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Antioxidant Activity and Stability of Radish Bulbs (*Raphanus sativus* L.) Crude Extract

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Abstract. Radish (*Raphanus sativus* L.) contain saponins, flavonoids, polyphenols, glycosides, essential oils, vitamin A, and vitamin C that potentially as antioxidants properties. Further studies of antioxidants properties of radish needs to be conducted in order to be applicable in food areas. The purpose of this study was to determine the best extraction conditions to extract radish (type of solvent and extraction time) and determine the stability of radish extract based on changes in pH and heating temperature. First of all, radishes were extracted with different polarity solvents (hexane [non-polar], ethyl acetate [semi-polar], and ethanol [polar] for 8, 16, and 24 hours. Ethyl acetate solvent and extraction time of 16 hours were determined to be the best extraction method based on sample yield (0.91%), antioxidant activity ($IC_{50}=127.96$ mg/l), total phenolic (37.37 mg GAE/g), and total flavonoids (5.74 mg QE/g); so it is used in the next stage of research to determine the antioxidant stability at pH (4, 5, 6, 7) with different heating temperature (70, 80, 90°C). Radish extract is unstable based on antioxidant activity, total phenolic, and total flavonoids; but with the heat treatment of 70°C at pH 4 was considered to be the best condition among other stability tests with the closest result of early radish extract condition (pH 4.58, room temperature ~25°C). Radish extract from the selected treatments are not toxic ($LC_{50}>1000$ ppm) which containing steroid compounds (β -sitosterol, β -sitostenone, campesterol), fatty acids (palmitic acid, linolenic acid), carboxylic acids (methyl esters, phthalic acid) and other hydrocarbons (acetic acid, squalene).

Keywords: antioxidant_activity, *Raphanus_sativus*_L., pH, stability, temperature

1. Introduction

Radish plant (*Raphanus sativus* L.) is potential as antibacterial, antiinflammation, and also as antioxidant because of its saponin, flavonoid, polyphenol, glycoside, essential oil, vitamin A, and vitamin C content [11, 16, 14]. The presence of antioxidant is needed to prevent oxidation process and maintain the quality of food products from rancidity, discoloration, and other physical spoilage (Tamat *et al.*, 2007). Several researches done by Barillari *et al.* (2008), Hanindita (2011), and Ghasemzadeh *et al.* (2012), showed that radish extract had 10.5% (w/v) glucosinolate glucoraphasatin (GRH) activity and 154.5% anti radical power (ARP) by the presence of 0.098 flavonoid and 24.32 μ g/ml phenolic compound.



In this research, radish extract was obtained from maceration method using solvent with different polarity (hexane [non-polar], ethyl acetate [semi polar], and ethanol [polar]) for 8, 16, and 24 hours. Solvent with different polarity in maceration could cause different yield and bioactive compounds that are potential as natural antioxidant [9, 5, 13]. Eight, sixteen, and fourteen hours maceration time were based on Sathishkumar *et al.* (2008) research which showed that extraction time for 4 hours didn't increase flavonoid yield, while Butsat *et al.*, (2016) gained the best yield for total flavonoid and phenolic in 12 hours extraction time. The best solvent and extraction time were then used in the next research step that was the determination of extract stability based on the change in pH (4.0, 5.0, 6.0, and 7.0) and heating temperature (70, 80, and 90°C). Analysis done in determining the best solvent and extraction time were antioxidant activity analysis, total phenolic, total flavonoid, total yield, and correlation analysis between phenolic flavonoid and antioxidant activity. Chosen extract was then tested for its toxicity and antioxidant compounds contained by using Gas Chromatography-Mass Spectrometry (GC-MS). Antioxidant activity and stability test of radish extract in this research hopefully could become a database for the next radish extract research; so that this extract could be applied in oxidation inhibitor product.

2. Materials and methods

2.1 Material

Main material used was radish sized 30 cm. Analysis material used were: ethanol (p.a), ethyl acetate (p.a), hexane (p.a), DPPH (2,2-Diphenyl-1-Picrylhydrazyl), Folin-Ciocalteu, gallic acid, Na₂CO₃, AlCl₃, sodium acetate, quercetin, CH₃COOH, NaOH, Na₂HPO₄, H₃PO₄, aquades, shrimp larvae, DMSO, ascorbic acid, K₂SO₄, selenium, H₂SO₄ 96%, H₂O₂ 35%, boric acid 4%, mixed indicator, petroleum benzene, Zn, HCl 2N, concentrated HCl, Mg, FeCl₃, dragendrof, CH₃COOH anhydrous, concentrated H₂SO₄, Pb acetate 5%, and chloroform.

