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Sequential Solvent Extraction of Red-Onion (*Allium cepa* L) Skin: Influence of Solvent Polarity on Antioxidant and Radical Scavenging Activity

S. Mahdian Kouchaksarayi [a], ***S.M. Vahdat*** [b *], ***M. Hejazi*** [c], ***M. Khavarpour*** [d], ***Z. Salimi*** [e]

[a] M. Sc of the Department of Chemistry, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran.
[b] Associate Professor of the Department of Chemistry, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran.
[c] Assistant Professor, Faculty of Chemistry, University of Kharazmi, Tehran, Iran.
[d] Assistant Professor of the Department of Chemical Engineering, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran.
[e] M. Sc of the Department of Chemical Engineering, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran.

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ABSTRACT: The present study was performed to evaluate the influence of solvent polarity on antioxidant and radical scavenging activity of the extracts of red onion skin. Extracts were obtained using sequential extraction method with various solvents namely ethyl acetate, n-butanol, methanol and water. Ethyl acetate and n-butanol extracts demonstrated the highest total phenolic (577.310±9.2 and 441.379±7.3 mg GAE/g extract, respectively) and flavonoids contents (36.188±5.1 and 33.844±4.6 mg QE/g extract, respectively), as well asthe highest antioxidant capacity compared to methanolic and waterextracts through all antioxidant assays.The results showed that all extracts exhibited antioxidant activity in a concentration-dependent manner. Significant correlation was obtained between antioxidant activities and total flavonoids.The highest DPPH and nitric oxide radical scavenging were acquired for n-butanol extract.Furthermore, ethyl acetate extract displayed the most reducing power.The results indicated that the components with antioxidant properties in onion skin were extracted in non-polar solvents more than polar solvents.

Keywords: *Antioxidant Activity, Red Onion Skin, Sequential Extraction, Solvent Polarity*.

Introduction

Excessive free radicals induce oxidative stresses in the body which potentially lead to damage in many kinds of biomolecules such as lipid, protein, DNA and RNA. Several degenerative diseases associated with oxidative damage are cardiovascular disease, diabetes, aging, cancer and hypertension (Mut-Salud *et al*., 2016). Meanwhile, lipid oxidation produced by accumulation free radicals results in shelf life reduction and food deterioration during heat treatment, storage and processing (Ye *et al*., 2013).

In order to inhibit the drawbacks of oxidative reactions, synthetic antioxidants are extensively used in the food products. Commonly, antioxidants are compounds which can scavenge free radicals and thus helping to inhibit the oxidation of lipids or other molecules. However, synthetic

* Corresponding Author: vahdat_mohammad@yahoo.com

antioxidants might be toxic, carcinogenic and inherently health harmful. To overcome the deficiencies associated with the synthetic antioxidants, growing attention has been paid to plant extracts as a source of safe, bioactive and natural antioxidants in recent years(Feng & Xu, 2014; Mtunzi *et al*., 2017; Sharifi *et al*., 2017).Large variety of plants has been demonstrated to contain phenolic and flavonoid compounds, thereby possessing antioxidant potential (Bharti*et al*., 2012; Boroomand *et al*., 2017).

Onion (*Allium cepa* L) has a long history of use in human diet as an ingredient and asource of health promotion compounds in the forms of cooked or fresh. Itis a natural medicinal remedy which exhibits the biological properties, such as antibacterial, anti-mutagenic, antioxidant, antiproliferative, anticancer, antiulcer, antispasmodic and antidiarrheal activities (Kim *et al*., 2017). It contains several phytonutrients as well as carotenoids, phenolics, copaenes, minerals, organosulfur compounds, terpenoids, amino acids, anthocyanins, vitamins and flavonols including mainly quercetin, kaempferol and tannins (Kwak *et al*., 2016). Many types of onion are existed which differ in color, flavor, flavonoids and phenolic compounds. The antioxidants and radical scavenging activities of onion are known to be attributed to the content of phenolic and flavonoid compounds (Jeong *et al*., 2009).

