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Research Paper

Column Chromatographic Fractionation and Evaluation of In vitro Antioxidant Activity of Flavonoid Enriched Extracts of Some Selected Citrus Fruit Peels

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Abstract

It is a long and tedious process to isolate pure and pharmacologically active constituents from plants. This study aimed to enrich flavonoids of crude extracts from some selected citrus fruit peels and evaluate in vitro antioxidant activity of the flavonoid enriched extracts. Enrichment was carried out by partitioning crude extract using silica gel column chromatography, testing presence of flavonoid in each fractions and pooling together positive fractions. Flavonoid contents were determined by colorimetric aluminum chloride. In vitro antioxidant properties were investigated by DPPH and Nitric Oxide radical scavenging, and reducing power activity assay. Lemon peel's flavonoid enriched extract showed significantly the highest flavonoid contents (9.57 ± 0.38). Orange peel's flavonoid enriched extract showed significantly the lowest flavonoid content (3.7 ± 0.45). Lemon peel's flavonoid enriched extract showed higher DPPH radical scavenging (75.60 ± 2.38%) at 100 µg/ml than Vitamin C. Orange peel's flavonoid enriched extract showed the highest Nitric Oxide scavenging (88.32 ± 1.37%) at 1000 µg/ml and reducing power (0.45 ± 0.5%) at 800µg/ml as compared to other flavonoid enriched extracts. A strong correlation between flavonoid enriched extracts of citrus fruit peel and antioxidant activity was failed to demonstrate by regression analysis. In conclusion, antioxidant activity of flavonoid enriched extracts of citrus fruit peels was confirmed.

Keywords: - *Column chromatography, flavonoid, orange, radical scavenging*.

1. Introduction

The search for effective, nontoxic natural compounds with anti-oxidative activity has been intensified in recent year as synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have recently been reported to be dangerous for human health (Lobo et al., 2010). It has been found that peels of fruit are the main sources of total phenols and flavonoid in citrus fruits (Ghasemi et al., 2009; Bind et al., 2015; Lim and Loh, 2016). The peel which represents almost one half of the fruit mass contains the highest concentrations of flavonoids in the Citrus fruit (Asjad et al., 2013). Citrus peels are rich in numerous active compounds like phenolic acids that have antioxidant properties (Manthey and Grohmann, 2001; Naczk and Shahidi, 2006). Peel residues from sweet and bitter oranges, lemons, and mandarins have proved to be an important source of phenolic acids, flavonoids, flavanones, and glycosylated flavanones (Xi et al., 2014; El Zawawy, 2015). Polar flavonoid structures were reported in mandarin and orange peels. Hesperidin is, for example, a polar bioflavonoids and is the major active constituent of tangerine peel *(Citrus reticulata)* (Fathiazad and Afshar, 2004; Aghel et al., 2008) and sweet orange peel *(citrus sinensis)* (Fathiazad

and Afshar, 2004; Belboukhari et al., 2015). Research done by Yusof et al. (1990) showed that the flavonoids content was richer in citrus peels instead of seeds. Flavonoids act on biological systems as antioxidants, antiviral, anti-inflammatory and anti-tumoral agents. They capture and neutralize the oxidative agents, and quench free radicals (Segev et al., 2010).

It is a long and tedious process to isolate pure and pharmacologically active constituents from plants. Thus, it is necessary to have the good methods available which eliminate unnecessary separation procedures (Kirankumar, 2015). Phytochemical screening enables in recognition of known metabolites present in extracts at earlier stages of separation and is thus economically important to know type of secondary metabolites before comprehensive separation procedure (Kumar and Pradeep, 2011). This study was aimed to enrich flavonoids of extracts from orange, mandarin, lime and lemon peels collected from Dire Dawa District, Ethiopia using a simple open column chromatography, and to evaluate antioxidant activity of flavonoid enriched extracts.

2. Materials and Methods

2.1. Plant Material Preparation

Fruits of orange (*Citrus sinensis*), mandarin (*Citrus reticulate)*, lime (*Citrus aurantifolium)* and lemon (*Citrus lemon)* were collected at the ripening stage from Dire Dawa District farmers. The fruits were carefully hand peeled. The peels were cut into small pieces and dried in a ventilated oven at 60°C for one day. After drying, the peel fragments were ground for a few minutes in blinder and were refluxed with petroleum ether at 60°C for 8 hours to remove oil and chlorophyll. Then, the marc was air dried to evaporate petroleum ether (Cai et al., 2010).

