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Antioxidant Potential of Flesh, Seed and Mace of Nutmeg (*Myristica fragrans* Houtt)

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Abstract: Antioxidant methanol extract potential of flesh, seed and mace of nutmeg (*Myristica fragrans* Houtt) were evaluated with methods of 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), ferrous ion chelating activity and antioxidant activity assay in a linoleic acid system with ferrothiocyanate reagent (FTC). Total phenol and phytochemical of the extract were also evaluated. Seed extract has the most powerful scavenging ability on free radicals based on test of DPPH and FRAP, i.e. 154,55 (IC_{50}=µg/ml) and 82,33 (mg GAE/g extract) respectively. DPPH and FRAP value of mace extract is 201,97 and 56,31 respectively, while in flesh are 1372,91 and 13,45 respectively. Total phenol of mace, seed and flesh extract are 4.630,62, 2.434,90 and 388,36 mg GAE/g D.W. Total phenol correlated to DPPH (r=0,87) and FRAP (0,63). Flesh, seed and mace extract well inhibit the linoleic peroxidation. Linoleic peroxidation on up to 8 days incubation – expressed as absorbance in control 1.4450 – in flesh is 0.0337; seed 0.0330; and mace 0.0310. Ferrous ion chelating activity orders are: flesh extracts > seed extracts > mace extracts. Tannin, flavonoid and terpenoid were also found in seed and mace extract, whereas flesh extract contain flavonoid and terpenoid.
Key words: antioxidant activity, free radical, methanol extract, nutmeg, scavenging.

Introduction

Indonesia has known well because it's various spices. Nutmeg (*Myristica fragrans* Houtt) is one of Indonesian popular spices. Average world nutmeg production is 10.000-12.000 ton/year. Indonesia and Granada dominate the production for 75% and 20% respectively [1]. North Sulawesi is one of the largest nutmeg producers in Indonesia [2]. North Sulawesi nutmeg especially that derived from Siauw and Sangihe, known well in East Indian Nutmeg trading, produce highly aromatic nutmeg [3]. Flesh, nutmeg and mace are three distinct parts of nutmeg. Seed of nutmeg is the inside nut, surrounded by a layer of hard shell and turns black when ripe. This nut seed is surrounded by a mace. Both seed and mace is the main product of *M. fragrans* which known as it's spice. Flesh is outer layer that covers mace and seed. During the harvesting time, farmer of North Sulawesi only takes seed and mace part, and discard the flesh. It means that only a small portion of nutmeg is used.

A number of spices and herbs contain antioxidants chemical compounds. Such compounds include: vitamins, carotenoids, terpenoids, alkaloids, flavonoids, lignin, simple phenols, phenolic acids and etc [4,5,6]. Likewise, nutmeg as a spice plants and herbs has antioxidant properties [7,8,9]. Antioxidants are necessary for body, serves to inhibit the damage caused by reactive oxygen species (ROS) on membrane lipid, DNA and

protein [10]. ROS comprise hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and free radicals: hydroxyl (•OH) and peroxyl (ROO•), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$) – unstable and highly reactive molecules. ROS damage cells by chain reactions like lipid peroxidation that contribute to chronic disease development, such as cancer, heart disease and cerebrovascular [11,12,13].

Mace and seed of nutmeg used in medicine and food, and used as a standard food seasoning in Netherlands. In Manado (North Sulawesi), seed and mace of nutmeg are also used as a seasoning in some foods Manado, but it is also used in some types of cookies. Nutmeg flesh made into sweets and syrup, while flesh of young harvested (6-7 months) nutmeg distilled into essential oils.

Nutmeg use in medicine is as stimulant and carminant. Nutmeg is used for carminative, stomachic, astringent, deodorant, narcotic, aphrodisiac, flatulence, vomiting and nausea. Nutmeg oil is used for urinary and bladder inflammation treatment, halitosis, dyspepsia, flatulence, impotence, insomnia and skin diseases. Seed and mace of nutmeg contain active compounds of narcotic myristicin. Nutmeg butter contains elemicin and myristicin which are narcotic and psychotropic [3].