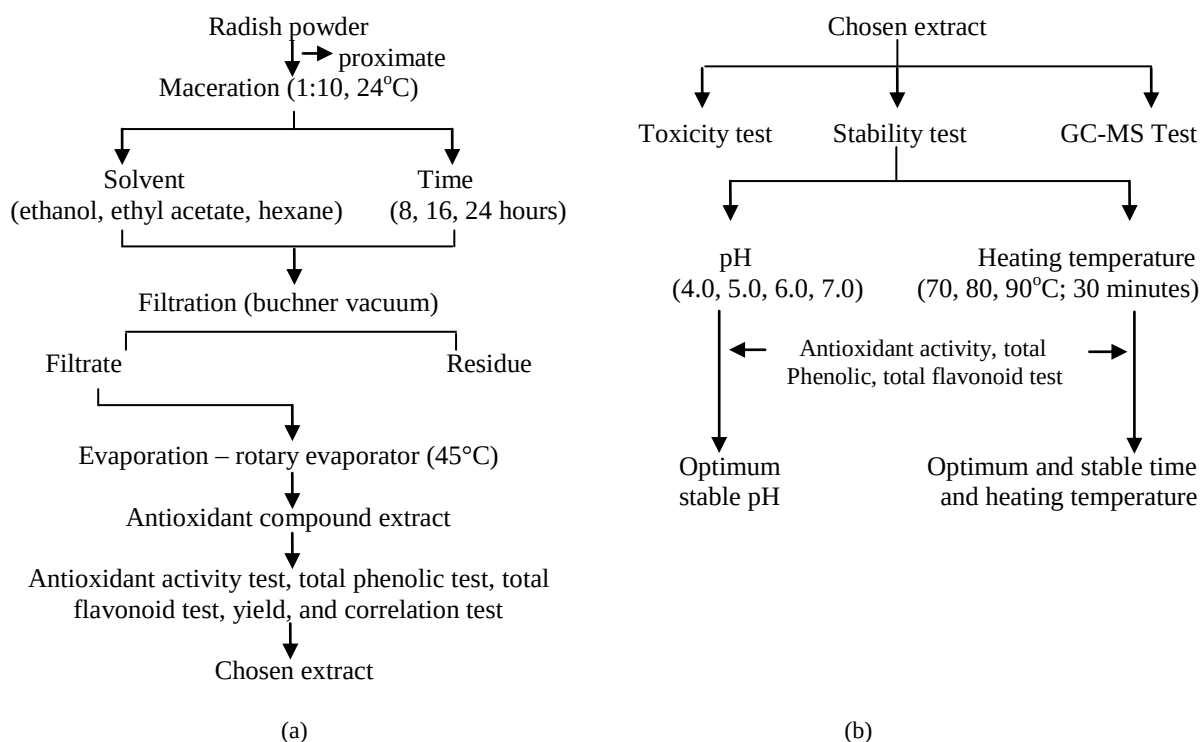


Figure 1. Research flowchart (a) step I; (b) step II

2.2 Methods

This research consisted of several steps, the production of radish powder, step I, and step II. The making of radish powder involved sorting, washing, cutting (~3 mm), drying (cabinet dryer; 60°C, 21 hours), size reduction (blender), and sifting (60 mesh). Step I research (Figure 1) started with maceration (1:10, room temperature [~24°C]) using three kinds of solvent (hexane, ethyl acetate, and ethanol) for 8, 16, and 24 hours. Filtrate filtration and evaporation (45°C) was done to produce antioxidant compound extract. The extract was then analyzed (antioxidant activity, total phenolic, total flavonoid, yield, and correlation of phenolic-flavonoid with antioxidant activity) to determine selected extract that was the result of determining the best solvent type and extraction time. Toxicity of selected extract was also analyzed as well as its antioxidant compound using GC-MS. In step II research (Figure 1) stability test against temperature (70, 80, and 90°C) and pH (4.0, 5.0, 6.0, and 7.0) was done in order to determine the extract stability based on antioxidant activity, total phenolic, and total flavonoid analysis.

2.3 Experimental Design

Experimental design in step I research was Completely Randomized Design with two factors. The first factor (solvent type) consisted of three levels (hexane [non-polar] A₁, ethyl acetate [semi polar] A₂, and ethanol [polar] A₃) with three repetition. The second factor (extraction time) consist of three levels (8 [B₁], 16 [B₂], and 24 hours [B₃]) with three repetition. Step II research used Completely Randomized Design with two factors that consisted of three levels (70 [A₁], 80 [A₂], and 90°C [A₃]).

