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Original Article

EVALUATION OF ANTIOXIDANT ACTIVITIES FROM VARIOUS EXTRACTS OF SWEET ORANGE PEELS USING DPPH, FRAP ASSAYS AND CORRELATION WITH PHENOLIC, FLAVONOID, CAROTENOID CONTENT

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ABSTRACT

Objectives: The objectives of this research were to study antioxidant capacity from various extracts of sweet orange peels using two methods of antioxidant testing which were DPPH (2.2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) and correlation of total phenolic, flavonoid and carotenoid content in various extracts of sweet orange peels with DPPH and FRAP antioxidant capacities.

Methods: Extraction was performed by reflux using various solvents. The extracts were vaporated using rotavapor. Then antioxidant capacities were tested using DPPH and FRAP assays. Determination of total phenolic, flavonoid and carotenoid content were performed by spectrophotometry UV-Vis and its correlation with FRAP and DPPH antioxidant capacities were analyzed by Pearson method.

Results: KT3 (ethanol peel extract of Kintamani orange) had the highest DPPH scavenging capacity with IC50 2.25 ppm and KT3 had the highest FRAP capacity also with EC50 131.7 ppm. KT3 contained the highest total phenolic (10.08 g GAE/100 g), KT2 (ethyl acetate peel extract of Kintamani orange) had highest flavonoid content (9.94 g QE/100 g) and BW1 had the highest carotenoid 2.33 g BET/100 g.

Conclusions: There were positively high correlation between total phenolic content in all of peels samples with their antioxidant activity using DPPH and FRAP assays. DPPH scavenging capacities in all of orange peels samples had positively high correlation with their FRAP capacities.

Keywords: Antioxidants, FRAP, DPPH, sweet orange peels, flavonoid, phenolic, carotenoid

INTRODUCTION

Antioxidant has potency to mobilize protective effects against oxidative stress on account of their high antioxidant activity [1]. Phenolic compounds such as phenolic acid, flavonoid and tannin are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity [1] [2] [3] [4] [5]. Many studies had revealed that phenolic content in plants could be correlated to their antioxidant activities. Plants contained phenolic and polyphenol compounds which have antioxidant activity [1] [6] [7] [8] [9].

Some of antioxidant methods such as DPPH (2,2-diphenyl-1 picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) were widely used to predict antioxidant capacity of fresh fruits, beverages and food [2]. In previous study [2] [10] [11] [12] exposed that DPPH and FRAP methods could be used to determine antioxidant activity in many plants extracts. The previous study [4] [10] [12] [13] [14] showed antioxidant activities of some plants including orange peels.

The objective of this research were to study antioxidant capacities of various extracts (n-hexane, ethyl acetate and ethanol) from sweet orange peels that were collected from three different growth location (Kintamani, Jember and Banyuwangi) using antioxidant testing DPPH and FRAP assays and correlations of their capacities with total flavonoid, phenolic, and carotenoid content in each extracts.

MATERIALS AND METHODS

Materials

TPTZ (2,4,6-tripyridyltriazine), DPPH (2,2-diphenyl-1 picrylhydrazyl), gallic acid, quercetin, beta carotene was purchased from Sigma-Aldrich (MO,USA), ferric chloride, methanol, ethanol. All other reagents were analytical grades.

Preparation of sample

Sweet orange (Citrus sinensis) fruits peels were collected from three different growth location: Kintamani (KT), Jember (JB) and Banyuwangi (BW) were thoroughly washed with tap water, wet sortation, cut, dried and grinded into powder.

Extraction

Three hundred grams of powdered samples were extracted by reflux using increasing gradient polarity solvents. The n-hexane extract was repeated three times. The remaining residue was then extracted three times with ethyl acetate. Finally the remaining residue was extracted three times with ethanol. So there were three n-hexane extracts (namely KT1, JB1, BW1), three ethyl acetate extracts (KT2, JB2, BW2) and three ethanolic extracts (KT3, JB3, BW3).

