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Agriculture and Agricultural Science Procedia 9 (2016) 378 – 385

Agriculture and Agricultural Science Procedia

International Conference on Food, Agriculture, and Natural Resources, FANRes2015

Antioxidant Activity of Various Kenitu *(Chrysophyllum cainito* L.) Leaves Extracts from Jember, Indonesia

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Abstract

Kenitu or star apple (*Chrysophyllum cainito* L.) is widely used as traditional remedy for inflammation, cancer, and diabetes mellitus. Leaves of four type of kenitu were extracted with different solvents, i.e., 96% of ethanol, 70% of ethanol, 50% of ethanol, 96% of acetonee, 70% of acetone, and 50% of acetone. The extracts have been screened for antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, total phenolic content, and total flavonoid content. The study showed that 70% of ethanol extracts exhibited the highest antioxidant activity. The type 2 samples exhibited the highest total phenolic content, while type 1 samples had the highest total flavonoid content.

Peer-review under responsibility of the organizing committee of IC-FANRes 2015

Keywords: Chrysophyllum cainito L., antioxidant activity, DPPH, total phenolic content, total flavonoid content;

1. Introduction

Antioxidant compounds play an important role as a health-protecting factor. The main characteristic of antioxidants is its ability to trap and stabilize free radicals (Prakash, 2001). Antioxidant compounds inhibit the autooxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions, so that the compounds can prevent cells damage. Free radicals cause a wide number of health problems, such as cancer, aging, heart diseases, and gastric problems. A free radical is defined as a molecule or molecular fragments containing one or more unpaired electrons in its outermost atomic or molecular orbital (Sen et al., 2010). The

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Peer-review under responsibility of the organizing committee of IC-FANRes 2015
doi:10.1016/j.aaspro.2016.02.153

antioxidative effect correlates with phenolic components, such flavonoids, phenolic acids, and phenolic diterpenes. The activity of phenolic compounds is mainly due to their redox properties in absorbing and neutralizing free radicals (Panovskaet al., 2005).

Chrysophyllum cainito L. (*Sapotaceae*) or star apple is locally known as kenitu in Indonesia. The plants are grown commercially in certain tropical and subtropical areas, such as southern Florida. It has been used as traditional remedy for laryngitis with inflammation, pneumonia, and diabetes mellitus (Morton, 1987). The decoction has empirical effect as an astringent and it is widely used to treat diarrhea, fever, and venereal disease in eastern Nicaragua (Coee & Anderson, 1996). Luo et al. (2002) identified nine polyphenolic antioxidants of *C. cainito* fruit, namely: (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, quercetin, quercitrin, isoquercitrin, myricitrin, and gallic acid. Another content of its fruits is cyanidin-3-*O*-*β*-glucopyranoside, an anthocyanin antioxidant (Einbond et al., 2004). *C. cainito* leaves contain triterpene antioxidants, include β-amyrin acetate and gentistic acid (Lopez, 1983; Griffiths, 1959).

There are four types of kenitu fruit from Jember, namely: big size, green color with round shape (type 1); small size, green color with round shape (type 2); medium size, green color with oval shape (type 3); and small size, red purplish color with round shape (type 4). Previous study showed that water, methanol, and ethyl acetate extracts of three types of kenitu fruit had antioxidant capacity toward DPPH radical (Hidayat & Umiyah, 2005; Hidayat & Ulfa, 2006; Amrun et al., 2007). In this study, leaves of each type of kenitu (type 1-4) were extracted with various solvents (96% of ethanol, 70% of ethanol, 50% of ethanol, 96% of acetone, 70% of acetone, and 50% of acetone), and were screened for their antioxidant capacity using DPPH method. The extract which showed the highest antioxidant capacity would be determined its total polyphenol content and its total flavonoid content, since polyphenol and flavonoid contributed to the antioxidant capacity of plant extract.