Given the extensive use of nutmeg in food, beverage and medicine, then we conduct research on the antioxidant potential of flesh, seed and mace of *Myristica fragrans*. Evaluation of antioxidant capacity was tested by DPPH, FRAP and chelating power and phenolic content to determine the content of phenolic compounds which are antioxidants. In addition, there is also a phytochemical test and application in inhibiting the peroxidation of linoleic. Research on antioxidant activity of nutmeg is still relative slight, especially potential comparison of flesh, seed and mace.

Materials and Methods

Samples

Nutmeg obtained from farmers in Karondoran, North Sulawesi, Indonesia. Part of flesh, seeds and mace are separated and oven-dried at a temperature of 45ºC, and then mashed with a coffee grinder. Each powder macerated 3 times with methanol with ratio of 1:4 for 20 hours. Methanol then evaporated with a rotary evaporator (R-3 Buchi) at 40ºC. Methanol extract of flesh, seed and mace were then tested for antioxidants activity, phytochemicals, total phenols and inhibition of linoleic peroxidation.

Reagents and standards

Methanol, etyl acetate, n-hexane, acetone, ethanol, Na_2CO_3, gallic acid, potassium phosphate, potassium ferricyanide, trichloro acetic acid, ferrous chloride, Iron (II) chloride tetrahydrate, ammonium thiocyanate, Si gel Merck kieselgel 60 F_{254} grade and purchased from Merck (Darmstadt, Germany). Folin Ciocalteu's, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferrozine, linoleic acid were purchased from Sigma Chemical Co. (Sigma-Aldrich Company Ltd, Great Britain).

DPPH Evaluation on Antioxidant activity

Evaluation of antioxidant activity were based on DPPH radical scavenging ability of methanol extract of nutmeg's flesh, seed and mace following Bandoneine *et al.* [14] procedure. A total of 0,1 ml of extract solution and 3,9 ml of DPPH solution (0,0025 g / 100 ml CH_3OH) inserted into cuvettes and homogenized. Absorbance was measured at a 515 nm wavelength using doeble-beam ultraviolet-visible spectrophotometer.

% Inhibition = 100 x $(A_o-A)/A_o$

A_o = absorbance on 515 nm wavelength of blank sample on t = 0 minute.

A = final sample absorbance on 515 nm wavelength.

FRAP Evaluation on Antioxidant activity

Evaluation of antioxidant activity by ferric ion reducing capability based on Chu *et al.* [15] procedure. 1 ml extract is taken with certain concentration mixed with 2,5 ml of 0,1 M potassium phosphate buffer in pH 6,6 and 2,5 ml of 1% potassium fericyanide. The mixture was incubated at 50°C for 20 minutes, then taken out 2.5

ml and add 2.5 ml of 10% trichloro acetic acid, 2,5 ml water and 0,5 ml of 0,1% $FeCl_3$. Incubation for 30 minutes to develop the color, absorbance read at 700 nm. FRAP values expressed in mg GAE / g of material.

Ferrous Ion Chelating Activity

Chelate ability of methanol extract of flesh, seeds and mace to ferrous ion by Dinis *et al.* [16] test. 1 ml extract with a particular concentration was mixed with 3,7 ml of methanol and 0.1 ml of 2 mM ferrous chloride, added with 0,2 ml of 5mM Ferrozine and fortexed. 10 minutes after the Ferrozine addition, absorbance is read at wavelength of 562 nm. The inhibition percentage of ferrozine-Fe^{2+} complexes calculated by formula:

% Inhibition = $[1\text{-}A_{sample} / A_{control}] \times 100\%$.