DPPH scavenging capacity

Preparation of DPPH solution were adopted from Blois [15] with minor modification. Each extracts 50 µg/mL was pipetted into DPPH solution concentration 50 µg/mL (1:1) to initiate the reaction. After 30 minutes incubation, the absorbance was read at wavelength 517 nm by using spectrophotometer UV-Vis Hewlett Packard 8435. Methanol was used as a blank and DPPH solution 50 □ g/mL as standard. Analysis was done in triplicate for standard and each extracts. Antioxidant activity of each extracts were determined based on the reduction of DPPH absorbance by calculating percentage of antioxidant activity [16].

FRAP capacity

Preparation of FRAP solution were adopted from Benzi [17]. FRAP solution were prepared in acetate buffer pH 3.6. Each extracts 50 µg/mL was pipetted into FRAP solution 50 µg/mL (1:1) to initiate the reaction. After 30 minutes incubation, the absorbance was read at wavelength 593 nm by using spectrophotometer UV-Vis Hewlett Packard 8435. Acetate buffer was used as a blank and FRAP solution 50 µg/mL was used as standard. Analysis was done in triplicate for standard and each extracts. Antioxidant capacity of each extracts were determined based on increasing in Fe (II) - TPTZ absorbance by calculating percentage of antioxidant capacity [17].

Total phenolic determination

Total phenolic content were measured using the modified Folin-Ciolcalteu method adapted from Pourmorad [18]. The absorbance was read at wavelength 765 nm. Analysis was done in triplicate for each extracts. Standard solutions of gallic acid with concentration 60-150 □ g/mL were used to obtain a standard curve. The total phenolic content was reported as percentage of total gallic acid equivalents per 100 g extract (g GAE /100 g).

Total flavonoid determination

Total flavonoid content was measured using adapted method from Chang et al [19]. The absorbance was read at wavelength 415 nm. Analysis was done in triplicate for each extracts. Standard solutions of quercetin with concentration 40-160 □ g/mL were used to obtain a standard curve. The total flavonoid content was reported as percentage of total quercetin equivalents per 100 g extract (g QE/100 g).

Total carotenoid determination

Total carotenoid content was measured using the modified carotene method adapted from Thaipong et al [2]. Each extracts were diluted in acetone. The absorbance was read at wavelength 470 nm. Analysis was done in triplicate for each extracts. Standard solutions of beta carotene with concentration 10-40 □ g/mL were used to obtain a standard curve. The total carotenoid content was reported as percentage of total beta carotene equivalents per 100 g extract (g BET/100 g).

Statistic

Each sample analysis was performed in triplicate. All results presented were the means (±SD) of at least three independent experiments. Statistical analysis (ANOVA with a statistical significance level set at $p < 0.05$ with post-hoc Least Significant Difference (LSD) procedure was carried out with SPSS 16.0 for Windows. Correlations between the total phenolic, flavonoid and total carotenoid content and antioxidant capacities were made using the Pearson method ($p < 0.01$).

RESULTS

Antioxidant capacities of various extracts from sweet orange peels using DPPH and FRAP assays

The antioxidant capacities using DPPH and FRAP assays of various peels extracts from sweet orange peels were shown in Table 1, Table 2, Table 3. In DPPH method, antioxidant capacities in the range of 50.13 – 55.46 %. KT2 peels extract (ethyl acetate extract of Kintamani orange) had the highest DPPH radical scavenging capacity (55.46 %), while the lowest antioxidant capacity (50.13 %) was given by JB1 peels extract.

In the FRAP method, free radical scavenging capacities of various peels extracts from sweet orange peels ranged from 0.13 – 20.07 %. KT3 (ethanolic extract of Kintamani orange) had the highest FRAP capacity (20.07%), while BW1 peels extract (0.13%) had the lowest FRAP capacity.