2. Materials and Methods

2.1. Materials

C. cainito fresh leaves of four plant types were collected from Jember District, East Java between March to April 2014. All of plant samples were properly authenticated by Indonesian Institute of Sciences at Purwodadi Botanical Garden, East Java, Indonesia. The mature dark green leaves were air-dried and powdered. All chemicals and reagents were analytical grade. DPPH, quercetin and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA), Ethanol, acetone, Folin-Ciocalteau reagent, Na_2CO_3, and $AlCl_3.5H_2O$ were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of Extracts

The dried ground plant material (500.0 mg for each sample) was extracted independently with various solvents: 96% of ethanol, 70% of ethanol, 50% of ethanol, 96% of acetone, 70% of acetone, and 50% of acetone (10 ml) – for 4 hours at 30°C using ultrasonicator (Elmasonic S180H, Germany). The extracts were centrifuged at 2,500 rpm for 30 min. Afterward, the supernatants were separated and concentrated to 2.0 ml at 60°C. The clear extracts were individually stored in well-capped tubes prior to antioxidant test at room temperature.

2.3. Determination of Total Phenolic Content

The total phenolic content was determined by the Folin-Ciocalteau method with some modifications (Wolfe et al, 2003). 0.1 ml of sample (0.08% w/v) was added to 1.0 ml of 1:10 v/v diluted Folin-Ciocalteau reagent and 0.8 ml Na_2CO_3 (7.5% w/v). The mixture was vortexes for 15 sec and kept at 25^{O}C for 30 min. Extraction solvents were used as blank. Absorbances of samples were read at 765 nm against the blank using UV-Vis spectrophotometer (U-1800, Hitachi Instruments Inc., Tokyo, Japan). The total polyphenol content was calculated by a standard curve prepared with 50.00-250.00 μg/ml gallic acid, and results were expressed as gallic acid equivalents (GAE). Samples were measured in triplicate analysis.

2.4. Determination of Total Flavonoid Content

Total flavonoid content was carried out according to the method reported by Ordonez et al. (2006) with slight modification. 0.5 ml of sample (0.01% w/v) was added to 0.5 ml $AlCl_3$ (2% v/v) and kept at 25^{O}C for 30 min. Discoloration to yellow indicated the presence of flavonoids. The absorbance of the samples was read at 420 nm against extraction solvents as blank by using UV-Vis spectrophotometer. With the solution of quercetin (20.00 – 100.00 µg/ml) as the standard, a calibration curve was plotted to calculate the content of total flavonoids and results were expressed as quercetin equivalents (QE). Samples were measured in triplicate analysis.

2.5. Evaluation of Antioxidant Activity

The DPPH radical scavenging assay was determined according to the previous methods (Santosa et al, 1998; Amrun et al, 2007; Hafid et al, 2014) with some modification. Initially, absorbance's of DPPH solution (0.004% w/v in methanol) were simultaneously read at 3 wavelengths: 495, 515, and 535 nm (equation 1). In a cuvette, 300 µl of sample solution (0.007% w/v) was added to 1200 µl of DPPH solution. After a 30 min incubation period at 25°C, absorbance of mixed solution was read simultaneously at 495, 515, and 535 nm. Gallic acid was used as positive control. Antioxidant capacity is defined by the ability of samples to inhibit initial DPPH absorbance and calculated according to equation 2. $A_{calc.DPPH}$ is calculated absorbance of initial DPPH solution. $A_{calc.sample}$ is calculated absorbance of mixed (DPPH and sample) solution.

$$\text{Acalc} = A_{515} - \frac{A_{495} + A_{535}}{2} \quad (1)$$

$$\% \text{ DPPH inhibition} = [1 - (A_{calc.sample}/A_{calc.DPPH})] \times 100\% \quad (2)$$

2.6. Statistical Analysis

The data obtained in this study were expressed as mean ± standard deviation of triplicate measurements. One-way analysis of variance (ANOVA) with Least Significance Different (LSD) method was carried out to test any significant differences among samples. Values of $P<0.05$ were considered to be significantly different ($\alpha=0.05$).