Antioxidant Activity Assay in Linoleic Acid System with FTC Reagent

Evaluation of antioxidant inhibits activity on linoleic peroxidation conducted with Osawa and Namaki procedure [17]. First, 4 mg of extract dissolved in 95% ethanol. Second, mix with 4,1 ml of 2,5% linoleic acid in 99,5% ethanol, 8 ml of 0,05 M phosphate buffer pH 7 and 3,9 ml distilled water, mixed homogeneous, store in dark and tightly closed container at 40ºC. Third, take 0,1 ml of this mixture, add 9,7 ml of 75% ethanol and 0,1 ml of 30% ammonium thiocyanate. After 3 minutes, add 0,1 mL of 0.02 M ferrous chloride in 3,5% hydrochloric acid, mixed homogeneous. Last, absorbance of the red color formed at 2 days intervals at 500 nm measured. BHT is used as a replacement for standard samples and control. Absorbance value indicates the occured level of linoleic peroxidation.

Determination of Total Phenolic Content

Total phenols content in methanol extract of flesh, seeds and nutmeg mace used procedure by Fang *et al.* [11]. Exactly 50 µl extract (80% ethanol extract), mixed with 5 ml of distilled water. Folin-Ciocalteu reagent was added (500 ml, 1M) and Na_2CO_3 (500 µl, 20%, w/v), mixed, left for 60 minutes at room temperature. Absorbance was measured with spectrophotometer at wavelength of 765 nm. Gallic acid standard curve made quantitatively. Total phenols expressed as mg gallic acid equivalents (GAE)/100 g dry weight, determined in triplicate.

Phytochemical Test

Phytochemical test in methanol extracts of flesh, seeds and mace guide were done using modified procedure by Harborne [18]. Types of analyzed phytochemicals are tannins, flavonoids, alkaloids, triterpenoids, steroids and saponins qualitatively. Analysis also estimated semiquantitatively based on extracts formed color density.

Tannin

A total of 2 g sample was extracted with 5 ml of methanol, filtered with cotton. Then it is moved to another tube and added 2-3 drops 1% $FeCl_3$. Positive samples contain phenolic if the color changes to green-black, purple, blue or solid black.

Flavonoid

2 g of sample extracted with 5 ml of methanol, filtered using cotton and transferred into three different tubes. For tube A, add 2 drops of concentrated HCl, shake vigorously, and add Mg powder. Flavonoids positive samples if there is intense froth and color changed into orange. For tube B, add 2 drops of H_2SO_4, shake vigorously. Flavonoids positive samples if there are a very striking color changes into yellow, red or brown. To tube C, add 10% NaOH 2 drops, shake vigorously. Flavonoid positive sample if there is a very striking color into yellow, red brown or green.

Alkaloid

Total of 2 g sample extracted with methanol, filtered with cotton and inserted into tubes A, B, C and D. Dragendorf reagent adds into tube A, positive alkaloids if there is a reddish sediment. Wagner reagent adds into Tube B, positive alkaloids if there is a brownish precipitate. Mayer reagent adds into tube C, positive alkaloids if there is a white precipitate. Hager reagent adds into Tube D, positive alkaloids if there is a yellow precipitate.

Triterpenoid dan Steroid

Total of 2 g sample was extracted with 5 ml of methanol, filtered by cotton. Then it dried heat and then extracted with chloroform and water (1:1). Next, 2 drops chloroform extract dripped on the plate and let it dry. 1 drop of concentrated sulfuric acid added; followed by 1 drop of acetic acid anhydride, observe the color change. Positive samples that contain terpenoids (triterpenoids) are if the color changes into red or brown. If formed color is blue, purple or green, samples contain steroids.

Saponin

A total of 2 g sample extracted with hot water, strongly shaken and left for 2 minutes. 2 drops of 2 N HCl was added, strongly shaken and observe whether the formed foam lasts for 10 minutes. Positive samples that contain saponins are if there is intense froth and consistent for 10 minutes.

Statistical Analysis

Obtained data (except water content, rendement and phytochemical data) were statistically analyzed using SPSS 16 software. We conducted Analysis of variance (ANOVA) and continued to analyze with the least significant difference test at $P \leq 0.05$ to determine the differences in each treatment. Water content and rendement data were analyzed using Microsoft Excel 2007 software.