The IC50 of DPPH scavenging capacities and EC50 of FRAP capacities in various extracts from sweet orange peels using DPPH and FRAP assays were shown in Fig 1 and Fig 2. Both of IC50 of DPPH scavenging capacities and EC50 of FRAP capacities of each extracts were compared to ascorbic acid as standard. The lowest IC50 or EC50 means had the highest antioxidant capacity.

The total phenolic content among the various extracts were expressed in term of gallic acid equivalent using the standard curve equation $y = 0.0044 x + 0.031$, $R2 = 0.993$. The total phenolic content in various extracts from sweet orange peels showed different result ranged from 3.29 to 10.08 g GAE/100 g. KT3 peels extract (ethanolic peels extract of Kintamani orange) had the highest phenolic content (10.08 g GAE/100 g) (Fig 3).

Total flavonoid in various sweet orange peels extracts

The total flavonoid content among the various extracts were expressed in term of quercetin equivalent using the standard curve equation $y = 0.00761355x + 0.00491857$, $R2= 0.998$. The total flavonoid content in various extracts from sweet orange peels showed different result in the range of 0.93 – 9.94 g QE/100 g (Fig 4). KT2 (ethyl acetate peels extract of Kintamani orange) had the highest total flavonoid content (9.94 g QE/100 g) and the lowest (0.93 g QE/100 g) for BW3 peels extract.

Total carotenoid in various sweet orange peels extracts

The total carotenoid content among the various extracts were expressed in term of beta carotene equivalent using the standard curve equation $y = 0.02764x - 0.00324857$, $R2 = 0.999$. The total carotenoid content in various extracts from sweet orange peels showed different result in the range of 0.021 – 2.33 g BET/100 g (Fig 5). The highest carotenoid content (2.33 g BET/100 g) for BW1 peels extract, while the lowest carotenoid (0.021 g BET/100 g) for JB3 peels extract.

Correlations between total phenolic, flavonoid, carotenoid content and DPPH scavenging capacities, FRAP capacities, in various sweet orange peels extracts

Pearson's correlation coefficient was positively high if $0.68 \leq r \leq 0.97$ [2]. The highest and positive correlation between total phenolic content and DPPH scavenging activity ($r = 0.984$, $p<0.01$) was given by sample KT. The positive and high correlation between phenolic content and FRAP capacities were given by sample JB ($r = 0.998$, $p<0.01$), followed by sample KT ($r = 0.984$, $p<0.01$) and sample BW ($r = 0.962$, $p<0.01$) (Table 4).

DISCUSSION

Some of tropical plants including sweet orange peels had antioxidant capacity using various antioxidant testing assays [2] [6] [10]. There were no study regarding antioxidant capacity of three various extracts (which were n-hexane, ethyl acetate and ethanol) of sweet orange peels from three different growth location using DPPH and FRAP assays.

DPPH is stable free radicals which dissolve in methanol or ethanol, and its colors show characteristic absorption at wavelength 517 nm. Colors of DPPH would be changed when the free radicals were scavenged by antioxidant [20] [21]. FRAP is $FeCl3$ that combined with 2,4,6-tripyridyltriazine (TPTZ) in acetate buffer pH3.6. Fe (III) will be reduced to Fe (II). Complex Fe(II) - TPTZ gives blue color and show characteristic absorption at wavelength 593 nm. Intensity of blue color is depend on amount of Fe (III) that is reduced to Fe (II). If a sample reduces Fe (III) to Fe (II),at the same time it will be oxidized, so that sample can act as antioxidant [2].

In the present study, the highest DPPH scavenging capacity was given by sample KT2 (ethyl acetate peels extract of Kintamani orange), followed by sample JB2 (ethyl acetate peels extract of Jember orange) and JB3 (ethanolic peels extract of Jember orange). Ethanolic peels extract of Kintamani orange (KT3), Jember orange (JB3) and Banyuwangi orange (JB3) had DPPH scavenging capacity 51.44 %, 54.03 % and 50.27 % respectively, while ethyl acetate peels extract of KT2, JB2 and BW2 had DPPH scavenging activity 55.46 %, 54.96 %, 53.99 % respectively. Study by Oboh [12] extracted orange peels in two parts of phenolic compound. Free phenolic compound was extracted by acetone and bound phenolic compound extracted by ethyl acetate. Acetone peels extract had DPPH scavenging capacity 48 %, while ethyl acetate peels extract had DPPH scavenging activity 45 %.