3. Results and Discussion

3.1. Antioxidant Assay

The potential antioxidant activity of various kenitu leaf extracts was determined using the DPPH method. The DPPH radical scavenging activity is affected by the presence of hydroxyl groups in the phenolic and flavonoid compounds. The reduction of DPPH radicals into the reduced form DPPH-H determined the ability of phenolic and flavonoid compounds to donate hydrogen atom or electron to the unpaired DPPH radicals. When DPPH reacted with radical scavenger, the purple color changed into pale purple or light yellow (Nurhanan et al, 2012). The free radical scavenging activity was evaluated by absorption reduction of the stable radical DPPH at 515 nm. Here, we used gallic acid as antioxidant standard (Fig. 1) to evaluate antioxidant activity of various kenitu leaf extracts. Moreover, gallic acid is one of the marker compounds in kenitu leaf which can be quantified by HPTLC as it reported by Shailajan and Gurjar (2014).

The antioxidant activity of various kenitu leaf extracts are shown in Table 1. Percentage of DPPH inhibition values were used for comparison. The 70% of ethanolic extract exhibited the highest antioxidant activity ($P<0.05$). Indeed, the DPPH inhibition percentage of type 1, type 2, and type 3 samples were greater than that of 10 µg/ml gallic acid solution. This study confirms that most antioxidant compounds of various kenitu leaf were extracted into the 70% of ethanolic extract. Our results were in accordance with that obtained by Abozed et al. (2014), in which the 70% of ethanolic extract of wheat had the highest antioxidant activity when compared with corresponding 70%

of methanolic and 50% of acetonic extracts. 70% of ethanol was the best solvent to extract anthocyanin of black pigeonpea shell (*Cajanus cajan*) observed from total anthocyanin content, total phenolic content, and DPPH radical scavenging activity (Al-Lawi, 2011). Therefore, the 70% of ethanolic extract was used for further study in determination of total phenolic content and total flavonoid content. In this study, aqueous alcohol solvents were more efficient in extracting antioxidant compounds. 96% of acetonic and 96% of ethanolic extracts showed lower level of scavenging activity compared to extracts of aqueous aceton and aqueous alcohol solvents. Anwar et al. (2013) also reported that aqueous alcohol solvents (80% of methanol and 80% of ethanol) were effective to extract antioxidants from cauliflower (*Brassica oleracea*) during the extraction process.

3.2. Total Phenolic Content

The major groups of phenolic substances have mechanism of primary antioxidant activity or free radical scavengers. Therefore, it was important to determine the total amount of phenolic compounds in the plant extracts. The correlation between antioxidant activity and total phenolic content had been proved in many studies (Nurhanan et al., 2012; Lou et al., 2014; Engida et al., 2015). The determination of total phenolic content was performed by the Folin-Ciocalteau method using gallic acid as standard, as it can be seen in Figure 2. In alkaline solution, the yellow color of Folin-Ciocalteau reagent would change to blue color. The increasing number of hydrogen donating groups in the phenolic compounds which showed high total phenolic content was characterized by the increasing intensity of blue colored complex (Kaur et al., 2008). According to the results of antioxidant assay, the total phenolic contents (expressed in mg GAE/g dry plant) of various kenitu samples were carried out using 70% of ethanol as the best solvent, as shown in Table 2. From the gallic acid standard curve, the highest amount of phenolic compounds was found in type 3 samples and the lowest total phenolic content was observed in type 4 samples (P<0.05). However, there was no significant difference between total phenolic content of type 1 and type 2 samples. These findings were supported by other studies in which total phenolic content of plants is affected by their varieties (Matthes et al., 2008; Rodrigues et al., 2011; Shian et al., 2012; Ariviani et al., 2013).

The correlation between total phenolic content (x) and % DPPH inhibition (y) of various kenitu samples was determined by using regression analysis. The correlation coefficient (R^2) was 0.921 (y = 0.187x + 59.10) (Fig. 3). This result indicated that antioxidant activity of samples was mainly determined by their polyphenol content (Javanmardi et al., 2003).

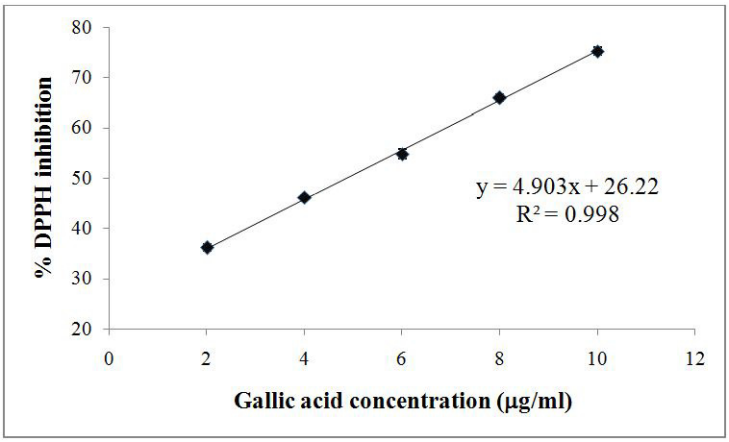

Figure 1. The percentage of DPPH inhibition by gallic acid (n=3).