2.2. Crude Extraction and Screening for Flavonoids

The powder was extracted using Soxhlet extractor with 80% ethanol at 78 °C for 6 hours (Cai et al., 2010). The extracts were concentrated over a rotary vacuum evaporator at 45°C until semi-solid extract were obtained. Ferric chloride method described by Ajayi et al. (2011) and lead acetate method described by Sofowora (1993) were used to detect presence or absence of flavonoids in crude extracts. Positive crude extracts were stored at –20°C for column fractionation.

2.3. Flavonoid Enrichment and Quantification

Slurry of silica gel (100-200 mesh size) was made using hexane and the columns were packed to the level of two third. Five gram of crude extracts were mixed with small amount of silica gel and loaded on the top of the column. Flavonoids are weakly polar and in this study crude extraction was done using highly polar ethanol and the expected extracts were enriched with polar phytochemicals. In order to enrich the crude extracts with flavonoid, mixture of hexane and ethanol was used as eluent to effectively separate non-polar photochemical. Therefore, elution was started with 70:30, and continued using 60:40, 50:50 hexane to ethanol, and finally 80:20 ethanol to distilled water. All the collected fractions were tested for flavonoid and the positive fractions were pooled together. The pooled fraction was concentrated at 45°C rotary evaporator until the formation of sediment.

The sediment freeze dried and represented the solid-state product of flavonoids. Freeze drying was conducted to make suitable mass measurement. Total flavonoid content of the purified extracts was determined. Colorimetric aluminum chloride method was used for flavonoid determination (Ghasemi et al., 2009; Asjad et al., 2013). Briefly, 1 mL of mg/mL of freeze-dried extracts in ethanol were separately mixed with 0.1 mL of 10% aqueous aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer. Distilled water was used as a blank. Total flavonoid contents were calculated as quercetin equivalent from a calibration curve. Regression linear line of $Y= 0.0005x+0.029$, $r^2 = 0.999$ of quercetin (200 -1200 µg/ml) was used as a reference standard curve (Kamtekar et al., 2014).

2.4. Evaluation of Antioxidant Activity

2.4.1. DPPH· scavenging activity

The stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity carried out following methods described by Ghasemi et al. (2009) and Asjad et al. (2013). Briefly, different concentrations of extracts and standards (100 µg/mL, 200 µg/mL, 400 µg/mL, 800

μg/mL and 1000 μg/mL) were prepared using ethanol. Four mL of each concentration and 4 mL DPPH **(**100μM in methanol) was added in 10 mL capacity test tubes. The solution were mixed and allowed to stay for 15 minutes at room temperature in dark place. The absorbance was recorded at 517nm. Mixture of ethanol and methanol was used as a blank, DPPH solution without extract used as a control and vitamin C as standard. The experiment was done in triplet. The percentage quenching of DPPH· was calculated as follows:

$$\%\ \text{inhibition of DPPH.} = \left(\frac{A_{br} - A_{ar}}{A_{br}}\right) \times 100$$

Where, A_{br} is the absorbance of DPPH· solution alone (without sample or standard solution addition) and A_{ar} is the absorbance after reaction was taken place.

In this DPPH assay of the flavonoid enriched extracts, the samples and DPPH were dissolved in different solvents. This is because the sensitivity of spectrophotometric measurements of DPPH in methanolic solutions is better than that of DPPH in ethanolic solutions (Om and Tej, 2008). Therefore, DPPH was dissolved in methanol. On the other hand, the soxhlet extraction was carried out using ethanol and column fractionation was using mixture of hexane and ethanol, and mixture of ethanol and water. As complete dissolution of extracts stabilizes UV reading, the flavonoid enriched extracts were dissolved in ethanol.

