and PE started to significantly reduce ferric at the concentration range between 0.5mg/mL to 2mg/mL. From the result, it was obvious that ME has the highest reducing power while PE has the weakest ferric-reducing power. The sequence of reducing power of extracts in decreasing order is ME> WE> CE> PE. The reducing power of the extracts was compared directly with reducing power of two standards, BHT and quercetin without interpreting the result in form of value of standards per milliliter extract like in ABTS assay. The absorbance itself can be used as reference to the value of reducing power of extracts.

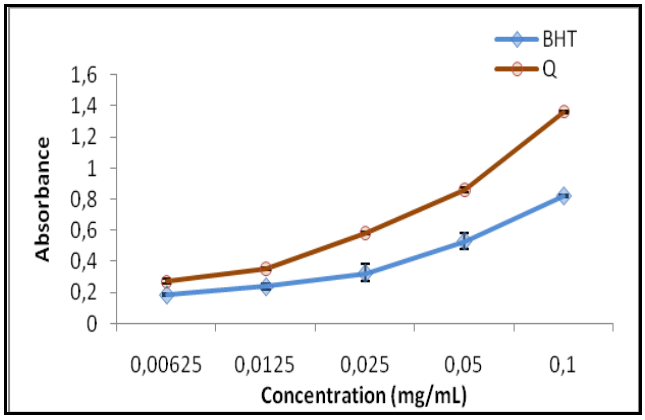

Figure 1. The ferric-reducing antioxidant powers of standards (BHT & quercetin) with concentration range from 0.00625mg/mL to 0.1mg/mL. Data are presented as mean±standard deviation.

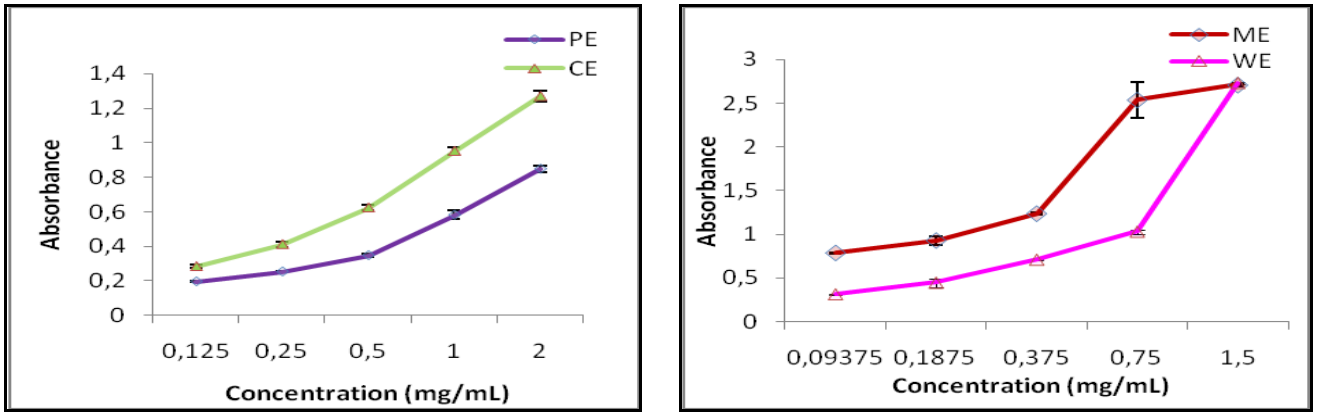

Figure 2. The ferric-reducing antioxidant powers of *E. polyantha* extracts and standards (BHT & quercetin) with different concentration. Data are presented as mean±standard deviation.
*** PE=petroleum ether extract, CE=chloroform extract, ME=methanol extract, WE=water extract.**

In DPPH antioxidant assay, 96-well microplate was used instead of cuvette for the determination of radical scavenging activity to ensure that more than one extract can be tested simultaneously at one particular time. Dark condition must be provided during the incubation of extracts and DPPH so that they can react naturally and give better absorbance value without being affected by other light sources. Result obtained from ABTS assay showed that it was slightly deviated from DPPH and FRAP assays. To achieve better accuracy, the ABTS working solution must be used within 4 hour. The absorbance of each sample might fluctuate and cause obvious difference between the two tests. To solve the problem, freshly prepared ABTS solution must be used in each assay to maintain the cation radical activity. Referring to the result from DPPH assay, *E. polyantha* in water should have better scavenging activity than same leaves extract in chloroform. But, this study showed that CE has higher TEAC value than WE which means that CE was better in scavenging ABTS cation radicals. Nevertheless, the antioxidant capacity of many compounds and foods had been determined extensively using the ABTS assay. This was due to its simple procedural operation. For FRAP assay, there was not much difference in the result obtained than result in DPPH assay. Both assays proved that ME was the best antioxidant and followed by WE compared to the other two extracts. But, contradiction was obvious when the result is compared to result obtained from ABTS assay. In FRAP test, tetrachloroacetic acid (TCA) and ferric chloride were the solutions that affect the reducing capacity of extracts. Therefore, they must not be kept for more than 48 hours or better prepared freshly prior to performing every assay. Incubation time was very critical in determining antioxidant capacity [25]. This was to prevent any obvious deviation in activity of our extracts on targeted free radicals. Advantages of FRAP assay include its inexpensiveness and its simple reduction principle. The ability of extracts to reduce ferric to ferrous indicated that the extract contained reductants. However, reductants do not act as antioxidants which make this assay to be less suitable for determining important antioxidant like thiols[26]. As the structures of free radicals differ from each other, they give different result in

different antioxidant test (Mermeilstein). Therefore, the inconsistent result obtained was negligible provided that we can notably see the difference in antioxidant capacity between each extract tested. Nevertheless, result obtained from this research was reliable as the three assays were comparable.

4.0 Conclusion

All *E. polyantha* leaf extracts showed antioxidant capacity. The assays shows ME as the strongest antioxidant.

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Author Contributions

The authors' contributions are as follows: Nurfifi Ashikin Bt Roslan conducted the research and drafted the manuscript. Tri Widyawati provided the extracts and revised the manuscript; Nor Adlin Yusoff helped collected the data. Mun Fei Yam, Khairul Niza bt Abdul Razak, Mariam Ahmad and Mohd Zaini Asmawi designed the research and responsible for the final content. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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