During onion cultivation and processing, onion skin waste is generated which contains significantly higher levels of polyphenols than the edible part (Jayaprakasha *et al*., 2008).Therefore, onion skin may be regarded as a safe, commercial and unique source of phenolic compounds that can be used for production of natural antioxidants. The produced natural antioxidants can be utilized not only for food nutritive but also for medicinal purposes.

Previous investigations demonstrated that red onion skin shows higher phenolics and flavonoids contents and therefore higher antioxidant activities as compared with white and yellow onions (Gorinstein *et al*., 2009). The recovery of antioxidant phytochemicals can be carried out by extraction. The extraction yield is influenced by solvent, time and temperature of extraction along with the chemical nature of the sample. Under the same process conditions, the selection of the solvent used is very vital (Li *et al*., 2017).

Some studies were conducted to investigate different solvent systems for extraction of phenolic and flavonoid compounds from onion skin and influence of solvent polarity on the antioxidant activities (Katsampa *et al*., 2015; Škerget *et al*., 2009). Skerget *et al.* (2009) used acetone, ethanol and mixtures of solvents with water to obtain crude extracts of red onion skin. They showed that antioxidant and radical scavenging activities of onion skin extracts were high. In addition, the amount of achieved phenolic compounds from onion skin was approximately 3 to 5 times higher as from the onion edible part. Katsampa *et al.* (2015) used mixture of water and glycerol for extraction of polyphenols from onion solid wastes. They indicated that the higher extraction yield was achieved by increasing the glycerol concentration due to the polarity of glycerol. Since, the polarity of solvent has influenced on total phenol and flavonoid contents and antioxidant activity, it is therefore of great interest to introduce properly the solvent which resulted to higher phytochemicals and antioxidant activity in the extract. The present study aimed at performing successive solvent extraction on the powdered-red onion skin using solvents of different polarity and compared the total phenol and total flavonoid contents and antioxidant potentials of various extracts obtained.

Materials and Methods

- *Materials*

Red onion (*Alliumcepa* L) was purchased from local market, Tabriz, Iran. Onion skins (the outer layer) were separated, cleaned and dried. They were ground to obtained powders for use (mesh 20). The HPLC-grade standard quercetin (> 95%) was acquired from Sigma-Aldrech, (USA). All organic solvents were regent grade and supplied by Scharlau, Spain. Ferrozine, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), and potassium ferricyanide were purchased from Sigma Chemical Co. (Germany). BHA, ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride and ferric chloride were provided by Merck, Chemical Company Germany.

- *Extraction procedure*

Extraction process was performed sequentially using solvents of different polarity, each time on sample residue obtained from previous extraction. Solvents used with respect of increasing polarity were: n-hexane, ethyl acetate, n- butanol, methanol and water. First, 150 g of powdered onion skin (mesh 20) was extracted by the percolation method using n-hexan (1:10 w/v) for 24 hrs at room temperature. The organic phase was then separated from the sample residue by filtration through Whatman No. 1 filter paper. This procedure was done triplicate to thoroughly extract the plant material. At the end, the extracts were combined and concen-trated under reduced pressure at 40°C using a rotary evaporator. In the second step, the remaining sample residue was consecutively extracted with ethyl acetate (1:10 w/v). The procedure was also repeated as being done by n-hexane. Sample residue extraction was continued with n-butanol, methanol and water successively to achieve extracts of various polarities. The extracts were concentrated separately on rotary evaporator (ethyl acetate and methanol at 45°C, n-butanol and water at 50°C under vacuum) at the end of each process. The extracts obtained were used to evaluate their phytochemical constituents and antioxidant potential. The extraction protocol is presented in Figure 1.

- *Determination of total phenolic contents*

Total phenolic contents of each extract were measured according to the Folin-Ciocalteau method, using gallic acid as a standard (Ebrahimzadeh *et al*., 2010).Briefly, 0.5 mL of each extracted samples (50 µg/mL)was mixed with 2.5 mL of 0.2 N Folin-Ciocalteau as an oxidizing agent. After a 5 min interval, 8 mL of 75 g/L sodium carbonate was added to the solutions. The solutions were allowed to stand for 2hrsat ambient temperature in dark place to stabilize the blue color. Then, the absorbance of reaction was measured at 760 nm by spectrophotometer (UV-Visible EZ201, Perkin Elmer, Norwalk, Connecticut). The blank involved of all reagents and solvents, but without the sample. The results were expressed as milligrams of gallic acid equivalents per g of extract (mg GAE/g extract).