2.4.2. Nitric Oxide scavenging activity

Nitric oxidant scavenging activity was conducted following methods described by Ebrahimzadeh et al. (2009) and Mahmoudi et al. (2009). Briefly, different concentrations of extract (100, 200, 400, 800 and 1000 μg/mL) were prepared dissolving in ethanol. Sodium nitroprusside (1 mL, 10 mM), in phosphate-buffered saline, was mixed with each of these concentrations (2 mL) separately and incubated at room temperature for 180 min. The same reaction mixture, without extract was served as control. After the incubation period, 3 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1- naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Vitamin C was used as standard. The percentage scavenging of NO. was calculated as follows:

$$\%\ \text{inhibition of NO.} = \left(\frac{A_{br} - A_{ar}}{A_{br}}\right) \times 100$$

Where, A_{br} is the absorbance of NO· solution alone (without sample or standard solution addition) and A_{ar} is the absorbance after reaction was taken place.

2.4.3. Reducing power determination

Different amounts of extract and standard (100, 200, 400, 800 and 1000 μg/mL) in ethanol were prepared. Two mL of each concentrations were mixed separately with phosphate buffer (2 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2 mL, 1%) in to centrifuge tube (10 mL). The mixture was incubated at 35^{o}C for 20 min. Trichloroacetic acid (2 mL, 10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. Two mL of upper layer of the solution was mixed with 2 mL of deionized water and $FeCl_3$ (0.25 mL, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Distilled water was used as a blank and Vitamin C as positive control (Ebrahimzadeh and Bahramian, 2009).

2.5. Statistical Analysis

Experimental results were expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means were separated by Duncan's multiple range tests.

3. Results

3.1. Extraction and Screening for Total Flavonoids

Ferric chloride and lead acetate tests for crude extracts were confirmed the presence of flavonoids in all extracts and some fractions of column chromatography

3.2. Flavonoid Enrichment and Quantification

The result of silica gel column chromatography separations was summarized in Table 1 and Figure 1(a), Figure 1(b), Figure 1(c) and Figure 1(d). The Number of column fractions of orange crude extracts was found 8 and three of them were positive for Ferric Chloride test. Most fractions eluted by 80:20 ethanol to water were found positive for all crude extracts of citrus fruit peels. All positive fractions were pooled together enriching flavonoid content of crude extracts. And the result of

determination of total content of flavonoid was summarized in Table 2. Lemon (*Citrus lemon*) flavonoid enriched extract showed significantly the highest values of total flavonoid (9.57 ± 0.375 mg quercetin equivalent/g of extracts).

Table 1: Column chromatography fractions of Citrus fruit peels' extract and flavonoid detection

Selected Citrus fruit	Solvent ratio	No. of Separated Fractions	Elution order and $FeCl_3$ test
Orange	70:30 Hexane to ethanol	1	Negative
	60:40 Hexane to ethanol	1	Negative
	50:50 Hexane to ethanol	2	Negative
	80:20 Ethanol to water	4	The first three positive
Mandarin	70:30 Hexane to ethanol	2	Negative
	60:40 Hexane to ethanol	1	Negative
	50:50 Hexane to ethanol	5	The fifth positive
	80:20 Ethanol to water	4	The first three positive
Lime	70:30 Hexane to ethanol	2	Negative
	60:40 Hexane to ethanol	1	positive
	50:50 Hexane to ethanol	2	Negative
	80:20 Ethanol to water	6	The first four positive
Lemon	70:30 Hexane to ethanol	1	Negative
	60:40 Hexane to ethanol	2	The second positive
	50:50 Hexane to ethanol	4	All are positive
	80:20 Ethanol to water	6	The first four positive

