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Potential of *Dyospirus khaki* Baverage as Sources of Natural Antioxidant

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Abstract: *Dyospirus khaki* is fruit belongs to the Ebenaceae family. Its beneficial properties are considered to be related to the various antioxidants, including vitamins, phenolic compounds and carotenoids, contained in this kind of fruit. Methanolic and ethanolic extract of *Dyospirus khaki* baverage were evaluated for their phenol and flavonoid content, antioxidant activities by using DPPH and bleacing of β-karoten method. Correlation between phenol and flavonoid content with pearson correlation. The result showed that methanolic extract of *Dyospirus khaki* baverage has higher phenol and flavonoid content than ethanolic extract of *Dyospirus khaki* baverage. Antioxidant activity of methanolic extract of *Dyospirus khaki* baverage was higher than that of ethanolic extract of *Dyospirus khaki* baverage based on DPPH and bleacing β-karoten. There were significant and positive correlation between antioxidant activity based on DPPH and bleacing β-karoten and phenol and flavonoid content on baverage from methanolic and ethanolic extract of *Dyospirus khaki*. These results indicated that methanolic extract and ethanolic extract of *Dyopsirus khaki* baverage might be used as potential source of natural antioxidants.

Key words: *Dyospirus khaki*, antioxidant, baverage

INTRODUCTION

Oxidative stress, caused by the imbalance of Reactive Oxygen Species (ROS) and antioxidative defense systems, is considered as a major etiological and/or pathogenic agent of most degenerative diseases such as cancer, Alzheimer's, diabetes and aging (Datta *et al*., 2000). The antioxidants are of interest in the treatment of several cellular degenerations and they inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions (Behera *et al*., 2006). Regular consumption of fruit and vegetables containing natural antioxidants is correlated with the decreased risk of diseases such as cancer and cardiovascular diseases (Michels *et al*., 2000).

Dyospirus khaki, which belongs to the Ebenaceae family, is originated from China. *Dyospirus khaki* is cultivated world widely, with 90% of production in Korea, China and Japan. *Dyospirus khaki* trees (*Diospyros khaki*) are mainly cultivated in the north-east Asian countries and their fruits are classified as sweet and astringent types (George and Redpath, 2008). Due to their nutritional and health benefit functional characteristics, the cultivation and production have been recently increased in Mediterranean countries, such as Spain and Italy (Ancos *et al*., 2000).

There are generally 2 types of *Dyospirus khaki* fruit: astringent and non-astringent. Astringent species cannot be eaten when firm because of high levels of soluble tannins, which can be removed naturally or artificially (Bubba *et al*., 2009). Nonastringent *Dyospirus khaki* are not actually free of tannins, but rather are far less astringent before ripening and lose more of their tannic quality sooner (Seong and Han, 1999). Non-astringent *Dyospirus khaki* may be consumed when still very firm and remain edible when very soft.

Dyospirus khaki fruit is known to contain many bioactive compounds including polyphenols and carotenoids, as well as dietary fiber and minerals (Veberic *et al*., 2010; Chen *et al*., 2008; Akter and Eun, 2009). Recent studies show that the Mopan *Dyospirus khaki* possesses antitumor and multidrug resistance reversal properties (Kawase *et al*., 2003), hypocholesterolemic and antioxidant effects (Gorinstein *et al*., 1998) and antidiabetic effects (Lee *et al*., 2006) and prevents the rise in plasma lipids (Matsumoto *et al*., 2006). These beneficial properties are considered to be related to the various antioxidants, including vitamins, phenolic compounds and carotenoids, contained in this kind of fruit. *Dyospirus khaki* have been used for their medicinal properties, such as their blood pressure-lowering and diuretic effects. They have been used to treat coughs and the seeds used for stopping hiccups.

The aim of this study was conducted to investigate the antioxidant activity of baverage as source of natural antioxidant from *Dyospirus khaki*, the relationship phenol and flavonoid content on baverage. Methods for evaluation antioxidant activity are using the b-carotene linoleate model system (b-carotene) (Singh *et al*., 2002) and radical scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Barros *et al*., 2007).

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MATERIALS AND METHODS

Dyospirus khaki fruits are from Batu Malang Indonesia, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-karoten, Gallic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All reagents were of analytical grade.