- *Determination of total flavonoid contents*

Total flavonoid contents were assessed based on calorimetric method using aluminium chloride (Ebrahimzadeh *et al*., 2010).First, to 0.5 mL solution of each extract (100µg/mL), 1.5 mL of methanol, 0.1 mL of 10% w/v aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water were added. The solutions were then incubated at room temperature for 30 min. The absorbance of the reaction mixture was immediately read at 415 nm by ultraviolet visible spectrophotometer. Total flavonoid contents were quantified using standard curve of quercetin and expressed asmilligramsof quercetin equivalents per g of extract (mg QE/gextract).

- *Antioxidant assay*

The antioxidant capacity can be determined based on two major methods: hydrogen atom transfer and electron transfer. Antioxidant assay is usually monitored through a change in color as the oxidant is reduced or through the competition of antioxidant and the substrate for free radicals. Initially, a stock solution of each extraction (800 µg/mL) were prepared, then serial dilutions of the stock solutions carried out to achieve appropriate concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL for each assay.

- *DPPH radical scavenging assay*

The antioxidant activity of each extract was estimated on basis of the scavenging potential of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical according to method described by *Ebrahimzadeh et al*. with a few modifications (Ebrahimzadeh *et al*., 2010).Two milliliters of each diluted solutions with various concentrations of 6.25, 12.5, 25, 50 and100µg/mL were added to 2 mL freshly prepared DPPH solution (100 µM prepared with methanol), mixed thoroughly and incubated at ambient