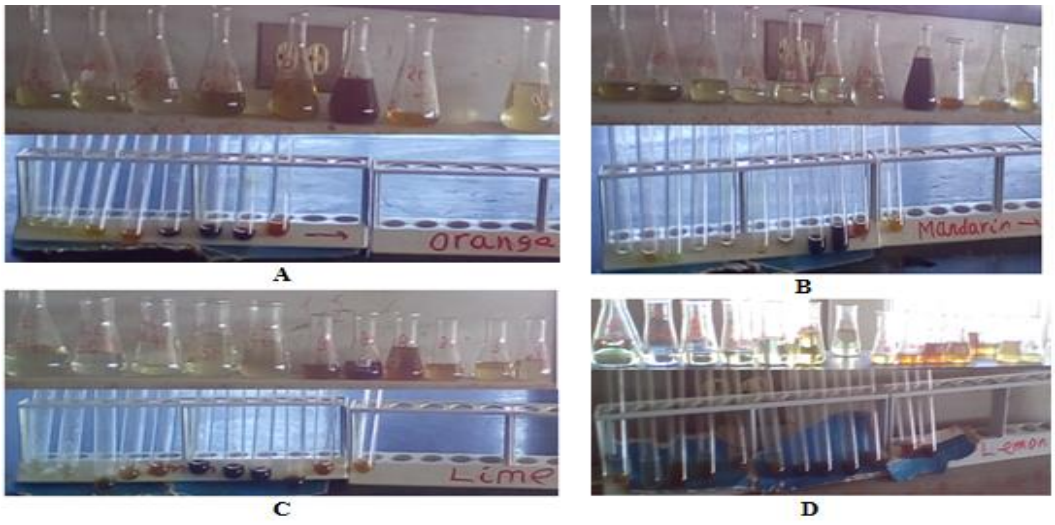

Figure 1: Column fractions and Ferric chloride test of citrus fruit peels' extracts

Table 2: Total flavonoid contents for citrus fruit peel extracts

Selected Citrus fruit	Flavonoid enriched extracts (in mg of quercetin equivalent/g of the extracts)
Orange	3.70 ± 0.448^{A}
Mandarin	5.24 ± 0.848^{B}
Lime	5.54 ± 0.564^{B}
Lemon	9.57 ± 0.375^{C}

The values are Mean ± Standard deviation (n=3). Superscript letters compares means. Means with similar letters show no significant difference, whereas means with different letters show significant difference at P<0.05.

3.3. Evaluation of Antioxidant Activity

The results of antioxidant activity evaluation were summarized in Table 3, 4 and 5. Lemon (*Citrus lemon*) peel's flavonoid enriched extracts showed significantly higher percentages of DPPH radical quenching ($P < 0.05$) at100 µg/ml than Ascorbic acid as a positive control. The flavonoid enriched extract of orange (*Citrus sinensis*) showed the lowest percentage of DPPH quenching at 100µg/ml. The flavonoid enriched extract of lime (*Citrus aurantifolia)* showed the lowest (74.36 ± 3.08) while the orange flavonoid enriched extract showed the highest (88.32 ± 1.37) Nitric Oxide radical scavenging at 1000 µg/mL. The flavonoid enriched extract of orange showed significantly highest reducing power activity at 100 and 800 µg/mL compared to other extracts. Reducing power of selected citrus fruit peel's flavonoid enriched extracts found to be increased as concentration of the extracts increased.

Table 3: Percentage of DPPH Scavenging by citrus fruit peel's flavonoid enriched extracts

Concentration	Orange	Mandarin	Lime	Lemon	Vitamin C
100µg/ml	57.86 ± 2.58^{a}	68.45 ± 0.54^{c}	68.93 ± 0.62^{c}	75.60 ± 2.38^{d}	69.64 ± 0.36^{c}
200 µg/ml	66.79 ± 0.36^{cd}	68.93 ± 4.21^{ed}	69.52 ± 0.54^{cd}	71.55 ± 1.35^{d}	72.62 ± 0.90^{d}
400 µg/ml	69.40 ± 1.09^{b}	69.40 ± 1.09^{ab}	70.12 ± 1.45^{ab}	72.97 ± 1.80^{ab}	75.95 ± 0.54^{b}
800µg/ml	72.98 ± 0.540^{c}	73.21 ± 1.56^{c}	66.43 ± 1.07^{ab}	71.90 ± 1.15^{c}	78.21 ± 2.34^{d}
1000µg/ml	76.31 ± 0.42^{d}	74.88 ± 1.76^{cd}	62.62 ± 0.21^{a}	71.67 ± 1.09^{b}	79.40 ± 1.03^{e}

Table 4: Percentage of Nitric Oxide scavenging citrus fruit peel's flavonoid enriched extracts