The highest FRAP capacitiy was given by KT3 (ethanolic peels extract of Kintamani orange), followed by JB3 (ethanolic peels extract of Jember orange) and BW3 (ethanolic peels extract of Banyuwangi orange). Ethanolic peels extract of Kintamani orange, Jember orange and Banyuwangi orange had FRAP capacity 20.07 %, 13.61 % and 10.05 % respectively, while ethyl acetate peels extract of KT2, JB2 and BW2 showed 6.06 %, 3.79 % and 4.03 % in FRAP capacity respectively. In contrast with previous study [12] demonstrated that FRAP capacities of acetone peels extract had FRAP capacity 55 % and ethyl acetate peels extract had FRAP activity 48 %.

IC50 of DPPH scavenging capacity is concentration of sample or standard that can inhibit 50 % of DPPH scavenging capacity, while EC50 of FRAP capacity is concentration of sample or standard that can exhibit 50 % of FRAP capacity. The lowest IC50 or EC50 means had the highest antioxidant capacity. IC50 or EC50 were used to

determine antioxidant capacity of sample compared to standard. Sample that had IC50 < 50 ppm, it was very strong antioxidant, 50-100 ppm strong antioxidant, 101-150 ppm medium antioxidant, while weak antioxidant with EC50 or IC50> 150 ppm [15].

KT3 (ethanolic peels extract of Kintamani orange) had the lowest IC50 of DPPH scavenging activity (2.25 ppm), while ascorbic acid standard gave IC50 of DPPH scavenging capacity 1.45 ppm. All of extracts (n-hexane, ethyl acetate and ethanol) of sweet orange peels (Kintamani orange, Jember orange, Banyuwangi orange) had the IC50 in the range of 2.25 – 72.65 ppm. Based on classification of potency of antioxidant by Blois [15], it could be classified as strong to very strong antioxidant. In the present study ethanolic peels extract of KT3 (Kintamani orange), JB3 (Jember orange) and BW3 (Banyuwangi orange) had IC50 of DPPH scavenging capacities was 2.25, 8.84 and 17.94 ppm, while ethyl acetate peels extract KT2, JB2 and BW2 gave 9.49, 8.67 and 72.65 ppm for IC50 of DPPH scavenging capacities respectively. The result of this study were different with research by Oboh [12], which showed IC50 of DPPH scavenging capacity of acetone peels extract of orange (Citrus sinensis), grapefruit (Citrus paradisii) and shaddock (Citrus maxima) were 1700 ppm, 1400 ppm and 1600 ppm, while in ethyl acetate peels extract had IC50 1000 ppm, 1800 ppm and 1900 ppm respectively.

Various extracts from sweet orange peels had EC50 of FRAP capacities ranged from 131.7 to 26,158 ppm. KT3 (ethanolic peels extract of Kintamani orange) had the lowest EC50 of FRAP capacity 131.7 ppm, while ascorbic acid standard gave EC50 of FRAP capacity 203 ppm and its exposed that antioxidant capacity of KT3 around two times of potency of ascorbic acid using FRAP method. Previous study [12] stated that EC50 of FRAP of acetone peels extract of Citrus sinensis, Citrus paradisii and Citrus maxima were 310 ppm, 630 ppm and 710 ppm, while in ethyl acetate peels extract were 480 ppm, 520 ppm and 1300 ppm respectively. The result in present study exposed that orange peels extracts from three different growth location gave different antioxidant capacities using DPPH and FRAP methods.