Preparation of extracts from *Dyospirus khaki*: *Dyospirus khaki* obtained from Batu Malang Indonesia. Washed and sliced thin, then dried using sunlight for 30 days. The dried *Dyospirus khaki* fruit was ground in a mill and passed through a 40-mesh sieve. Then extracted by maceration 1:5 (w/w) for 3 days with methanol and ethanol at room temperature and filtered through a Whatman No. 1 filter paper. The filtrate was concentrated to dryness under reduced pressure on a rotary at 37°C. Each dried extract was dissolved in DMSO with concentration of 50 mg/mL for the experiments. All samples were place in a glass bottle and stored at 4°C until used.

Preparation of *Dyospirus khaki* beverages: *Dyospirus khaki* beverage created by making three formulations. First formulation contains 750 mg of methanolic extract or ethanolic extract. Second formulation contains 1500 mg of methanolic or ethanolic extracts. While third formulation contains 3000 mg of extract ethanol or methanol. In each formula there were addition of citric acid, sucrose, aspartase, sodium bicarbonate and sodium carbonate. Each formulation added with 100 ml distilled water and keep on low temperature (4°C) and used for further analysis.

Total Phenolic Contents (TPC) TPC of each baverage: Total phenolic content of each baverage was estimated by Follin-Ciocalteu method (Singleton and Rossi, 1965). To 6.0 ml triple distilled water, a 75 ul methanolic or ethanolic baverage of *Dyospirus khaki* and 0.5 ml Follin ciocalteu reagent was mixed followed by addition of 1.5 ml Na_2CO_3 (20 g/100 ml water) and the volume was made up to 10.0 ml with distilled water. The reaction mixture was kept in dark for 30 min at 25°C, the absorbance was measured at 760 nm and the phenolic content was calculated using the gallic acid standard curve and expressed as gallic acid equivalents.

Total Flavonoid Contents (TFC) TFC of each baverage: Flavonoid contents in baverage of methanolic and ethanolic extract of *Dyospirus khaki* were determined by a colorimetric method described by Jia *et al.* (1999). 200 µl of each baverage sample was taken and made up to 5 ml with distilled water and 0.3 ml of 5% $NaNO_2$ solution was added. After 5 min, 0.3 ml 10% $AlCl_3.H_2O$ solution was added. After 6 min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml distilled water. The solution was mixed well and the absorbance was measured against a blank at 510 nm. Flavonoid contents were calculated using a standard calibration curve, prepared from Quercetin. The flavonoid contents were expressed as mg quercetin g^{-1} of extract.

DPPH radical scavenging activity The DPPH radical: Three formulation of 0.3 ml baverage of methanolic or ethanolic extract of *Dyospirus khaki* were mixed with 2.7 ml of methanolic solution containing DPPH radicals ($6x10^5$ mol/l). The mixture was vortexed and incubated in dark for 60 min. The reduction of the DPPH radical was determined by reading the absorbance at 517 nm. The Radical-Scavenging Activity (RSA) was calculated as percentage of DPPH discoloration. Using the equation: % RSA = [(ADPPH-AS)/ A DPPH] x 100, where AS is the absorbance of the solution when the sample extract is added at a particular level and A DPPH is the absorbance of the DPPH solution (Barros *et al.*, 2007).

Antioxidant assay using the b-carotene linoleate model system: b-carotene (0.2 mg) in 0.2 ml chloroform, linoleic acid (20 mg) and Tween-40 (polyoxyethylene sorbitan monopalmitate) (200 mg) were mixed. Chloroform was removed at 408C under vacuum. The resulting mixture was diluted with 10 ml water. To this emulsion was added 40 ml oxygenated water. Four milliliter aliquots of the emulsion were added to 0.2 ml of the sample of baverage of methanolic or ethanol extracts of *Dyopsirus khaki* (Singh *et al.*, 2002).
The absorbance at 470 nm was taken at 50°C at zero time (t/0). Measurement of absorbance was continued during 180 min at an interval of 15 min. A mixture prepared as already described, but without b-carotene, served as the blank. Antioxidant Activity (AA) was expressed as percent of inhibition relative to the control, using the following formula:

$$AA = \left(\frac{DE_{control} - DR_{sample\ or\ standard}}{DR_{control}} \right) x\ 100$$

Statistical analysis: Experiment data were analyzed using Excel (Microsoft Inc.) and SPSS version 17.0 software. Significant differences between samples were analyzed using Analysis of Variance (ANOVA) and Duncan's multiple-range test ($p<0.05$). Pearson's correlation was used to determine the correlation of data between DPPH free radical-scavenging activity or bleaching β-karoten to phenol or flavonoid content. All treatments were run in triplicate.