Concentration	Orange	Mandarin	Lime	Lemon	Vitamin C
100µg/ml	78.63 ± 2.60^{ab}	85.47 ± 0.43^{c}	83.76 ± 1.28^{bc}	86.61 ± 0.89^{cd}	91.68 ± 7.41^{d}
200 µg/ml	79.91 ± 1.13^{a}	85.19 ± 1.08^{ab}	82.91 ± 2.67^{ab}	87.32 ± 1.08^{bc}	91.80 ± 1.54^{c}
400 µg/ml	81.05 ± 0.99^{ab}	83.90 ± 2.15^{b}	77.49 ± 6.34^{ab}	86.04 ± 0.65^{cd}	91.92 ± 4.58^{d}
800µg/ml	85.76 ± 0.65^{c}	81.34 ± 2.43^{b}	76.64 ± 2.46^{a}	82.91 ± 2.60^{bc}	93.01 ± 3.24^{d}
1000µg/ml	88.32 ± 1.37^{c}	82.62 ± 0.65^{b}	74.36 ± 3.08^{a}	81.77 ± 1.37^{b}	93.43 ± 4.71^{d}

Table 5: Reducing power of citrus fruit peel's flavonoid enriched extracts

Concentration	Orange	Mandarin	Lime	Lemon	Vitamin C
100µg/ml	0.36 ± 0.01^{c}	0.21 ± 0.02^{a}	0.26 ± 0.00^{b}	0.26 ± 0.00^{b}	0.20 ± 0.03^{a}
200 µg/ml	0.36 ± 0.03^{a}	0.29 ± 0.02^{a}	0.27 ± 0.00^{a}	0.26 ± 0.01^{a}	0.76 ± 0.01^{b}
400 µg/ml	0.38 ± 0.03^{ac}	0.31 ± 0.02^{ab}	0.27 ± 0.03^{ab}	0.27 ± 0.01^{a}	0.77 ± 0.06^{d}
800 µg/ml	0.45 ± 0.05^{b}	0.34 ± 0.01^{a}	0.32 ± 0.00^{a}	0.32 ± 0.03^{a}	0.94 ± 0.04^{c}
1000 µg/ml	0.41 ± 0.05^{b}	0.43 ± 0.03^{ab}	0.41 ± 0.05^{b}	0.35 ± 0.03^{ab}	0.96 ± 0.03^{c}

The values are Mean ± Standard deviation (n=3). Small letters superscript compares between means in row, and means with similar small letters show no significant difference, whereas means with different small letters show significant difference at P<0.05.

3.4. Correlation of Flavonoid Content and Antioxidant Activity

Linear association between flavonoid contents of selected citrus fruit peel's flavonoid enriched extracts and antioxidant activity was analyzed using linear regression analysis and the results were presented in Figure 2, 3 and 4. From the Figure, linear association was weak (the correlation coefficient (R) value is near to 5).

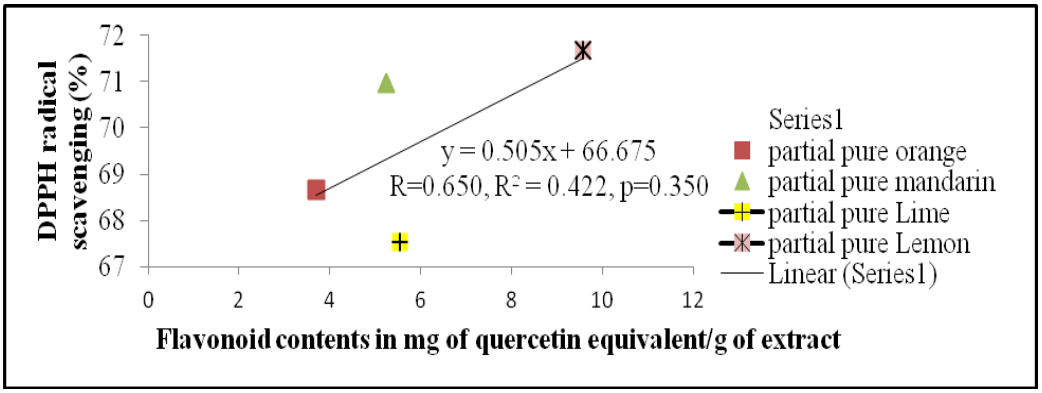

Figure 2: Correlation between flavonoid contents and DPPH radical scavenging activity of flavonoid enriched extracts