The presence of total phenolic might contribute to antioxidant activity [6]. Phenolic acid might contributed in antioxidant activity. Phenyl acetic acid and benzoic acid had lower antioxidant capacity than cinnamic acid [22]. In present study total phenolic of ethanolic peels extract of Kintamani orange, Jember orange and Banyuwangi orange were 10.08 g GAE/100 g, 8.85 g GAE/100 g and 9.54 g GAE/100 g, while in ethyl acetate peels extract were 4.23 g GAE/100 g, 5.76 g GAE/100 g and 3.95 g GAE/100 g for KT2, JB2 and BW2 respectively. Oboh [12] exposed that total phenolic in acetone extract of orange peels was 10.5 mg GAE/g and ethyl acetate extract was 6.8 mg GAE/g. Study by Karoui [4] demonstrated that methanolic extract of Tunisian bitter orange (Citrus aurantium) was 71.25 %, while Londono [23] revealed that total phenolic in water extract of Tahiti lime (Citrus latifolia), sweet orange (Citrus sinensis) and oneco tangerine (Citrus reticulata) peels were 74.80 mg GAE/g, 66.36 mg GAE/g and 58.68 mg GAE/g respectively.

Total flavonoid of ethanolic extract in the present study exposed that Jember orange peels had the highest total flavonoid (1.50 g QE/100 g) compared to Kintamani orange (1.22 g QE/100 g) and Banyuwangi orange (0.93 g QE/100 g), while total flavonoid in ethyl acetate peels extract of KT2, JB2 and BW2 were 9.94 g QE/100 g, 3.37 g QE/100 g and 5.64 g QE/100 g respectively. It was different with previous study [4] exposed that total flavonoid in methanolic extract of orange peels was 23.13 %. Oboh [12] demonstrated that total flavonoid in acetone extracts (free flavonoid) and ethyl acetate extract (bound flavonoid) of orange peels were 1.3 mg/g and 0.21 mg/g respectively.

The data in Table 4 exposed that there were positively high correlation between total phenolic content in all of orange peels sample (Kintamani orange, Jember orange and Banyuwangi orange) and antioxidant capacities using two methods DPPH and FRAP assays. Total phenolic content in Kintamani orange, Jember orange and Banyuwangi orange had high and positive correlation with DPPH scavenging capacity that were r = 0.984, p<0.01, r = 0.730, p<0.05, r = 0.976, p<0.01, while with FRAP capacity were r = 0.984, p<0.01, r = 0.998, p<0.01 and r = 0.962, p<0.01 respectively. Based on this data it could be concluded that antioxidant capacities in all orange peels sample by DPPH and FRAP methods might be estimated indirectly by determining their total phenolic content.

Phenolic acid had the lower antioxidant capacity than flavonoid [22]. Flavonoid would give higher antioxidant capacity if flavonoid had OH in ortho C 3',4', OH in C3, oxo function in C4, double bond at C2 and C3. The OH with ortho position in C3'-C4' had the highest influence to antioxidant capacity of flavonoid. The flavonoid glycosides would give lower antioxidant capacity than flavonoid aglycones [22]. Fig 4 showed that total flavonoid in KT2 (ethyl acetate peels extracts of Kintamani orange) was higher than the other extracts and its DPPH scavenging capacities (55.46 %) was higher than the other extracts. Based on this data it can predicted that many flavonoids in ethyl acetate peels extract of Kintamani orange were flavonoid that had OH in ortho C3',4', OH in C3, oxo function in C4, double bond at C2 and C3.

Carotenoid with more double bonds would give higher scavenging free radical capacity [24]. Carotenoid that consisted of maximum 7 double bonds gave lower scavenging radical free capacity than more double bonds [25]. In previous study [26] revealed that increasing in lipophilicity of carotenoid would increase scavenging radical capacity. Lycopene was effective to reduce Fe (III), because of it had 11 conjugated double bonds. Carotenoid such as phytoene, phytofluene, neurosporene that consisted of 3, 5 and 9 conjugated double bonds respectively, did not show significant capacity to reduce Fe (III) [27]. Beta carotene was used as standard because of it had conjugation double bonds doe to its ability to scavenge free radicals [28]. Based on the above data, it could be seen that many carotenoid in all orange peels sample were lower than 7 double bonds, that had no or low antioxidant capacity.