Results and Discussion

Rendement Fruit Parts and Extract of *Myristica fragrans*

Moisture content of flesh, seeds and mace of nutmeg after oven-dried are: 5,33% ± 0,34, 0,11 ± 6,91% and 5,61% ± 0,77 respectively (results are expressed in means ± SD, n=3). The yield parts then weighed and counted respectively. Seed is the biggest fruit part of *M. fragrans*, yield of 8,85% ± 0,97 and mace has the lowest yield, 1,62% ± 0,06 while the flesh yield is 7,34% ± 0,20. Fruit parts that oven-dried, extracted with methanol to get the antioxidant compounds. Mace has the greatest extract yield, 30,92% ± 1,09, followed by flesh 23,04% ± 0.52 and seeds 18,23% ± 1,38.

Phytochemical

Result shows the seed and mace extract of nutmeg contain quite high tannins, flavonoids and terpenoids (based on intensity of formed color, which formed fairly strong green-black color for tannins, a fairly strong yellow color for flavonoids and fairly strong red brown for terpenoids). Nutmeg's flesh contains few flavonoids and terpenoids due to less intense formed color. Phytochemical test were conducted because these compounds contribute antioxidant activity of flesh, seed and mace extract of nutmeg.

Antioxidant Activity Based on DPPH Test

DPPH is a recent commonly used method. This method is simple, rapid and sensitive for antioxidant activity [19]. In addition, BHT and ascorbic acid also measured to compare antioxidant activity of methanol extract of nutmeg's flesh, seeds and mace. DPPH Measurement expressed as IC_{50}, i.e. amount of extract concentration (µg/ml) which scavenge DPPH radicals by 50%.

IC_{50} value of methanol extract of nutmeg's flesh, seed and mace are: 1372,91; 154,55; and 201,97 µg/ml respectively (Figure 1). Results on statistical tests ($P \leq 0.05$) show significant differences in seed extract, mace and flesh of nutmeg with BHT and ascorbic acid on DPPH radical scavenging. Seed extract has the highest scavenging ability to 50% DPPH radical, required 154,55 µg/ml extract concentration. Ascorbic acid and BHT as comparators has IC_{50} of 9,15 and 21,29 µg/ml respectively.

High antioxidant capacity of seed extract is due to the quite high presence of tannin, flavonoids and terpenoids compounds. These compounds contribute to extracted antioxidant to serve as electron donors as described by Buhler and Miranda [20]; Joshis *et al.* [21]; and Das *et al.* [22]. Flesh of nutmeg has the lowest antioxidant capacity, because there are only small amounts of flavonoids and terpenoids. The basic structure of flavonoids is the two hydroxyl groups attached to the benzene ring. Both hydroxyl group act as an electron donor group and may increase antioxidant activity [23].