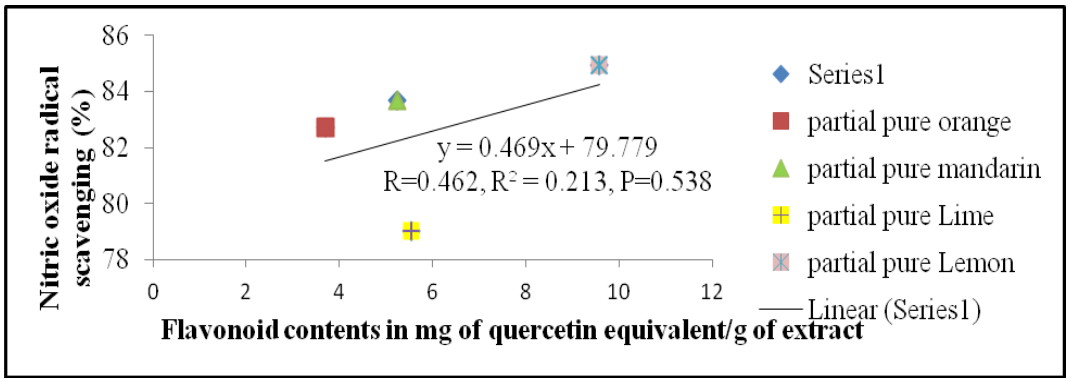

Figure 3: Correlation between flavonoid contents and nitric oxide scavenging activity of flavonoid enriched extracts

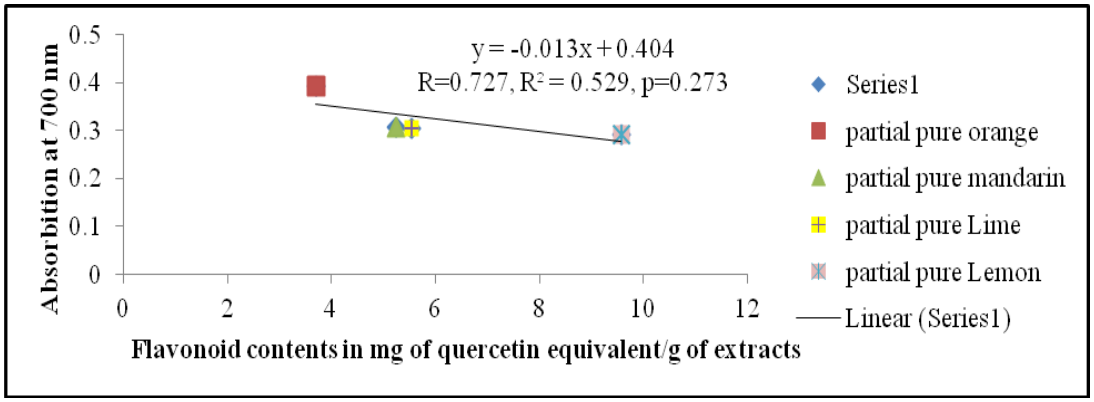

Figure 4: Correlation between flavonoid contents and reducing power of flavonoid enriched extracts

4. Discussion

4.1. Enrichment, Screening and Quantification of Total Flavonoids

It is necessary to have the good methods available which eliminate unnecessary separation procedures for phytopharmaceuticals from plant extracts. Kumar and Pradeep (2011) concluded that phytochemical screening is economically important as it enables recognition of known chemical constituents present in plant extracts at earlier stages of isolation and characterization. In this study, Ferric Chloride and Lead Acetate chemical screening methods were used to detect flavonoids in crude extracts of citrus fruit peels and column fractions. All crude extracts were tested positive and they were subjected to silica gel column chromatographic fractionation. The result of chemical screening showed that Ferric chloride test confirmed the presence of flavonoids in some fractions of column chromatography (Figure 1). Some fractions that were confirmed the presence of flavonoids by Ferric chloride test showed the absence of flavonoid by Lead Acetate test. In line with this result, Andrew and Kurt (2006) described that the Lead Acetate procedure is often unsatisfactory since some phenolics do not precipitate while ferric chloride is a general cover reagent for phenolic compounds and gives a blue-black coloration with flavonoids.