FRAP and DPPH methods had different mechanism reaction. Mechanism of DPPH that was electron transfer assays [29] and FRAP was redox assays. The results of this study demonstrated that DPPH scavenging capacity all of orange peels sample were linear with FRAP capacity. Sample will act as antioxidant in FRAP assays if sample had reduction potential was lower than reduction potential of Fe (III)/Fe (II) that was 0.77 V, so the sample had reducing power to reduce Fe (III) to Fe(II) and this sample will be oxidized.

The Pearson's correlation coefficient of various extracts from sweet orange peels indicated that all of sample had positive and high correlation between DPPH scavenging capacities and FRAP capacities. It could be seen that antioxidant capacities in all of orange peels sample by DPPH assays were linear with FRAP assays.

Table 1: DPPH scavenging capacities and FRAP capacities of n-hexane peels extracts

Sample	DPPH scavenging capacitiy (%)	FRAP capacity (%)
KT1	50.31 ± 0.25 a	0.21 ± 0.03 a
JB1	50.13 ± 0.22 a	1.96 ± 0.51 b
BW1	50.25 ± 0.20 a	0.13 ± 0.08 a
Ascorbic acid	98.49 ± 0.33	39.65 ± 0.28
P value	< 0.05	< 0.05

Note: a –b = means within a column with the different letter were significantly different (p<0.05)

Table 2: DPPH scavenging capacities and FRAP capacities of ethyl acetate peels extracts

Sample	DPPH scavenging capacitiy (%)	FRAP capacity (%)
KT2	55.46 ± 0.89 a	6.06 ± 0.15 a
JB2	54.96 ± 0.55 b	3.79 ± 0.21 b
BW2	53.99 ± 0.83 a	4.03 ± 0.18 b
Ascorbic acid	98.49 ± 0.33	39.65 ± 0.28
P value	< 0.05	< 0.05

Note: a –b = means within a column with the different letter were significantly different (p<0.05)

Table 3: DPPH scavenging capacities and FRAP capacities of ethanolic peels extracts

Sample	DPPH scavenging capacitiy (%)	FRAP capacity (%)
KT3	51.44 ± 0.38 a	20.07 ± 0.15 a
JB3	54.03 ± 0.74 a	13.61 ± 0.31 b
BW3	50.27 ± 0.20 a	10.05 ± 0.25 c
Ascorbic acid	98.49 ± 0.33	39.65 ± 0.28
P value	< 0.05	< 0.05

Note: a –c = means within a column with the different letter were significantly different (p<0.05)

Table 4. Pearson's correlation coefficient of total phenolic, flavonoid, carotenoid of peels extract from sweet orange peels and DPPH scavenging capacities, FRAP capacities

	Total Phenolic	Total Flavonoid	Total Carotenoid	DPPH KT	DPPH JB	DPPH BW
DPPH KT	0,984**	-0,658^{ns}	-0,701*			
DPPH JB	0,730*	-0,092^{ns}	-0,971**			
DPPH BW	0,976**	-0,789*	-0,544^{ns}			
FRAP KT	0,984**	-0.603^{ns}	-0,770*	0,975**		
FRAP JB	0,998**	-0,701*	-0,669*		0,977**	
FRAP BW	0,962**	-0,512^{ns}	-0,835**			0,965**

Note: DPPH = DPPH scavenging capacity, FRAP = FRAP capacity, KT = sample Kintamani, JB = sample Jember, BW = sample Banyuwangi, ns = not significant, * = significant at p < 0.05, ** = significant at p < 0.01