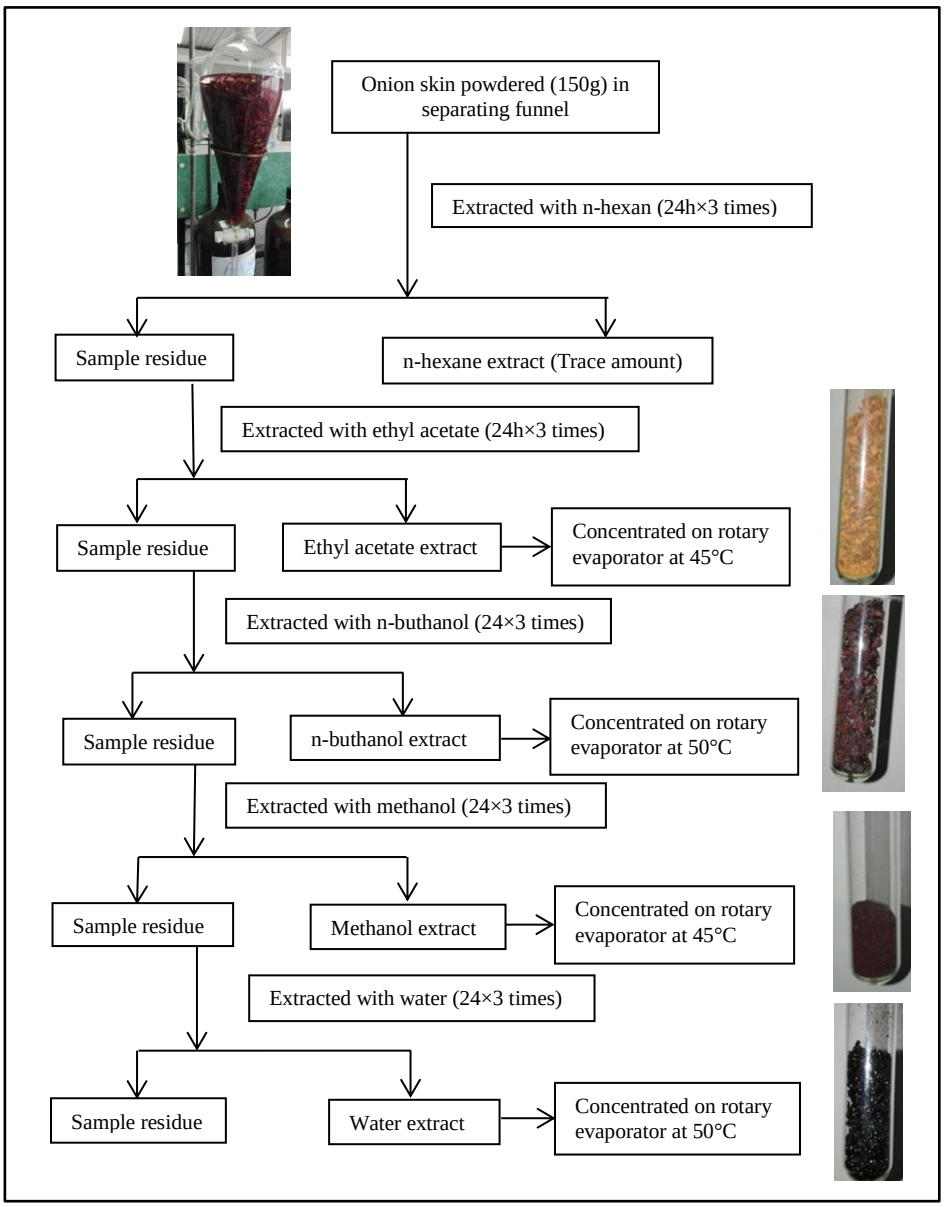

Fig. 1. Successive extraction protocol of onion skin

temperature under dark conditions for 30 min. The absorbance of the reaction mixtures was recorded at 517 nm. BHA was used as standard controls. The percentage of DPPH radical scavenging potential was calculated by the following equation:

$$\%\ DPPH\ radical\ scavenging\ activity = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (1)$$

where, $A_{blank}$is the absorbance of the control reaction (including all reagents except the extract sample) and $A_{sample}$is the absorbance of the solution while adding the extract sample. The IC_{50} value was determined from the plots as the effective concentration of extract required scavenging 50% of DPPH free radicals. All tests were run in duplicate and analyses of the samples were run in triplicate and averaged, as with the total antioxidant activity.

- *Reducing power assay*

The reducing power of each fractions and ascorbic acid were determined based on the method previously used by Ebrahimzadeh *et al*. (2010). Different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/mL) of each extract (1 mL) were combined with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% potassium hexacyanoferrate. Then, the reaction mixture was incubated at 50°C of water bath for 20 min and1 mL of trichloro acetic acid (10% w/v) was added to the mixture to terminate the reaction. After that, 1 mL of the upper portion of the solution was mixed with 1 mL of distilled water and 0.2 mL of 0.1% ferric chloride solution. The absorbance was measured at 700 nm against the blank (containing all reagents except the extract sample). A higher absorbance reflects a stronger potential antioxidant capacity. Ascorbic acid was used as a standard.

- *Nitric oxide scavenging assay*

The nitric oxide scavenging activity was assayed using sodium nitroprusside method as described by Ebrahimzadeh *et al*. (2010).Briefly, 1 mL of 10 mM sodium nitroprusside was added to 3 mL of each extract with different concentrations(100, 200, 400, 800 µg/mL), dissolved in water, and allowed to stand at ambient temperature for 150 min. Then, the solutions were combined with 0.5 mL of the Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) and shaken evenly. The absorbance was recorded at 546 nm, while quercetin was applied as a positive standard. The percentage of inhibition of nitric oxide production was expressedas below:

$$\%\ Nitric\ oxide\ scavenging\ activity = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (2)$$

- *Statistical analysis*

Extraction yield, total phenolic and total flavonoid contents and IC_{50} value were determined by linear regression analysis method. All of the experiments were carried out in triplicate order, and the results are offered as the means±standard deviations (SDs). The statistical analyses were conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed by one-way analysis of variance (ANOVA), and the significance of the differences between the mean values was determined by Duncan's test at a significance level of $P<0.05$.

Results and Discussion

The extraction process was carried out with the sequential solvent procedure using solvents of various polarities. The extraction yield was obtained for each extract. Non-considerable extract was achieved for non-polar phase of n-hexane. As shown in Table 1, methanolic extract was found to be the best extractant, achieving the highest

extraction yield of the plant component (9.3%), more than any other solvents.