3. Results

3.1 Step I

Step I research was done in order to determine the solvent type (hexane [non-polar], ethyl acetate [semi polar], and ethanol [polar]) and extraction time (8, 16, and 24 hours) that could give the best antioxidant activity from antioxidant activity, total phenolic, total flavonoid, yield, and correlation of phenolic-flavonoid with antioxidant activity analysis. Radish used in this research had been analyzed proximately with results of 12.56% water content; 11.43% ash content; 0.68% fat content; 11.11% protein content; and 64.23% carbohydrate content (by difference). The water content was intentionally dried until ~12% for extraction necessity, while according to Bangash *et al.* (2011), water content of fresh spring onion is 92.50%.

Table 1. Step I Test Result

Solvent Type	Extraction Time (Hours)	Yield (%)	Antioxidant Activity (mg/l)	Total Phenolic (mg GAE/g extract)	Total Flavonoid (mg QE/g extract)
Ethanol	8	14.32±1.82 ^b	68.93±295.96 ^c	7.33±0.10 ^b	0.80±0.01 ^c
	16	16.89±0.49 ^c	4064.1±991.23 ^b	7.93±0.01 ^b	0.93±0.01 ^c
	24	22.42±0.68 ^d	5337.22±447.50 ^b	8.14±0.21 ^b	0.75±0.02 ^b
Ethyl Acetate	8	0.67±0.04 ^a	1948.70±64.43 ^a	26.94±0.27 ^c	3.56±0.23 ^d
	16	0.91±0.07 ^a	1275.96±13.95 ^a	37.37±1.27 ^d	5.74±0.14 ^f
	24	1.24±0.09 ^a	1407.02±144.81 ^a	37.98±1.51 ^d	5.27±0.13 ^e
Hexane	8	0.65±0.05 ^a	29450.34±515.48 ^f	1.34±0.23 ^a	0.31±0.005 ^a
	16	0.93±0.09 ^a	27362.04±1480.05 ^e	1.86±0.06 ^a	0.35±0.02 ^a
	24	1.65±0.13 ^a	21918.3±1753.6 ^d	2.02±0.06 ^a	0.43±0.02 ^a

Note: - Different notation showed there was a significant difference (p<0.05)

- No comparison between parameter analysis

Yield value showed that the number of radish component dissolved in solvent stated in percentage. Its value was determined using extract weight and radish powder weight when extracted. The statistical test of extract yield showed that there was an interaction between solvent and extraction time ($p < 0.05$), solvent type and extraction time each had an influence toward extract yield ($p < 0.05$) table 1 showed that ethanol solvent (polar) gave the highest yield compared to other solvents. This result indicated that most of the extracted compound was polar compound [6, 13]. Table 1 also tell that 24 hours maceration gave the highest yield compared to 8 and 16 hours maceration time. The longer the extraction time, solvent penetration to the material would increase and bind component with the same polarity [32, 6].

Antioxidant activity value was determined based on the sample concentration needed to decrease 50% of free radical DPPH activity and stated as IC_{50} . The smaller IC_{50} value shows that antioxidant activity is better (Lim, 2014). The statistical test result of IC_{50} showed an interaction between the solvent and extraction time ($p < 0.05$), each solvent type and extraction time affect IC_{50} value. In Table 1 could be seen that ethyl acetate gave the highest antioxidant activity compared to ethanol and hexane. Huliselan *et al.* (2015) also experienced the same result in his experience that ethyl acetate was good solvent for high effective antioxidant compound. Ethyl acetate is a semi-polar compound that is able to dissolve bioactive component, therefore this solvent is more reactive in neutralize free radical. Table 1 also shows that the longer the extraction time, the higher the relative antioxidant activity. Contact between solvent and material will increase so that active component in the extract will increase until the saturation point of the solution, but overlong extraction using compatible solvent can also give negative effect for the extract. Maslukhah *et al.* (2016) added that overlong extraction time could trigger more oxygen exposure and increase the possibility of oxidation of antioxidant compound such as flavonoid.

Total phenolic count was done by using folin ciocalteu method that would change phenolic color from yellow to blue. The more concentrated the phenolic content in sample, the blue color would be more visible (Alasalvar *et al.*, 2011). The statistical test result showed an interaction between solvent type and extraction time that affect total phenolic ($p < 0.05$). Both solvent and extraction time also affect total phenolic ($p < 0.05$). Ethyl acetate gave higher total phenolic compared to other solvents. According to Widyawati *et al.* (2010), polarity difference of each solvent decides the extracted chemical structure of phenolic compound. Rafsanjani *et al.* (2015) and Putranti (2013) said that ethyl acetate was a polar solvent that was able to extract phenol, terpenoid, aglycone, and glycoside that are polar and semi-polar in characteristic easily. Table 1 also showed that the longer the extraction time, the more extracted phenolic compound gained. Jaya (2015) in his research that used 4-12 hours extraction time also gave similar result which showed that longer extraction time would increase total phenolic.