RESULTS AND DISCUSSION

Extraction yields: The extraction yields of *Dyospirus khaki* used methanolic and ethanolic solvent were 49.89±1.47%; 47.53±0.09%, respectively. Relatively higher extraction yields were obtained from methanolic

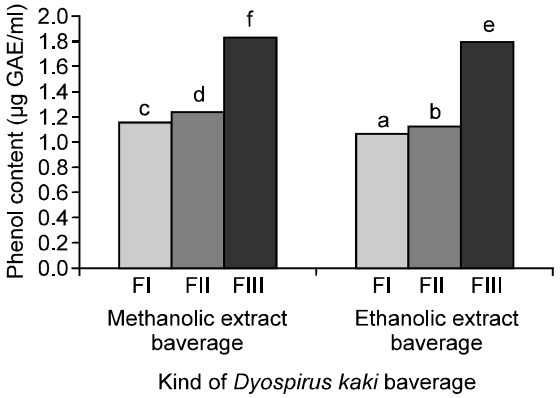

Fig. 1: Phenol content of methanolic and ethanolic extract of *Dyopsirus khaki* baverage on variation formulation. Data are presented as mean from three independent experiments. Means with different letters are significantly different at level of $p<0.05$

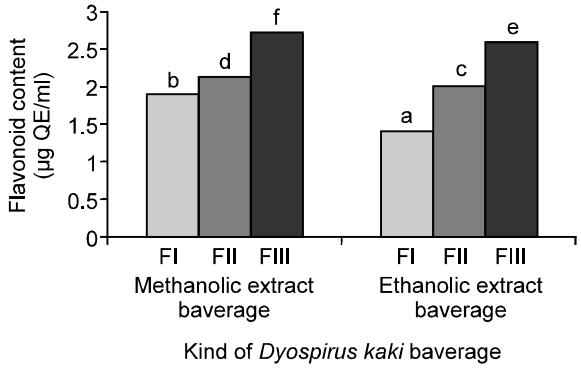

Fig. 2: Flavonoid content of methanolic and ethanolic extract of *Dyopsirus khaki* baverage on variation formulation. Data are presented as mean from three independent experiments. Means with different letters within the same sample are significantly different at level of $p<0.05$

solvent than ethanolic solvent. Among solvents, methanol was a most effective solvent on the extraction. These results showed that the extraction yield varied by solvents.

Total phenol and flavonoid content: The Total Phenolic Content (TPC) values was quantified based on the linear equation obtained from gallic acid standard calibration curve. Thus, TPC values were expressed as gallic acid equivalent (mg GAE/100 g samples). The amount of phenol on methanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 1.16±0.01 (ug/ml), 1.24±0.1 ug/ml and 1.84±0.01%, respectively. The amount of phenol on ethanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 1.04±0.04%, 1.13±0.01% and 1.79±0.01%, respectively (Fig. 1). Results of ANOVA analysis indicated that there was significant difference ($p<0.05$) between methanolic extract of *Dyopsirus khaki* baverage and ethanolic extract of *Dyopsirus khaki* baverage. It is considered that the phenolic compounds contribute to overall antioxidant activities of *Dyospirus khaki* baverage from methanolic and ethanolic extracts. The extraction yield of phenolics content varied depending on the extraction solvent with the following order: methanol > ethanol extracts.

Flavonoids are naturally occurring substances in plants that are thought to have positive effects on human health (Montoro *et al*., 2005). The most important function of flavonoids is the antioxidants properties. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Bravo, 1998).

Flavonoid distribution in plants depends on the several factors including variation according to plant phyla/order/family and population variations within species (Harborne, 1986). The antioxidant property of flavonoids was the first mechanism of the action studied, particularly with regard to their protective effects against cardiovascular diseases. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Bravo, 1998) that are probably involved in several diseases.

Harborne and Williams (2000) suggested that additional benefit of flavonoids is their ability to stabilize membranes by decreasing membrane fluidity.