- *Total phenolic and flavonoid contents of extracts*

Phenolic and flavonoid compounds demonstrate a significant role as antioxidant because of performing as free radical scavenging potential, hydrogen donor and reducing agent (Khan *et al*., 2016). The total phenolic and total flavonoid values are shown in Table 1. According to the results, ethyl acetate and n-butanol extracts had the highest total phenolic contents, specifically, 577.31 and 441.379 mg GAE/g extract, respectively. In total, 1218.688 mg GAE/g extract was achieved. In addition, ethyl acetate and n-butanol extract had the highest total flavonoid with the values of 36.188 and 33.844 mg QE/g extract, respectively. Thus, ethyl acetate and n-butanol, non-polar solvents, were identified as the best solvents for extracting the phenolic and flavonoid compounds from onion skin. On the other hand, water extract presented the lowest phenolic and flavonoid contents showing that onion skin contains components which have lower solubility in water phase. In general, the results depicted that the solvent with a lower polarity yielded higher phenolic and flavonoid contents. However, Skerget *et al*. (2009) reported that the best results regarding the amounts of isolated phenolic compounds and flavonoids were obtained when 35%acetone and 60% ethanol aqueous mixtures were used as solvents for extraction. The difference between our results and previous work could be due to genetic variations, environmental conditions, or extraction methods. In addition, although the solubility of phenols in different solvents can be based on their polarities; solubility is a complicated phenomenon which varies with the degree of conjugation of the aromatic rings, the stereochemistry of phenols and intermolecular forces between them and the solvent (Galanakis *et al*., 2013).Furthermore, high correlation between total phenol and total flavonoid contents were found (R=0.9475).

- *Antioxidant activity of extracts acquired by DPPH radical scavenging assay*

The antioxidant capacity of onion skin extracts was obtained using sequential solvent extraction as a function of their concentration through different mechanisms of action. DPPH is regarded as a stable free radical which by accepting an electron or hydrogen radical is commonly used for examination of the antioxidant potential of food and vegetables (Padhi *et al*., 2015).When an antioxidant encounters the DPPH solution, the color changes from violet to yellow is because of its scavenging action on free radical (Bratzadeh *et al*., 2013). The free radical DPPH has absorbance at 517 nm, but the absorption spectrum intensity decreases with the increase of antioxidants. A lower reaction mixture absorbance indicates a higher DPPH radical-scavenging activity.

Figure2 shows DPPH radical scavenging of various extracts obtained by sequential extraction of powdered onion skin. The DPPH radical scavenging activity increased significantly as the extract concentration was

Table 1. Comparative analysis of extraction yield, total phenolic and total flavonoids content of red-onion skin extract acquired using various solvents

Fraction	Extraction yield (%)	TPC (mg GAE/g extract)	TFC (mg QE/gextract)
Ethyl acetate	3.3 ± 0.4^{c}	577.310 ± 9.2^{a}	36.188 ± 5.1^{a}
n- butanol	3.4 ± 0.3^{c}	441.379 ± 7.3^{b}	33.844 ± 4.6^{b}
Methanol	9.3 ± 0.6^{a}	151.724 ± 4.5^{c}	17.906 ± 1.4^{c}
Water	5.2 ± 0.1^{b}	48.275 ± 2.1^{d}	6.187 ± 0.5^{d}

Values are mean ± standard deviation of three replicate analyses. Means with different letters (a–d) within the same column differed significantly ($P < 0.05$). TPC, total phenolic content; TFC, total flavonoids content.

increased from 6.25 to 100 µg/mL. It was found to be the highest for n-butanoland ethyl acetate extracts as compared to those obtained by methanolic and water extracts. In addition, IC_{50} values of n-butanol and ethyl acetate extracts achieved 14.122 and 17.269 µg/mL, respectively which demonstrated the marked antioxidant activity of red onion skin (Table 2). This finding is in accordance with research carried out by Skerget *et al.* (2009) which showed that the antioxidant and radical scavenging activities of red onion skin extracts were generally high, comparable to that of BHT. However, the lowest value of antioxidant activity was observed for water extract (IC_{50}=35.99 µg/mL). Furthermore, our results depicted that DPPH radical scavenging activity has high correlation with total flavonoid content (R=0.9475). Lower correlation was observed between this activity and total phenolic contents (R=0.8295). The differences between the DPPH radical scavenging capacities of the two sample scan be explained by solvents polarity which may be resulted to the differences in the components of phenols and flavonoids (Jayaprakasha *et al*., 2008).Thus, phenolic compounds, particularly flavonoids are the major constituents in onion skin which can scavenge the DPPH radical, due to the presence of the hydroxyl groups in their structure and their electron donating ability.