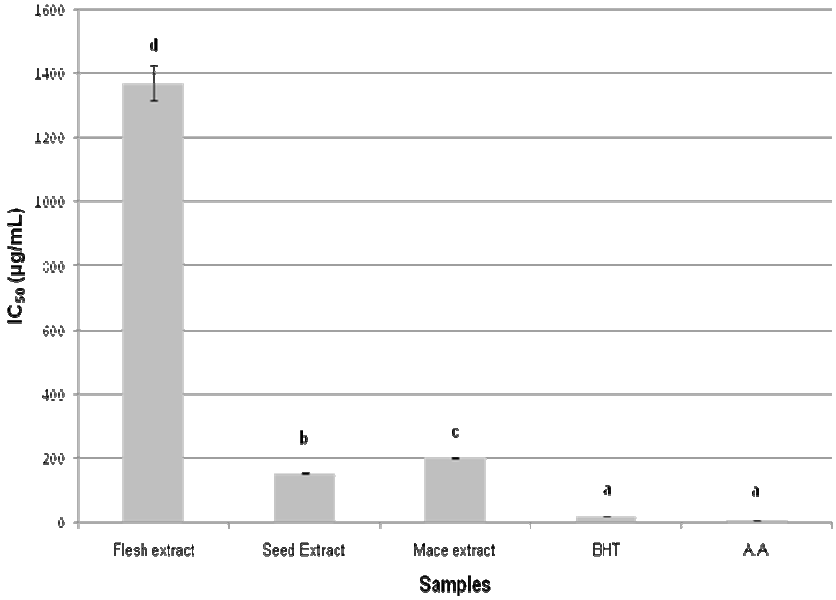

Figure 1. Antioxidant activity of the nutmeg's extract based on DPPH test (IC_{50})

Vertical lines stated standard deviation, the value of the same notation means that there are no significant differences ($P \leq 0.05$) between samples. BHT and AA (Ascorbic acid) is positive control.

Min *et al.* [24] isolated some pure compounds of nutmeg which active as anti-inflammatory, i.e. guaiacin. Guaiacin generally included in phenolic groups that having hydroxyl groups attached to the benzene ring and is active as an antioxidant. Its antioxidant role donates electrons (in the form of hydrogen radicals) to DPPH radical into non radical DPPH and unstable radical guaiacin. In the next process, guaiacin delocalized the electron to stabilize itself (Figure 2).

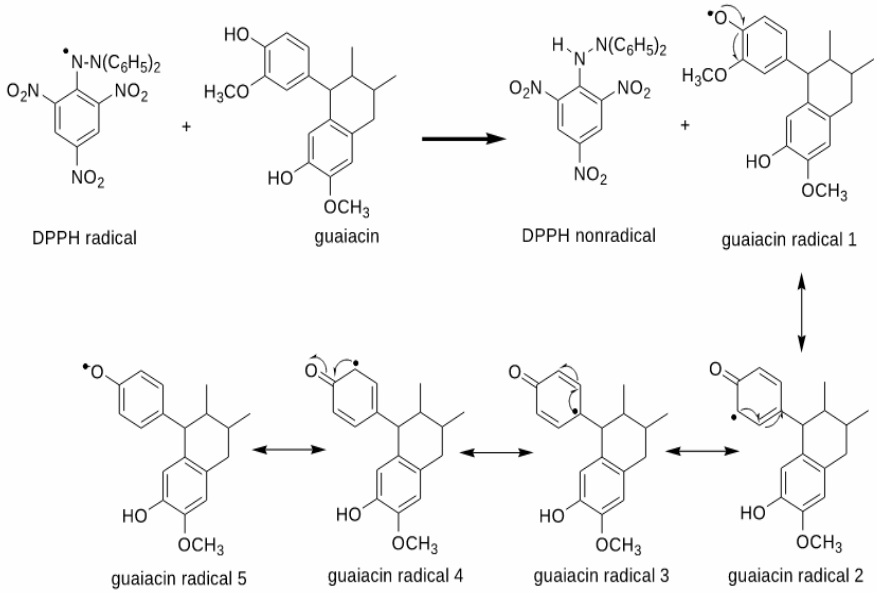

Figure 2. Guaiacin Mechanism on DPPH radical scavenging

Antioxidant Activity Based on FRAP Test

This antioxidant activity testing are based on the reduction of Ferric (III) to Ferrous (II) for receiving one electron [25]. Ferric into Ferrous reducing activity is being expressedin Gallat Acid Equivalent (mg GAE/g extract). Reduction power is associated with the potential standard of one-electron reduction. According to the Lee *et al.* [26], to act as an antioxidant – in lipid oxidation – reduction potential should 600 mV lower (which is PUFA potential reduction). For example, ascorbic acid and tocopherol has the standard reduction potential of 1-

electron 282 mV and 480 mV respectively [27]. Both used as PUFA and other oxidation inhibitors because it's low standard reduction potential.