Isolation of pure and pharmacologically active constituents like flavonoids from plants is a long and tedious process. In this study, crude extracts of selected citrus fruit peels was fractionated using a simple open column chromatography, each fractions were tested for presence of flavonoids and positive fractions were pooled together to enrich flavonoid contents of extracts. Kirankumar (2015) stated that it is necessary to have the good methods available which eliminate unnecessary separation procedures. Andrew and Kurt (2006) described that there is no single isolation strategy for the separation of flavonoids and conventional open-column chromatography is still widely used because of its simplicity and value as an initial separation step. In this study, column chromatography elution was started with 70:30, and continued using 60:40, 50:50 hexane to ethanol, and finally with 80:20 ethanol to distilled water. The results showed that the most fractions obtained by using 80:20 ethanol to distilled water were tested positive for flavonoid showing the highest extractive ability of flavonoid by aqueous ethanol. This is in line with the result reported by Kirankumar (2015). Bharathi (2016) found the presence of more flavonoid content in ethanol extracts than the hexane and ethyl acetate extracts.

One fraction of mandarin and lime crude extract eluted by 50:50 and 60:40 hexane to ethanol, respectively, were confirmed the presence of flavonoid. In addition, one fraction of lemon crude extract eluted by 60:40 and three fractions eluted by 50:50 hexane to ethanol were found positive for flavonoid. This might be showed that peel's crude extracts contain both less polar aglycones flavonoids and glycosides. Andrew and Kurt (2006) stated that less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures.

Colorimetric aluminum chloride method was used for total flavonoid determination. In increasing order, the total flavonoid contents were 3.70 ± 0.448, 5.24 ± 0.848, 5.54 ± 0.564 and 9.57 ± 0.375 for orange, mandarin, lime and lemon peels' flavonoid enriched extracts, respectively (Table 2). Lemon peel's flavonoid enriched extract showed significantly the highest values of total flavonoid while orange peel's flavonoid enriched extract showed significantly the lowest values of total flavonoid. Lower content of flavonoid in orange peel's flavonoid enriched than lemon peel's flavonoid enriched extract was reported by Singh and Immanuel (2014) and El zawawy (2015).

4.2. Evaluation of Antioxidant Activity

Antioxidant activity should not be concluded based on a single antioxidant test model. Antioxidant test models vary in different respects and are difficult to compare fully one method to other one (Alam et al., 2012). In this study, antioxidant activity of the fruit peel's flavonoid enriched extracts was assessed by three different methods: DPPH radical quenching, Nitric Oxide radical scavenging and reducing power determination.

DPPH method is based on hydrogen atom transfer (HAT) and electron transfer (ET) antioxidant activities (Bae et al., 2015). When a solution of DPPH is mixed

with Antioxidant (AH) that can donate a hydrogen atom, DPPH is reduced and loses violet color (Alam et al., 2012). DPPH radical scavenging assay showed that lemon peel's flavonoid enriched extract had significantly higher DPPH radical scavenging (75.60 ± 2.38%) at concentration of 100 µg/mL than Vitamin C as positive control (Table 3). In line with this result, Singh and Immanuel, (2014) reported DPPH scavenging of 75.9% for orange peel. In the present study, Vitamin C showed significantly higher DPPH radical scavenging activity than lemon fruit peel's flavonoid enriched extracts only at 1000 µg/mL. Orange and mandarin peel's flavonoid enriched extracts showed quenching activity of DPPH radical in a dose dependent manner. But, lime and lemon peel's flavonoid enriched extracts did not show DPPH radical quenching in a dose dependent manner. In line to this result, El Zawawy (2015) reported dose dependent DPPH radical quenching activity of orange peel extracts and found dose independent DPPH radical quenching activity of lemon peel extracts. DPPH radical quenching of tangerine (*Citrus tangerine*) was found increase along concentration (Won et al., 2014*).*

Nitric Oxide (NO•**)** scavenging assay is based on the Sodium Nitroprusside decomposition in aqueous solution at physiological pH (7.2) producing NO•. Under aerobic conditions, NO• reacts with Oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent (Marcocci et al., 1994). The Nitric Oxide radical scavenging assay showed that lemon peel's flavonoid enriched extract had similar scavenging activity ($p > 0.05$) with vitamin C at 100, 200 and 400 µg/mL (Table 4). Nitric Oxide radical scavenging values for orange fruit peel's flavonoid enriched were found increased as the concentration of extracts increases. Munmar et al. (2015) reported Nitric Oxide scavenging value (79.42%) for orange varieties, which is similar with this study results at 100-800 µg/mL.

