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Research Article

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In vitro antioxidant activities, phenolic, flavonoid and carotenoid content from different polarity extracts of five citrus peels using DPPH and Cuprac method

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ABSTRACT

The objectives of this research were to study antioxidant activities from various extracts of five Citrus peels using two methods of antioxidant assays which were DPPH (2.2-diphenyl-1-picrylhydrazyl) and CUPRAC (Cupric ion Reducing Antioxidant Capacity); and correlation of total flavonoid, phenolic, and carotenoid content in various extracts of five Citrus peels with IC_{50} of DPPH antioxidant activities and EC_{50} of CUPRAC antioxidant capacities. Extraction was performed by reflux apparatus using different polarity solvents. The extracts were evaporated using rotary evaporator. Antioxidant capacities were tested using DPPH and CUPRAC assays. Determination of total phenolic, and carotenoid content performed by UV-visible and their correlation with IC_{50} of DPPH antioxidant activities and EC_{50} of CUPRAC antioxidant capacities were analyzed by Pearson's method. Ethyl acetate extract of C.sinensis peels (MA2) had the lowest IC_{50} of DPPH scavenging activity 11.04 µg/ml, while n-hexane extract of C.sinensis peels (MA1) had the lowest EC_{50} of CUPRAC capacity 22.93 µg/ml. Ethyl acetate extract of C. sinensis (MA2) the highest total phenolic content (431.3 mg GAE/100 g), MA1 had the highest total flavonoid content (2037.1 mg QE/100 g), and the highest total carotenoid content (509.5 mg BE/100 g). There was a negatively high correlation between total phenolic content in C. aurantifolia peel extracts with their IC_{50} of DPPH scavenging activity and EC_{50} of CUPRAC assays. The IC_{50} of DPPH scavenging activities in C. aurantifolia, C. limon, C. maxima, C. sinensis peel extracts gave linear result with EC_{50} of CUPRAC capacities.

Keywords: Antioxidants, DPPH, CUPRAC, peel, five Citrus, phenolic, flavonoid, carotenoid

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INTRODUCTION

Oxidative stress has related with many degenerative diseases. Antioxidant has potency to mobilize protective effects against oxidative stress. Phenolic compounds are commonly found in plants, and they have revealed to have multiple biological effects, including antioxidant activity [1-2]. Many studies had revealed that phenolic content in plants could be correlated to their antioxidant activities. Plants contained phenolic and polyphenol compounds can act as antioxidant [3-5].

Some of antioxidant methods such as CUPRAC (Cupric ion Reducing Antioxidant Capacity) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were widely used to predict antioxidant capacity of fresh fruits, beverages, and food [2,6]. revious studies [2,6-7] revealed that DPPH and CUPRAC methods could be used to determine antioxidant activity in many plants extracts. The previous studies [8-11] exhibited antioxidant capacities of some plants including Citrus sp.

The objectives of this research were to study antioxidant activities of various different polarities extracts (n-hexane, ethyl acetate and ethanol) from five Citrus (*Citrus aurantifolia, Citrus limon, Citrus hystrix, Citrus maxima, Citrus sinensis*) peels using DPPH and CUPRAC assays; and correlations of their antioxidant capacities with total phenolic, and carotenoid content in each extract.

EXPERIMENTAL SECTION

Materials: Neocuproine, DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid, quercetin, beta carotene purchased from Sigma-Aldrich (MO, USA), cupric chloride, peels from five Citrus. All other reagents were analytical grades.

Preparation of sample: Peels from five Citrus: *C. aurantifolia* sample NI from Subang, *C. limon* as sample LE, *C. hystrix* as sample PU, *C. maxima* sample BA and *C. sinensis* as sample MA from Bandung were thoroughly washed with tap water, sorted while wet, cut, dried, and grinded into powder.

Extraction: Three hundred grams of powdered samples were extracted by reflux apparatus using increasing polarity of solvents. The extraction using n-hexane was repeated three times. The remaining residue was then extracted three times using ethyl acetate. Finally the remaining residue was extracted three times using ethanol. So there were five n-hexane extracts (NI1, LE1, PU1, BA1 and MA1), five ethyl acetate extracts (NI2, LE2, PU2, BA2 and MA2) and five ethanolic extracts (NI3, LE3, PU3, BA3 and MA3).

IC_{50} of DPPH scavenging activity: Preparation of DPPH solution was adopted from Blois [12] with minor modification. Various concentration of each extract were pipetted into DPPH solution 50 µg/ml (1:1) to initiate the reaction for obtaining a calibration curve. After 30 minutes incubation, the absorbance was read at wavelength 515 nm by using spectrophotometer UV-Vis Hewlett Packard 8435. Methanol was used as a blank. Ascorbic acid was used as standard. Analysis was done in triplicate for standard and each extract. Antioxidant activity of each extract was determined based on the reduction of DPPH absorbance by calculating percentage of antioxidant activity [13]. IC_{50} of DPPH scavenging activity of each extract can be calculated using its calibration curve.