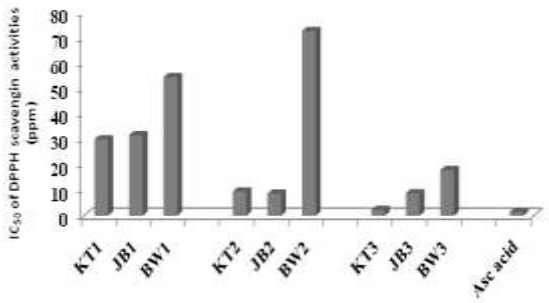

Fig 1: IC_{50} of DPPH scavenging capacities in various sweet orange peels extracts

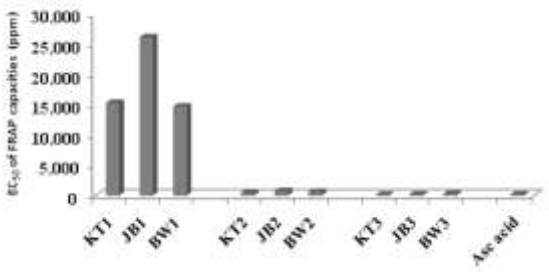

Fig 2: EC_{50} of FRAP capacities in various sweet orange peels extracts Total phenolic in various sweet orange peels extracts

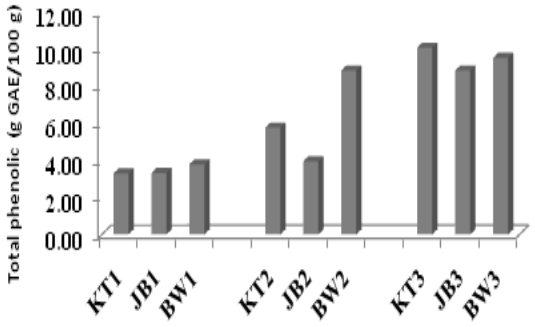

Fig 3: Total phenolic content in various sweet orange peels extracts

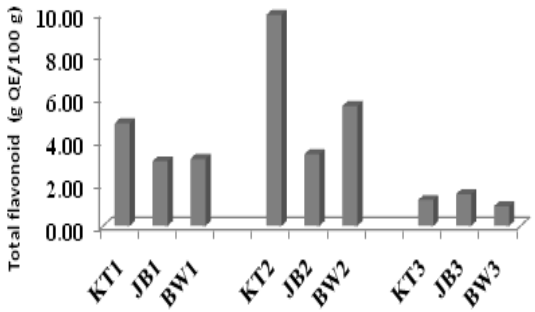

Fig 4: Total flavonoid content in various sweet orange peels extracts

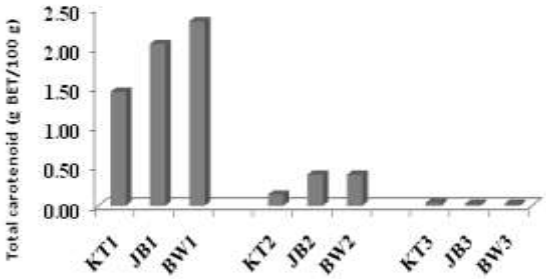

Fig 5: Total carotenoid content in various sweet orange peels extracts

IC50 of DPPH scavenging capacity and EC50 of FRAP capacity

CONCLUSION

To assess the antioxidant capacity of sample, variety of methods must be used in parallel, because different methods often give different results. The ethanol extracts of Kintamani orange peels had the lowest IC50 of DPPH scavenging capacities that was very strong antioxidant and also gave the lowest EC50 of FRAP capacities. All of orange peels sample had positively high correlation between total phenolic content with DPPH and FRAP capacities. Antioxidant capacity using FRAP and DPPH assays in all of orange peels sample might be estimated indirectly by using total phenolic content. Phenolic compounds were the major contributor in antioxidant capacity in all of orange peels sample. There were all of DPPH scavenging capacities in peels extracts from sweet orange sample linear with FRAP capacities. Kintamani orange, Jember orange, Banyuwangi orange peels extracts may be exploited as natural antioxidant in food applications as well as for health supplements or functional food, to alleviate oxidative stress.

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