Reducing power assay is based on the reduction of Fe^{3+} to Fe^{2+} by an electron donation from reductants (antioxidants) in the samples. Amount of Fe^{2+} complex formed can be monitored by measuring Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability (Mahmoudi et al., 2009). Reducing power assay results showed that mandarin peel's flavonoid enriched extract had similar (0.21 ± 0.02) reducing power activity with vitamin C as positive control (0.20 ± 0.03) at 100 µg/mL (Table 5). Orange peel's flavonoid enriched extract showed the highest reducing power at 800 µg/mL compared to other citrus fruit peel's flavonoid enriched extracts. A higher reducing power than this study result was reported by Mahmoudi et al. (2009) and Cardeñosa et al. (2015). Vitamin C showed significantly higher reducing power than evaluated citrus fruit peel's flavonoid enriched at 200-1000 µg/mL ($p < 0.05$). Reducing power of selected citrus fruit peel's flavonoid enriched extracts found to be increased as concentration of the extracts increased. In agreement with this study result, Al-Anbari and Hasan (2015) reported increased reducing power of some citrus leaves and seeds ethanolic extracts as concentration increases.

4.3. Correlation of Flavonoid Content and Antioxidant Activity

The correlation between total phenol contents and antioxidant activity of food, fruit and vegetables has been widely studied (Klimczak et al., 2007; Jayaprakasha et al., 2008; Calado et al., 2015; Ghasemi et al., 2015). As they reported, antioxidant activity of food samples, fruits and vegetables significantly increases with the presence of high concentration of total polyphenol content. In the present study, the correlation between total flavonoids contents and radical scavenging activity of citrus fruit peel's flavonoid enriched extracts were analyzed.

A strong correlation between citrus fruit peel's flavonoid enriched extracts and antioxidant activity was failed to demonstrate by regression analysis (Figure 2, 3, 4). The correlation coefficients were 0.65, 0.46 and 0.73 for DPPH and Nitric Oxide radical scavenging and reducing power, respectively. This showed that the correlation between flavonoid contents of citrus fruit peel's flavonoid enriched extracts and anti-oxidant activity is a weak. The correlations were insignificant for all assay methods ($p > 0.05$). Supporting this result, lack of correlation between crude methanolic extracts of citrus fruit peel and DPPH radical scavenging activity was reported by Ghasemi et al. (2015). Opposing this result, Calado et al. (2015) reported good correlation between flavonoid content and antioxidant activity in

most food samples analyzed. About 42.2 %, 21.3 % and 52.9% variability of DPPH and Nitric Oxide scavenging, and Reducing power, respectively, was accounted for the difference in flavonoid contents (R^2=0.422, 0.213, 0.529 respectively). It was known that the presence or absence of certain structure of flavonoid is important in antioxidant activity. Hydroxyl group in the molecule, for example, can act as proton donating and show radical scavenging activity and reducing power (Mensor et al., 2001; Hou et al., 2003).

5. Conclusion

Orange, mandarin, lime and lemon fruit peel's fractions tested positive for flavonoid and pooled together showed antioxidants properties. The highest amount of total flavonoid content was found in flavonoid enriched extracts of lemon peel. Maximum antioxidant activity was found in different citrus fruit peel's flavonoid enriched extracts for different antioxidant assay methods. Lemon showed highest DPPH and Nitric Oxide scavenging and mandarin the highest reducing power. A strong correlation between citrus fruit peel's flavonoid enriched extracts and antioxidant activity could not been demonstrated by regression analysis. It can be concluded from the study that the flavonoid enriched extracts had antioxidant properties that did not depend on the concentration of flavonoid and different antioxidant assay methods give different antioxidant activity results. It is recommended that lemon and peel's flavonoid enriched extract can be used as a potential DPPH scavengers and orange peel's flavonoid enriched as Nitric Oxide inhibitors and reducing power. Further isolation and purification of distinct flavonoid from the investigated citrus fruit peels will be required.

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