EC_{50} of CUPRAC capacity: Preparation of CUPRAC solution was adopted from Apak [6]. The CUPRAC solution was prepared in ammonium acetate buffer pH 7. Various concentration of each extract were pipetted into CUPRAC solution 50 µg/ml (1:1) to initiate the reaction for obtaining a calibration curve. After 30 minutes incubation, the absorbance was read at wavelength 450 nm by using spectrophotometer UV-Vis Hewlett Packard 8435. Ammonium acetate buffer was used as a blank. Ascorbic acid was used as standard. Analysis was done in triplicate for standard and each extract. Antioxidant capacity of each extract was determined based on increasing in Cu (I)-neocuproine absorbance by calculating percentage of antioxidant capacity [6]. EC_{50} of CUPRAC capacity of each extract can be calculated using its calibration curve.

Total phenolic content (TPC): Total phenolic content were measured using the modified Folin-Ciolcalteu method adapted from Pourmorad [14]. The absorbance was read at wavelength 765 nm. Analysis was done in triplicate for each extract. Standard solutions of gallic acid 30-180 µg/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 (mg GAE/100 g).

Total flavonoid content (TFC): Total flavonoid content was measured using adapted method from Chang *et al* [15]. The absorbance was read at wavelength 415 nm. Analysis was done in triplicate for each extract. Standard solutions of quercetin 20-120 µg/ml were used to obtain a standard curve. The total flavonoid content was reported as percentage of total quercetin equivalents per 100 g extract (mg QE/100 g).

Total carotenoid content (TCC): Total carotenoid content was measured using the modified carotene method adapted from Thaipong *et al* [2]. Each extract were diluted in n-hexane. The absorbance was read at wavelength 470 nm. Analysis was done in triplicate for each extract. Standard solutions of beta carotene 5-70 µg/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 (mg BE/100 g).

Statistical Analysis: Analysis of each sample 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 $p < 0.05$ and post-hoc Tukey procedure) was carried out with SPSS 22 for Windows. Correlations between the total phenolic, and carotenoid content with antioxidant capacities were made using the Pearson's method ($p < 0.01$).

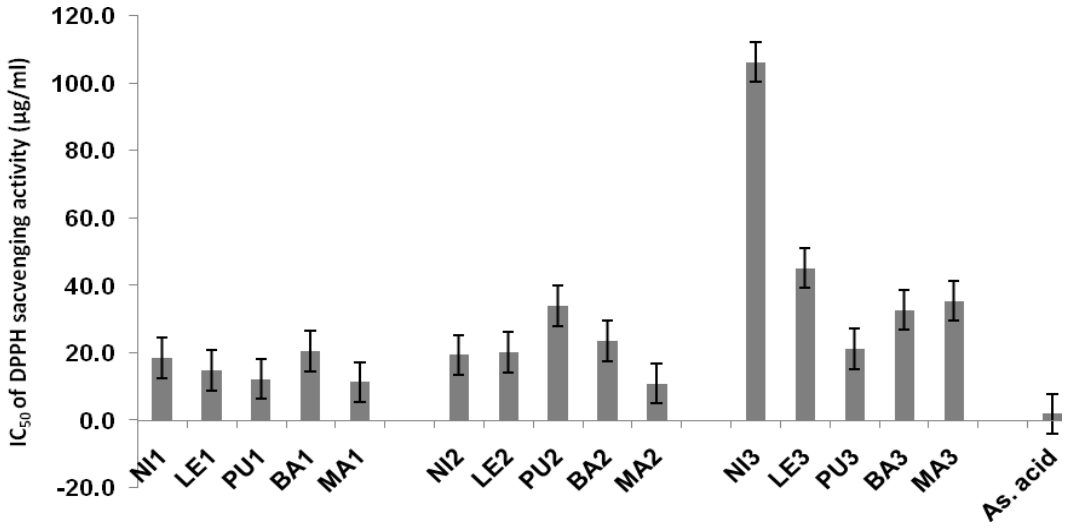
RESULTS AND DISCUSSION

The previous study [3,10,16-17] that Citrus sp had antioxidant capacity. There were no stud regarding antioxidant capacity of three various polarities extracts (which were n-hexane, ethyl acetate and ethanol) of peels from five Citrus using DPPH and CUPRAC assays.