Table 1. DPPH scavenging activity of various *C. cainito* leaf extracts (%).

Extracts	Type 1	Type 2	Type 3	Type 4
96% acetone	19.936± 0.333[a]	38.774± 0.681[g]	43.031± 0.592[f]	39.534± 0.358[f]
70% acetone	60.305± 0.868[b]	53.081± 0.838[m]	40.254± 0.647[m]	25.030± 0.271[s]
50% acetone	52.507± 0,806[c]	75.135± 1.015[t]	56.699± 1.008[m]	32.624± 0.386[t]
96% ethanol	25.175± 0,402[u]	44.873± 0.809[j]	53.251± 0.719[b]	62.203± 1.224[u]
70% ethanol	91.088± 0,881[e]	89.097± 1.600[k]	90.914± 1.717[p]	66.549± 0.616[v]
50% ethanol	68.099± 0,900[i]	52.228 ± 0.496[m]	59.233 ± 0.445[q]	61.980 ± 1.130[u]

Values (mean ± SD) are average of each sample, determined in triplicate (n=3). Different superscript letters indicate a significant differences of means using post hoc test (P<0.05).

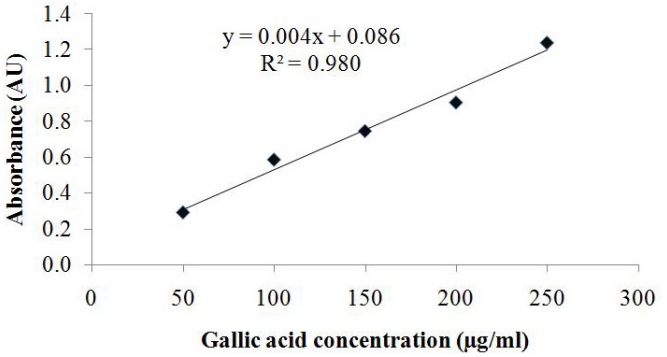

Fig.2. Calibration curve for total phenolic content measurement (n=3).

Table 2. Total phenolic content of the 70% ofethanolic extracts of various *C. cainito* leaf extracts.

Variants	Total phenolic content (mg GAE/g extract)
Type 1	150.073 ± 0.335[a]
Type 2	152.339 ± 1.660[a]
Type 3	190.132 ± 0.457[b]
Type 4	47.533 ± 0.493[c]

Values (mean ± SD) are average of each sample, determined in triplicate (n=3). Superscript letters indicate significant differences of means using post hoc test (P<0.05).

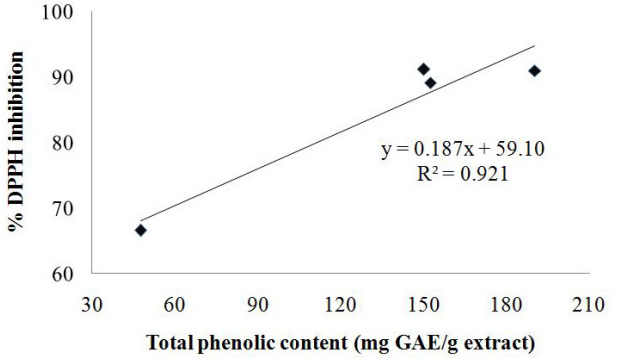

Fig. 3. Linear correlation of total phenolic content (x) versus % DPPH inhibition (y) of various kenitu samples.