The flavonoids content was quantified based on the linear equation obtained from quercetin standard calibration curve. Thus, flavonoid values were expressed as quercetin equivalent (ug QE/ml). The amount of flavonoid on methanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 1.92±0.07 ug/ml, 2.14±0.04 ug/ml and 2.74±0.01 ug/ml, respectively. The amount of flavonoid on ethanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 1.41±0.09 ug/ml, 2.02±0.03 ug/ml and 2.60±0.01 ug/ml, respectively (Fig. 2). Results of ANOVA analysis indicated that there was significant difference ($p<0.05$) between methanolic extract *of Dyospirus khaki* baverage and from ethanolic extract of *Dyopsirus khaki* baverage.

DPPH radical scavenging activity of *Dyospirus khaki* baverage: DPPH is a free radical which is stable and consists of nitrogen centered in its chemical structure. The reducing purple color 2,2-diphenyl-1-picrylhydrazyl

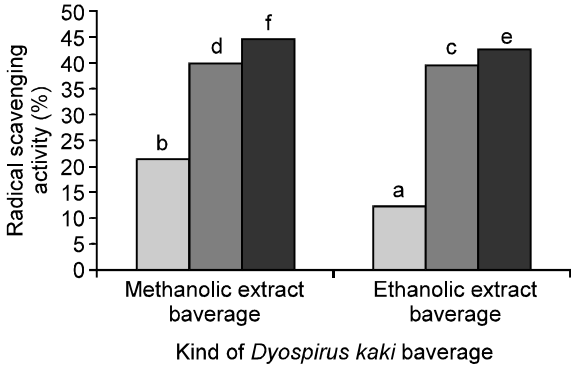

Fig. 3: Radical scavenging activity of methanolic and ethanolic extract of *Dyospirus khaki* baverage on variation formulation. Data are presented as mean from three independent experiments. Means with different letters within the same sample are significantly different at level of $p<0.05$

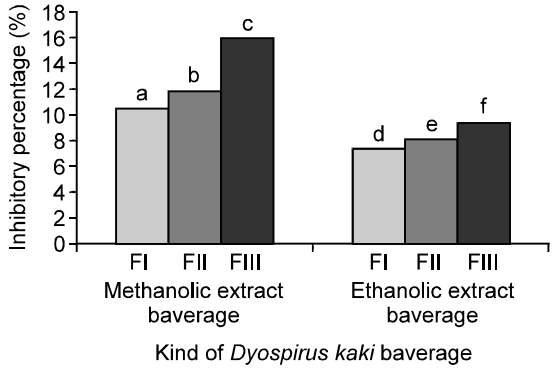

Fig. 4: Inhibitory percentage of ß-karoten bleaching on methanolic and ethanolic extract of *Dyospirus khaki* baverage. Data are presented as mean from three independent experiments. Means with different letters within the same sample are significantly different at level of $p<0.05$

(DPPH) to pale yellow *hydrazine* occurs due to reduction process by antioxidant whether in term of hydrogen or electron donation (Pokorny *et al*., 2001). The substances that are able to act as donor to DPPH free radical was identified as an antioxidant and free radical scavenger. DPPH free radical scavenging activity has been reported to show high correlation with inhibition capacity towards lipid peroxidation process (Rekka and Kourounakis, 1991).

Radical scavenging activity of methanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 21.57±0.29%, 39.97±0.15% and 44,63±0.18%, respectively. Radical scavenging activity of ethanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 12.35±0.25%, 39.56±0.09% and 42.55±0.15%, respectively (Fig. 3).

Evaluation of antioxidant using DPPH method proves that methanolic and ethanolic extracts of *Dyospirus khaki* baverage were dose dependent manner. Increasing the concentration of hydroxyl groups will increase hydroxyl groups. It impacts the ability of scavenging free radicals DPPH as the ability to donate hydrogen atoms greater (Manthey, 2004).

There were significant differences between these values at $p<0.05$. The antioxidant activity of methanolic extract of *Dyopsirus khaki* baverage on all formula higher than ethanolic extract of *Dyopsirus khaki* baverage. The methanolic extract *Dyospirus khaki* baverage has higher DPPH radical scavenging activity than the ethanolic extract one. The DPPH radical scavenging activity of *Dyospirus khaki* baverage nearly coincided with the result of TPC. The percentage of radical scavenging activity (% RSA), which suggests that the ability of baverage from methanolic extract is greater in scavenging free radicals than the ethanolic extract. This is presumably related to differences in content of phenolic compounds and flavonoids in methanol extracts (Fig. 1 and 2).