Our results were consistent with the outcomes reported by many research groups, confirming that extraction of phenolic compounds greatly depends on the solvent and its polarity and phenols and flavonoids are mainly responsible for the radical scavenging activity of extracts (Narkhede & Jagtap, 2015).The capacity of phenolic compounds to scavenge free radicals may be due to their possession of many phenolic hydroxyl groups; Therefore, they transfer hydrogen to radicals and produce phenoxide radical, which stabilizes products.

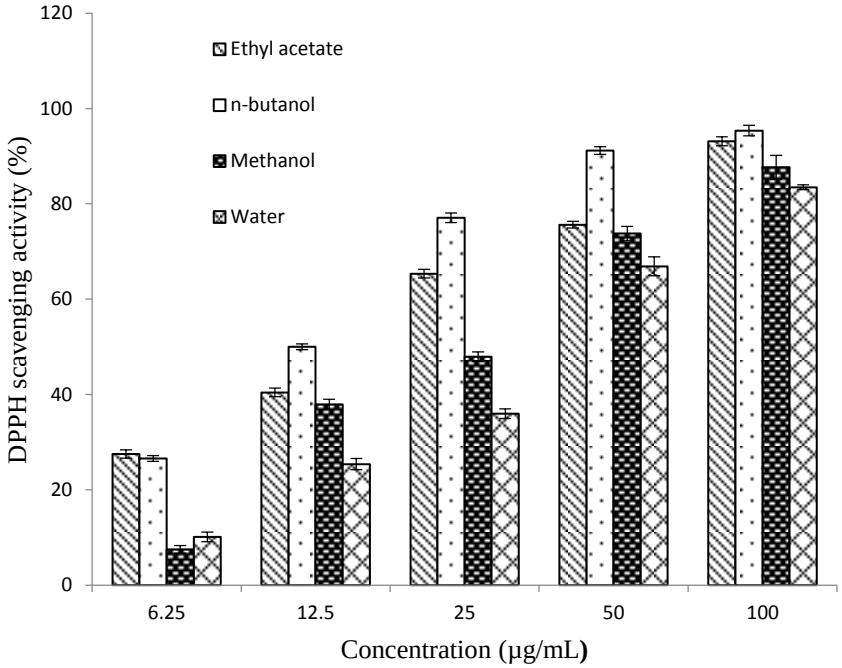

Fig. 2. DPPH radical scavenging activity of different extracts from onion skin versus various concentrations. The values represent mean ± S.D. of three independent experiments

The results revealed higher DPPH radical scavenging in the n-butanol extract as compared to the ethyl acetate extract suggesting that antioxidant action does not only depends on the concentration and polarity but however, depends on the structure and interaction between the enclosed antioxidants (Kannan*et al*., 2016). Furthermore, variation in trend of different extracts versus concentrations may be due to the capability of solvents to extract different types of compounds and propose the fact that different concentration of compounds in the extracts might affect the ability of donating hydrogen atom and reaction with free radicals (Hamid*et al*., 2016).

- *Antioxidant activity of extracts acquired by reducing power assay*

The reduction capacity of a compound may assist as a significant indicator of its potential antioxidant activity. Reducing power can be used as an antioxidant activity index of plants. In general, the presence of reducing agents plays a major role in regenerative property. These compounds exert their activity by donating an electron. Once the compound has these properties, it can reduce the amount of oxidized intermediate made during the lipid peroxidation process. Therefore, it will break the chain of reaction and can act as a primary and secondary antioxidant. In the reducing power assay, the reducing of Fe^{3+} to Fe^{2+} occurs by donating an electron in the presence of reductants in the samples. The amount of Fe^{2+} complex can be then examined by measuring the formation of Perl's Prussian blue at 700 nm. A higher absorbance value indicates a stronger reducing power and so higher antioxidant activity of the sample.