3.3. Total Flavonoid Content

The antioxidant activity of flavonoids depends on functional groups on its basic structure. Several mechanisms of antioxidant activity, such as by scavenging free radicals and chelating metal ions, are affected by the configuration, substitution, and total number of functional hydroxyl groups. The most significant determinant in scavenging reactive oxygen and reactive nitrogen species is the B ring hydroxyl configuration of flavonoids. The configuration can donate hydrogen and an electron to hydroxyl, peroxyl, and peroxy nitrite radicals and result a relatively stable flavonoids radical (Kumar & Pandey, 2013). The antioxidant activity will increase with an increase in the number of hydroxyl groups of flavonoid compounds (Rice-Evans et al., 1996). Previous studies showed that total flavonoid content in plants associated with DPPH free radical-scavenging ability (Nurhanan et al., 2012).

As shown in Table 3, the flavonoid content of 70% of ethanolic extract decreases in the following order: type 1>type 3>type 2>type 4 (P<0.05). Ghasemzadeh et al. (2010) reported that two varieties of Malaysia young ginger (*Zingiber officinale*), namely Halia Bara and Halia Bentong had significantly different total flavonoid content (P<0.001).

In this study, regression analysis was carried out to determine the correlation between total flavonoid content (x) and % DPPH inhibition (y) of various kenitu samples. The correlation coefficient (R^2) was 0.596 (y = 4.126x + 38.58) (Fig. 3) indicated that the correlation degree was weak. However, this result showed that flavonoid compounds also contributed to antioxidant capacity of kenitu samples.

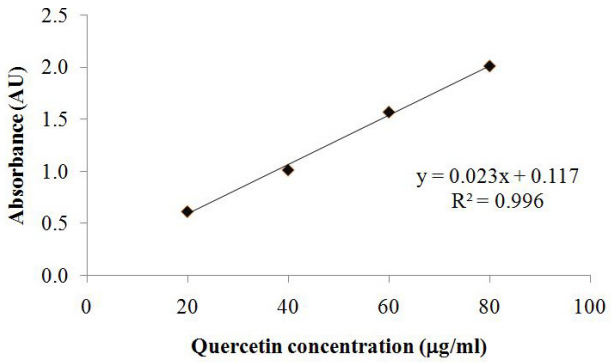

Fig. 4. Calibration curve for total flavonoid content measurement (n=3).

Table 3.Total flavonoid content of the 70% ethanolic extracts of various *C. cainito* leaf extracts.

Variants	Total flavonoid content (mg QE/g extract)
Type 1	14.039 ± 0.030^{a}
Type 2	10.643 ± 0.109^{b}
Type 3	11.116 ± 0.176^{c}
Type 4	8.623 ± 0.105^{d}

Values (mean ± SD) are average of each sample, determined in triplicate (n=3). Superscript letters indicate significant differences of means using post hoc test (P<0.05).

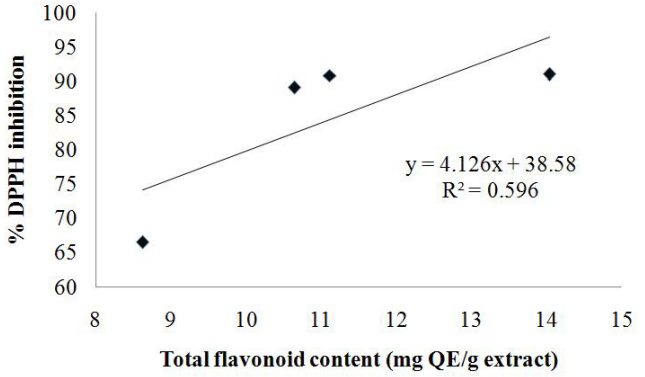

Fig. 5. Linear correlation of total flavonoid content (x) versus % DPPH inhibition (y) of various kenitu samples.

4. Conclusion

The present study shows that solvent selection was important for obtaining kenitu leaf extract with high level of total phenol content, total flavonoid content, and antioxidant activity. Further studies like comparing the extraction techniques are needed for obtaining antioxidant extracts which can give health benefit and higher commercial value.

Acknowledgements

The authors are grateful to Directorate of Research and Community Service, Directorate General of Higher Education of Ministry (DIKTI), Ministry of Research, Technology and Higher Education for supporting this research through BOPTN funding of University of Jember under contract number: 354/UN25.3.1/LT.6/2014.

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