Seed extract has the highest ability to reduce Ferric (82,33 mg GAE/g extract) compared to flesh and mace extract (Figure 3). This means that each gram of seed extract has ferric ion reducing ability equivalent to 82.33 mg of gallic acid. The reducing ability influenced by contained phenolic and terpenoids compounds. Seed extract contain rather high tannins, flavonoids and terpenoids, while flesh extracts contain less flavonoids and terpenoids, and low reduction power (FRAP = 13.45 mg GAE/g extract). Otherwise, reduction power of mace extract is 56.31 mg GAE/g extract. Comparison of the reducing ability to ferric ions from flesh, seeds and mace extracts of nutmeg described in Figure 3. The statistic test showed significant differences in each extract ($P \leq 0.05$) for the reduction of ferric ions.

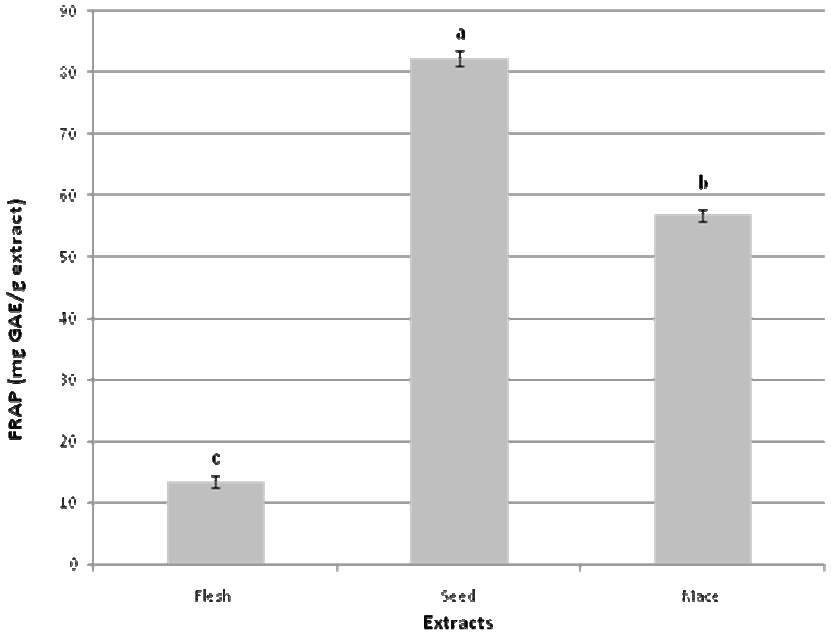

Figure 3. The antioxidant ability of extracts on reducing ferric

Vertical lines expressed the standard deviation. FRAP values with different notation means that there is a difference between the extract ($P \leq 0.05$) .

Ferrous Ion Chelating Activity

Antioxidant activity assay was carried out as the act of bivalent or polyvalent metals such as Fe, Cu and Mn on lipid oxidation. These metals increase lipid oxidation by acting as catalyst in free radical reaction [28]. In this test, formed complex between ferrozine-Fe^{2+}, chelating agent will bind the ferrous ion first before ferrozine-Fe^{2+} complex formed. Pure state of ferrozine-Fe^{2+} complex is when ferrous ion unchelated yet.

Antioxidants on Nutmeg's flesh extract have the highest chelating power, 52,65%±0.45. This means that these antioxidants 52.65% inhibit the formation of ferrozine-Fe^{2+} complex with a concentration of 10.000 µg/ml extract. The lowest chelating power is mace extract, i.e. 12,85%±2,33 in a concentration of 10^4 µg/mL. Chelating power of mace extract is the lowest, 12.85 ± 2.33%. Otherwise, seed extract had the chelating power of 38.48 ± 2.24%. EDTA is often used as a positive control to evaluate the FIC. EDTA chelating power is 68.89 ± 0, 64% at 100 mg/mL concentration. On the inhibition of lipid oxidation, antioxidants will chelate bivalent or polyvalent metals and prevent the metals action such as pro oxidant on the formation of free radicals in the initiation stage. Comparison of the ferrous ions chelating ability of nutmeg extract described in Figure 4.