β-carotene bleaching activity: In the b-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50°C. The presence of antioxidants in the extract will minimize the oxidation of b-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by the antioxidants from the extracts. Thus, the degradation rate of carotene depends on the antioxidant activity of the extracts. There was a correlation between degradation rate and the bleaching of b-carotene; where the extract with the lowest b-carotene degradation rate exhibited the highest antioxidant activity.

The averaged values of inhibition β-karoten bleaching for methanolic extract of *Dyopsirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 10.56±0.16%, 11.877±0.17% and 15.99±0.20%, respectively. Inhibition β-karoten bleaching activity of ethanolic extract of *Dyospirus khaki* baverage at formulation I (750 ug/ml), formulation II (1500 ug/ml) and formulation III (3000 ug/ml) were 7.39±0.16%, 8.18±0.65% and 9.44±0.06%, respectively (Fig. 2). ANOVA test showed significant differences exist between these samples values at $p<0.05$. The antioxidant activity of methanolic extract of *Dyospirus khaki* baverage was higher than ethanolic extract. The mechanism of β-carotene bleaching method involved activity of linoleic acid free radical on unsaturated β-carotene until the β-carotene became oxidized and split into a few parts that resulted in the loss of chromophore (orange color) that could be

detected by spectrophotometer. However, this mechanism can be inhibited in the presence of antioxidants (Abdille *et al*., 2005) which inhibit the bleaching of β-carotene via neutralization of linoleic acid free radical and other free radicals (Jayaprakasha *et al*., 2001).

By considering the results of phytochemical screening and total phenolics and flavonoids content, the activity of the methanol extract would be mostly attributed to these compounds. The key role of phenolic compounds as antioxidant and scavengers of free radicals is emphasized in several reports (Theriault *et al*., 2006). Based on Figure 2 show that methanol and ethanol extracts of persimmon fruit baverage have the ability as an antioxidant with beta-carotene bleaching method, though not strong. Nevertheless, this study proves that methanol and ethanol extracts have the ability to inhibit bleaching of beta carotene which is a non-polar system. Based on this information, suggests that the bioactive compounds contained in the functional beverage can prevent the oxidation processes in biological systems tend to be lipophilic. This interesting phenomenon formulated as the "polar paradox" has been reported earlier (Frankel *et al*., 1994; Koleva *et al*., 2002). The polar antioxidants remaining in the aqueous phase of the emulsion are more diluted in lipid phase and are thus less effective in protecting the linoleic acid. On the other hand if polar compounds (ascorbic acid, rosmarinic acid, caffeic acid etc.) are tested only by the bleaching beta-carotene method they would be considered as weak antioxidants. However, the strong antioxidant activity of these compounds can be proven by other testing methods (Koleva *et al*., 2002).

Methanolic extract or ethanolic extract of *Dyospirus khaki* baverage or ethanolic extract have antioxidant activity to be related to the various antioxidants, including vitamins, phenolic compounds and carotenoids. Another reason for this antioxidant activity was this baverage added citric acid that can act as antioxidant. Wahyudi (2006) proved that the addition of citric acid in curcumin can increase the antioxidant capacity compared with only curcumin alone. The same thing is also explained by Pujimulyani (2006) that white turmeric blanching syrup with citric acid for five minutes to have high antioxidant activity.

Gill *et al*. (2000) proved that there is a significant action by hydroxy acids, particularly citric acid on the antioxidant activity of pomegranate juice. There is a synergy between the sugars, hydroxy acids (citric acid) and polyphenols in capturing the hydroxyl radical (Falchi *et al*., 2006). Roberto (2010) explains that the sugar and the hydroxy acid (citric acid) have the ability to capture a significant hydroxyl radical. This is proven by using the Electronic paramagnetic resonance measurements.

Dyospirus khaki L was source of pectin. Recent studies indicated, pectin can interacts directly with oxidants and free radicals. It has been suggested pectin extracted from Chickpea (CAP) that pectin interacts directly with oxidants and free radicals (Khasina *et al*., 2003). The antioxidant activity in CAP could be related to the high galacturonic acid content. It has been reported that a relatively low molecular weight and a high uranic acid content in polysaccharides appeared to increase the antioxidant activity. However, the mechanism of free-radical scavenging of polysaccharides is still not fully understood (Chen *et al*., 2004). The scavenging activity of CAP on DPPH radicals is related to the polysaccharide concentration.