As shown in Figure 3, the reduction power of all the extracts increased when their concentration increased. The highest absorbance values were obtained for ethyl acetate and n-butanol extract (0.334 and 0.326, respectively) at a concentration of 100 µg/mL. Ethyl acetate and n-butanol extracts had also higher amount of phenolic and flavonoid content showing that the reducing power of all the extraction is highly related to the amount of phenolic and flavonoid compounds in the extracts, that can serve as electron donor to terminate the radical chain reaction (Liu*et al*., 2011).At lower doses, the reduction effect was not comparable with that of ascorbic acid which was used as positive control. However, in higher doses (25, 50 and 100 µg/mL), ethyl acetate and n-butanol extract showed better activity than ascorbic acid. In all concentration, water extract showed lower reduction power compared with the other extractions. The results also showed that the reducing power increased with concentration. The obtained results depicted a correlation with the amount of phenolic and flavonoid constituents and reducing capacity. This finding indicates that phenolic and flavonoid compounds directly contribute to the reducing power.

- *Nitrite oxide scavenging activity*

Nitric oxide is a free radical with a single electron which is produced physiologically from L-arginine under the effect of nitric oxide synthase (NOS) enzyme. It plays an important role in vital physiologic functions such as blood pressure regulation, smooth muscle relaxation of vessels and immune system. Many disorders such as stroke, headache, inflammation and disorders like Alzheimer's occur due to the function of this compound (Khalili & Ebrahimzadeh, 2015). In the nervous system, nitric oxide plays a role in neurotransmitter release, neuronal excitability, and learning and memory as amodulator (Donner *et al*., 1997).Overproduction of nitric oxide is known to be toxic. It may cause oxidative and nitrosative stresses resulted to DNA degradation, variation of protein performance and lipids peroxidation

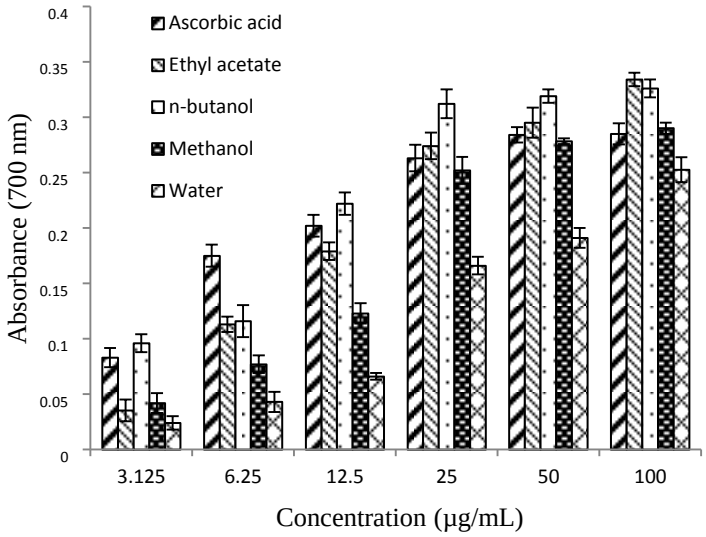

Fig.3. Reducing power of different extracts from onion skin versus various concentrations. The values represent mean ± S.D. of three independent experiments

(Ebrahimzadeh*et al*., 2015). Free radical nitric oxide rapidly reacts with free radical superoxide (O_2^-) and produces peroxynitrite ($ONOO^-$) which is an oxidant and nitrating agent. The half-life of peroxynitrite is very low and is about 1 second. However, this short time is enough for its reaction with biological molecules including DNA and proteins (Khalili & Ebrahimzadeh, 2015). In addition, low concentration of peroxynitrite can cause initiation of apoptosis and high concentration may result to necrosis. Therefore, the presence of antioxidants to scavenge nitric oxide and prevent the formation of reactive oxygen and nitrogen particles has a significant effect on avoiding the oxidative and nitrosative stresses. The most important of antioxidant agent which reduces the degradation effect of peroxynitrite is polyphenols, specifically flavonoids (Shan *et al*., 2005).