Total flavonoid was determined by aluminum chloride. At 415 nm wavelength, quercetin was used as standard in this test. The statistical test result showed that solvent type and extraction time interacted and affected total flavonoid ($p < 0.05$). Solvent type and extraction time separately also affect total flavonoid ($p < 0.05$). Table 1 showed that ethyl acetate extracted more flavonoid compared to ethanol and hexane. According to Fans (2015), flavonoid compound has a polar and non-polar characteristic so that it can easily be bound by ethyl acetate that has semi-polar characteristic. An extraction time of 16 hours tends to produce higher flavonoid compared to 8 and 24 h extraction time. Overlong extraction time can cause oxidation as the outcome of oxygen exposure during the extraction process.

Correlation analysis using Spearman method was used in order to know how strong phenolic and flavonoid compound contribute to radish extract antioxidant potential. According to Leblanc (2004), correlation is stated as correlation coefficient (r) which is categorized as: $r \leq 0.35$ = weak

correlation, $0.36 \leq r \leq 0.67$ = medium correlation, and $0.68 \leq r \leq 1.00$ = strong correlation. Test result showed that total phenolic against IC_{50} had r value of -0.50 and flavonoid against IC_{50} was -0.78. phenolic component in radish extract was normally contribute to antioxidant activity, while flavonoid gave more contribution to antioxidant activity. Fans (2015) and Maslukhah (2016) said that hydroxy group in phenolic and flavonoid provide ability to stabilize free radical, so both of them contribute in antioxidant activity. Negative symbol in correlation value r mean that the correlation between both variables (phenolic/flavonoid and IC_{50}) was inversely proportional. This result indicated that the higher the phenolic or flavonoid content, the smaller the IC_{50} value which means that antioxidant activity also increased.

Based on all of solvent and extraction time against several analysis parameters, it could be deduced that ethyl acetate was able to extract antioxidant compound more than alcohol and hexane. Extraction time of 16 h was declared as the best extraction time because it gave the highest total flavonoid even though total phenolic was not too different from 24 hours extraction with ethyl acetate as solvent.

3.2 Step II

Step II research was done to determine the stability of extract against change in heating temperature (70, 80, and 90°C) and pH (4.0, 5.0, 6.0, and 7.0) based on antioxidant activity, total phenolic, and total flavonoid analysis. In this step, toxicity test and compound identification test with GC-MS of selected extract from step I research were also done.

Statistical test showed that there was an interaction between change in pH and heating temperature against antioxidant activity ($p < 0.05$). Change in pH significantly ($p < 0.05$) affect antioxidant activity value, as well as change in heating temperature that affects antioxidant activity value ($p < 0.05$). Table 2 also showed that compared to control, radish extract was not stable against changes in pH and heating temperature. Radish extract that was close to control's IC_{50} was radish extract with a pH of 4 and temperature of 70°C. Ramdani *et al.* (2013) and Settharaksa *et al.* (2012) explained that heating temperature variance tends to cause antioxidant activity degradation, but there were specific pH and temperature that would produce high antioxidant activity. Fathinatullabibah *et al.* (2014) in her research found that pH 4 was the condition to generate higher antioxidant activity compared to pH 5-7. It happened because in low pH, the density of hydrogen ion raises so when antioxidant donates hydrogen ion to reduce free radical, antioxidant compound would be regenerated. High pH would cause hydrogen ion concentration in the media to decrease, and antioxidant would release hydrogen ion (without regeneration).