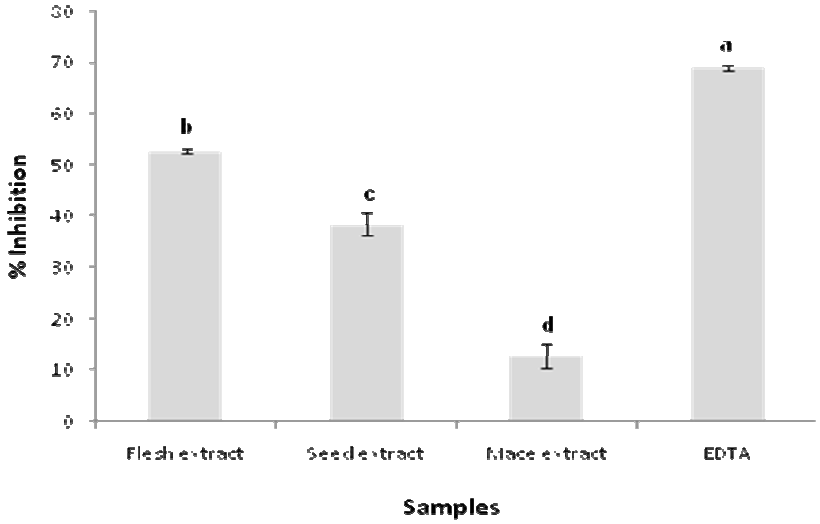

Figure 4. Ferrous ion chelating activity

Vertical lines expressed the standard deviation Values with different notation means that there are differences in the chelating ability ($P \leq 0.05$) .EDTA is a positive control.

Contents of Phenolic Compounds

Mace extract contain high phenolic compounds, i.e. 4630.62 mg GAE/100 g DW. It implied that every 100 g dry weight (dry mace powder) contains phenolic compounds of 4.630,62 mg Gallic Acid Equivalent. Mace extract obtained from the dried mace powder which was extracted with methanol, yield rendement of 30.92%. The content of phenolic compounds in seed extracts is 2.434,90 mg GAE/100 g DW, whereas the flesh extract contain low phenolic compound, i.e. 388.36 mg GAE/100g DW (Table 1).

Table 1. Total Phenol of Nutmeg's flesh, seed and mace extract

Sample	Total Phenol (mg GAE/g D.W)
Flesh Extract	$388,36 \pm 30,85^{c}$
Seed Extract	$2434,90 \pm 216,89^{b}$
Mace Extract	$4630,62 \pm 116,83^{a}$

D.W = *dry weight*.
Total phenol, average of 3 times replication ± standard deviation
LSD values with different letters indicate significant difference at $p \leq 0,05$.

Many studies focused on the correlation of antioxidant activity to phenolic compounds content. The results of Kumar *et al.* [13]; Yao *et al.* [29] and Hinnenburg *et al.* [30], stated a strong correlation between phenol and antioxidant activity. Kumar *et al.* [13] reported a very strong correlation (r = 0.937) between DPPH and total phenol contents in the extracts of *Kappaphycus alvarezii*. Yao *et al.* [29] explained moderate strength correlation (r = 0.701) in the 11 celeries cultivars extract. Evaluation on the antioxidant activity of flesh, seeds and mace of nutmeg showed a very strong correlation (r= 0.862) between the total phenols with DPPH radical scavenging capabilities. Increased content of phenolic compounds in the extracts also increased the DPPH radical scavenging ability.

The content of phenolic compounds in the nutmeg extracts of flesh, seeds and mace was correlated with FRAP. Increased content of phenolic compounds in the extracts also increased the ferrous ions reducing ability. The presence of FRAP correlation with the content of phenolic compounds was also based on the results of Hinnenburg *et al.* [30]. Phenolic compounds and FRAP were closely correlated (r= 0.887) in extracts of basil, laurel, parsley, juniper, aniseed, fennel, cumin, cardamom and ginger.