Commonly, sodium nitroprusside can be decomposed in an aqueous solution at physiological pH (7.2) and produce nitric oxide. Under aerobic conditions, nitric oxide reacts with oxygen to produce stable products such as nitrate and nitrite which can be quantified with Griess reagent using spectrophotometer at a wavelength of 546 nm. Nitric oxide Scavenging in competition with oxygen reduces the nitrite ions. Reduction in nitrite ions results to the lower color intensity in the solution; consequently, lower absorbance will be observed.

Figure 4 shows the percentage of nitric oxide radical scavenging using various solvents at concentrations of 100, 200, 400 and 800 µg/mL. The IC_{50} values are also depicted in Table 2. The results revealed that n-butanol and ethyl acetate showed the highest activity to inhibit nitric oxide production (IC_{50} of 178.476±7.07 and 188.684±3.506 µg/mL, respectively). Ethyl acetate and n-butanol hadalso the highest content of phenol and flavonoid. The water extract (polar phase) indicated the lowest activity to inhibit nitric oxide production. In addition, increasing the concentration of onion skin extracts resulted to an increase in the radical scavenging activities. Acceptable correlation was found between nitric oxide scavenging activity and total flavonoid contents (R = 0.8619) but lower correlation was found between this activity and total phenolics content (R = 0.6728). In general, it is evident that the extracts are effective scavengers of nitric oxide and could serve as free radical scavengers.

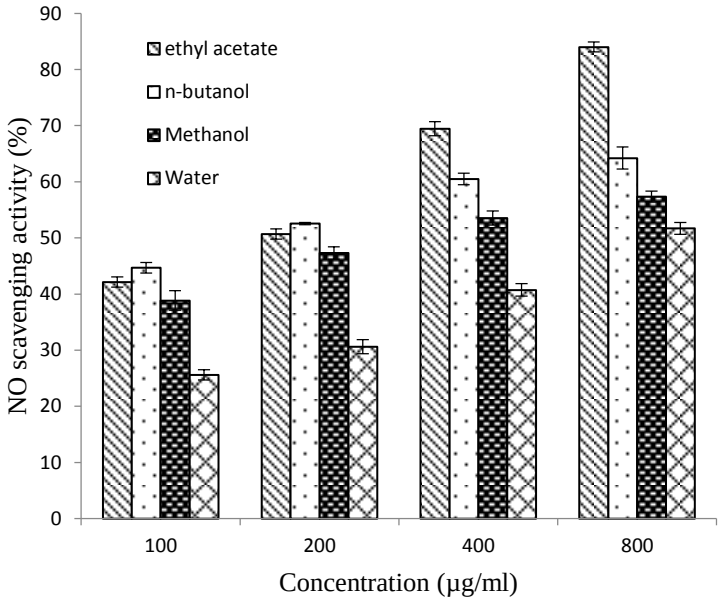

Fig. 4. Nitric oxide scavenging of different extracts from onion skin versus various concentrations. The values represent mean ± S.D. of three independent experiments

Table 2. IC_{50} value in DPPH and nitric oxide radical scavenging assay obtained for each extract

Extract	IC_{50} (µg/mL)	
	DPPH	Nitric oxide
Ethyl acetate	17.269±0.44	188.684±3.506
n- butanol	14.122±0.112	178.476±7.07
Methanol	26.47±0.45	307.503±13.83
Water	35.99±0.84	723.721±10.86
*BHA	53.79±0.70	-
*Quercetin	-	17.35±4.3

*Standard control
Results are means ± standard deviation of duplicate analysis of three replications

Conclusion

This study assessed the effect of solvent polarity on total phenolic and flavonoid contents and antioxidant activities of red onion skin using sequential extraction method. The results reveal that non-polar extracts exhibited a potential antioxidant activity which is beneficial in inhibition or treatment of diseases accompanying with free radicals. In addition, significant correlation between antioxidant activities and the total flavonoids proposes that these compounds are important contributors for the antioxidant properties of red onion skin. In conclusion, thered-onion skin extracts represent a ready accessible and valuable bioactive source of natural antioxidants.

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