Table 2. Step II Test Result

pH	Temperature (°C)	Antioxidant Activity (mg/l)	Total Phenolic (mg GAE/g extract)	Total Flavonoid (mg QE/g extract)
Control 4.58	~25	770.78±99.91	37.02±0.34	2.04±0.05
4	70	1071.93±45.71 ^a	28.65±0.64 ^h	1.15±0.03 ^f
	80	1143.72±40.82 ^{ab}	24.76±0.50 ^f	1.08±0.03 ^{de}
	90	1318.12±99.02 ^{bc}	11.60±0.18 ^c	0.98±0.01 ^{bc}
5	70	1359.63±112.05 ^{bc}	27.18±0.45 ^g	1.55±0.19 ^f
	80	1391.82±19.14 ^c	25.29±0.74 ^f	1.04±0.04 ^{bcd}
	90	1314.54±3.81 ^{bc}	11.04±0.24 ^c	1.02±0.02 ^{bcd}
6	70	1912.42±138.17 ^e	25.03±0.41 ^f	1.16±0.02 ^e
	80	1666.12±103.04 ^d	22.58±0.23 ^e	1.02±0.03 ^{bcd}
	90	2420.67±27.71 ^f	9.87±0.18 ^b	0.95±0.02 ^{ab}
7	70	2052.05±232.39 ^e	23.11±0.52 ^e	1.11±0.02 ^{de}
	80	1968.18±233.41 ^e	19.54±0.54 ^d	0.92±0.03 ^{ab}

90	2484.18±104.86 ^f	6.40±0.14 ^a	0.87±0.01 ^a
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Note: - Different notation showed there was a significant difference ($p < 0.05$)

- No comparison between parameter analysis

Statistical test showed that there was an interaction between change in pH and heating temperature against total phenolic ($p < 0.05$). Both of the change in pH and heating temperature also affect total phenolic ($p < 0.05$). Table 2 showed that there was a decrease in total phenolic of control extract by the change of pH and heating temperature, so it can be deducted that radish extract was not stable against change in pH and heating temperature. Radish extract had higher phenolic content in acid pH. It was confirmed by Jaya (2015) that extract had higher phenolic content in acid treatment (pH 4-5) and going through reduction in neutral pH. The presence of temperature also causes total phenolic of the material to decrease (Table 2). According to Settharaksa (2013), the decrease of total phenolic could be caused by degradation of several phenolic components along the heating process.

Statistical test done toward total flavonoid indicated an interaction between the change in pH and heating temperature ($p < 0.05$). Both factors also affect total flavonoid of radish extract ($p < 0.05$). Table 2 showed a decrease in total flavonoid of radish extract when there was a change in pH and heating temperature. Acid pH tends to give higher flavonoid value compared to base pH. Decrease in flavonoid content could be caused by degradation of flavonoid component in base pH [13]. Rahmawati *et al.* (2013) research showed that heating temperature could damage flavonoid structure, so flavonoid content tends to decrease along with the increase of heating temperature. pH 4 and 5 in heating temperature of 70°C gave the smallest change in flavonoid content compared to control extract.

Antioxidant activity, total phenolic, and total flavonoid test result showed that radish extract was unstable against temperature and pH change, indicated by decreasing antioxidant activity, total phenolic, and total flavonoid of radish extract when exposed to heat and pH. Extract with pH 4 and heating temperature of 70°C gave the smallest decrease compared to control.

Toxicity value is stated as LC_{50} , that is the concentration when the death of shrimp larvae reaches 50% (Ramdhini, 2010). Radish extract toxicity test in this research was done in Laboratorium Pusat Studi Biofarmaka. The test result showed that LC_{50} value from chosen radish extract was >1000 ppm (non-toxic). According to Juniarti (2009), toxicity value of a material is classified into three: non-toxic ($LC_{50} > 1000$ ppm), light toxic (LC_{50} 30-1000 ppm), and toxic ($LC_{50} < 30$ ppm).

Chosen extract from step I research also analyzed by using GC-MS. Steroid compounds (β -sitosterol, campesterol), fatty acids (palmitic acid, linolenic acid), carboxylic acids (methyl esters, phthalic acid) and other hydrocarbons (acetic acid, squalene) were detected in majority of the extract. All of those compounds could act as antioxidant [31, 17, 20, 26, 22].

4. Conclusions

Ethyl acetate solvent (semi-polar) was chosen as the best solvent to extract radish. Extraction time that produced the best extract (the extract with the highest value of antioxidant activity, flavonoid, and phenolic) was 16 h. The best extract could produce 0.91% yield, antioxidant activity $IC_{50} = 127.96$ mg/l, total phenolic 37.37 mg GAE/g, and total flavonoids 5.74 mg QE/g. Radish extract was not stable against pH and heating temperature change. pH value of 4 at 70°C treatment produces the lowest decrease in antioxidant activity. Ethyl acetate solvent (semi-polar) was chosen as the best solvent to extract radish. The best extraction time didn't show any toxic compound ($LC_{50} > 1000$ ppm) with antioxidant steroid compound (β -sitosterol, campesterol), fatty acids (palmitic acid, linolenic acid), carboxylic acids (methyl esters, phthalic acid) and other hydrocarbons (acetic acid, squalene).

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