The content of phenolic compounds in nutmeg extract has the opposite correlation with chelating power. There is a negative correlation (r = -0.984) between the total phenolic extract of nutmeg to the chelating ability on ferrous ions. Flesh extract of nutmeg have much smaller total phenols than mace and seed extract, which has a stronger chelating ability.

Inhibition of Linoleic Acid Peroxidation

Linoleic is fatty acids with 18 C atoms with 2 double bonds. This fatty acid is often used to describe lipid peroxidation. Peroxidation occurred since incubation day 0, although in a low level (Table 2). During sample preparation, it has been in contact with oxygen as the initial oxidation of linoleic acid.

Table 2. Effect of nutmeg extract on linoleic acid peroxidation, 40^{o}C incubation

Sample	Absorbance				
	Day-0	Day-2	Day-4	Day-6	Day-8
Control	0,0057 ± 0,0006^{a}	1,2323 ± 0,0176^{a}	1,3003 ± 0,0121^{a}	1,3773 ± 0,0110^{a}	1,4450 ± 0,0191^{a}
Flesh	0,0033 ± 0,0115^{ab}	0,0103 ± 0,0153^{b}	0,0153 ± 0,0115^{b}	0,0260 ± 0,0017^{b}	0,0337 ± 0,0029^{b}
Seed	0,0023 ± 0,0006^{b}	0,0090 ± 0,0100^{b}	0,0163 ± 0,0058^{b}	0,0203 ± 0,0025^{b}	0,0330 ± 0,0044^{b}
Mace	0,0020 ± 0,001^{b}	0,0060 ± 0,0200^{b}	0,0117 ± 0,0015^{b}	0,0150 ± 0,0020^{b}	0,0310 ± 0,0056^{b}
BHT	0,0017 ± 0,0006^{b}	0,0053 ± 0,0115^{b}	0,0113 ± 0,0032^{b}	0,0147 ± 0,0025^{b}	0,0283 ± 0,0015^{b}

Data are average of 3 times replication ± standard deviation.
LSD values with different letters indicate significant difference at p≤0,05.

Day-2 of incubation, occur a high oxidation increase on control with absorbance value of 1.2323 compared to Day-0 of 0.0057. The addition of flesh, seed and mace extract of Day-2 inhibit linoleic peroxidation with absorbance of 0.0103; 0.009 and 0.006 respectively. Peroxidation increased steadily until Day-8, especially in control, while increase of extract and BHT is low. This means each extract contains effective antioxidants that inhibit linoleic peroxidation with similar capabilities to the BHT.

Well antioxidants had a significant potential difference to the oxidizing agent. For example, standard reduction potential of 282 mv ascorbic acid and peroxyl radical ($ROO^{\bullet}$), should have 1000 mv oxidizing agent and 1600 mv alkoxy ($RO^{\bullet}$) [27]. Ascorbic acid easily releases electrons into peroxyl and alkoxy radicals into non-radical products. Standard reduction potential of ascorbic acid is low, making it an effective antioxidant that inhibits oxidation.

Antioxidant in flesh, seeds and mace extracts, has strong scavenging capability of free radicals on linoleic peroxidation. Peroxyl and alkoxy radicals are have major influence on propagation stage of linoleic peroxidation. Based on inhibition data of linoleic peroxidation, concluded that antioxidant in flesh, seeds and mace extracts has quite large difference of reduction potential with formed radical species during the linoleic peroxidation.

Methanol extract of nutmeg's seed has the best ability to scavenge free radicals showed by DPPH and FRAP tests, while the flesh extract has the best ability in ferrous ion chelating activity. Flesh, seeds and mace extracts are very effective in inhibiting linoleic peroxidation and have relative similar ability to BHT. The three parts, especially nutmeg's seed and mace has the potential to be a source of natural antioxidants.

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