Correlation between total phenolic, flavonoid content and antioxidant assays: There were high correlations between total phenolic content and all antioxidant activity assays using Pearson correlation. β-carotene bleaching activity and scavenging activity of methanolic extract of *Dyospirus khaki* baverage showed high correlation with total phenolic and flavonoid content. Correlation between phenolic content to radical scavenging activity of methanolic extract of *Dyospirus khaki* baverage were 0.907 and 0.918, respectively (Table 1). Correlation between flavonoid content to radical scavenging activity of methanolic extract of *Dyospirus khaki* baverage were 0.978 and 0.970, respectively (Table 1). Correlation between phenolic content to beta-carotene bleaching activity of methanolic extract of *Dyospirus khaki* baverage were 0.981 and 0.963, respectively (Table 2). Correlation between flavonoid content to beta-carotene bleaching activity of ethanolic extract of *Dyospirus khaki* baverage were 0.972 and 0.959, respectively (Table 2).

Table 1: Correlation between phenol and flavonoid content to antioxidant activity of methanolic and ethanolic of *Dyospirus khaki* baverage based on DPPH method

Kind of bioactive compound	Antioxidant activity of methanolic extract of *Dyospirus khaki* baverage	Antioxidant activity of ethanolic extract of *Dyospirus khaki* baverage
Phenol	0.967**	0.918**
Flavonoid	0.978**	0.970**

Means with * were significantly different at level of p<0.05. **Were significantly different at level of p<0.01

Table 2: Correlation between phenol and flavonoid content to antioxidant activity of methanolic and ethanolic extract of *Dyospirus khaki* baverage based on β-karoten bleaching method

Kind of bioactive compound	Antioxidant activity of methanolic extract of *Dyospirus khaki* baverage	Antioxidant activity of ethanolic extract of *Dyospirus khaki* baverage
Phenol	0.981**	0.963**
Flavonoid	0.972**	0.959**

Means with * were significantly different at level of p<0.05. **Were significantly different at level of p<0.01

The antioxidant activity of methanol and ethanol extracts of *Dyospirus khaki* baverage based on DPPH and bleaching β-karoten related to levels of bioactive compounds contained therein, such as phenols and flavonoids. Several studies (Shan *et al*., 2005; Wu *et al*., 2006; Wong *et al*., 2006) reported that phenolic compounds in spices and herbs significantly contributed to their antioxidant properties.

The relationship between the content of total phenolics and the radical scavenging activity of the baverage from *Dyospirus khaki* was investigated. The statistical analysis showed a positive and highly significant relationship between the content of total phenolics and radical scavenging activity against DPPH radicals and bleaching of β-karoten. Although many other natural compounds, including carotenoids, vitamin E and vitamin C, may also contribute to the radical, the present results suggest that the total phenolics are mainly responsible for the observed antioxidant activities.

Consistent with this research, previous experiments conducted by Kaur and Kapoor (2002) showed that phenolic compounds might mainly contribute to the radical scavenging activity of these fruit and vegetable extracts. The radical scavenging activity determined by DPPH assays using discoloration of these radicals has been applied due to their reproducibility (Katsube *et al*., 2004; Kondo *et al*., 2004). According to our data, the correlation coefficient between the Folin-Ciocalteu assay and the DPPH radical scavenging assay is high. These results correspond with the data of Katsube *et al*. (2004), who reported that the correlation between DPPH radical scavenging activity and total phenol content as estimated by the Folin-Ciocalteu method was significant and varied from 0.70 to 0.90. Norhaiza *et al*. (2009) explained that flavonoid of *Labinisia pumila* from Malaysia have high correlation with DPPH activity. Lelono *et al*. (2009) reported that flavonoid from Eugenia polyantha Wigh grown in Indonesia have correlation to beta-carotene bleaching activity.

Conclusion: The result from this study showed the level of natural antioxidant on methanolic and ethanolic extract of *Dyopsirus khaki* baverage. Methanolic extract of *Dyospirus khaki* baverage showed higher phenol, flavonoid and antioxidant activities based on DPPH and bleaching β-karoten than ethanolic extract of *Dyospirus khaki* baverage. Antioxidant activity of methanolic extract of *Dyospirus khaki* and ethanolic extract of *Dyospirus khaki* baverage in a dose-dependent manner. This research proves that *Dyopsirus khaki* baverage potential as source of natural antioxidant.

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