IC_{50} of DPPH scavenging activity and EC_{50} of CUPRAC capacity: The DPPH is stable free radicals which dissolve in methanol or ethanol, and its colors show characteristic absorption at wavelength 515-520 nm. Colors of

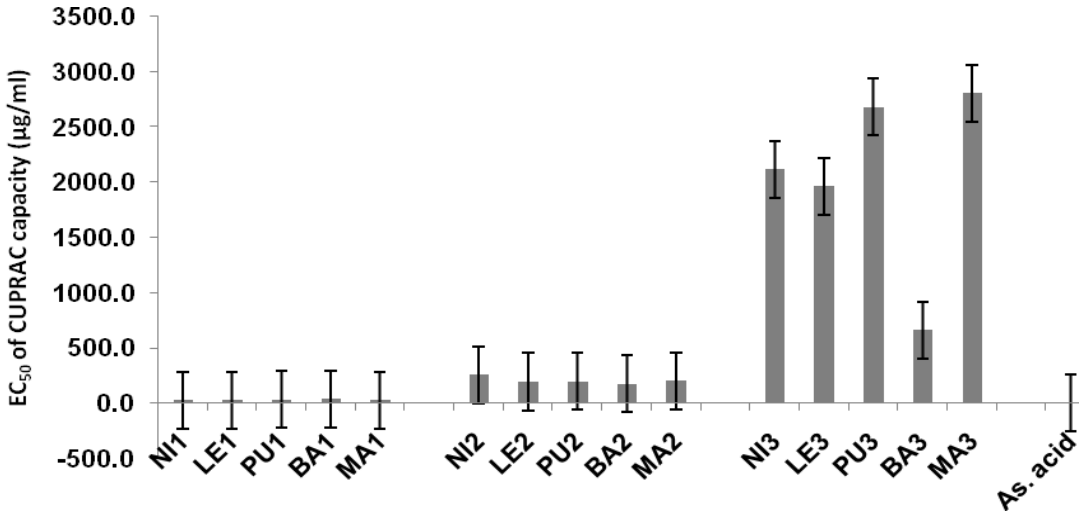
DPPH would be changed when the free radicals were scavenged by antioxidant [6,18]. Reagent of CUPRAC is $CuCl_2$ that combined with neocuproine in ammonium acetate buffer pH 7. Cu (II) will be reduced to Cu (I). Complex Cu (I) – neocuproine gives yellow color and show characteristic absorption at wavelength 450 nm. Intensity of yellow color depends on amount of Cu (II) that is reduced to Cu (I). If a sample reduces Cu (II) to Cu (I), at the same time it will be oxidized, so that sample can act as antioxidant. Sample will act as antioxidant in CUPRAC assays if sample had reduction potential lower than reduction potential of Cu (II)/Cu (I) which was 0.46 V, so the sample can reduce Cu (II) to Cu (I) and this sample will be oxidized.

The IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities in various peel extracts from five Citrus using DPPH and CUPRAC assays were shown in Fig 1 and Fig 2. The half minimum inhibitory concentration (IC_{50}) of DPPH scavenging activities and EC_{50} of CUPRAC capacities compared to IC_{50} ascorbic acid standard and EC_{50} ascorbic acid standard.



n=3

Fig 1: IC_{50} of DPPH scavenging activities in various peel extracts from five Citrus



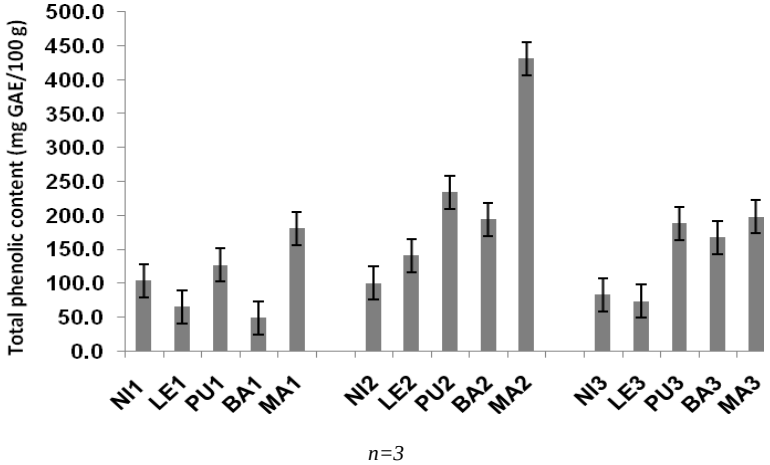
n=3

Fig 2: EC_{50} of CUPRAC capacities in various peel extracts from five Citrus

The half minimum inhibitory concentration of DPPH scavenging activity is the concentration of sample or standard that can inhibit 50% of DPPH scavenging activity, while EC_{50} of CUPRAC capacity is the concentration of sample or standard that can exhibit 50% of CUPRAC capacity. The lowest IC_{50} or EC_{50} means had the highest antioxidant capacity. The IC_{50} or EC_{50} were used to determine antioxidant capacity of a sample that compared to standard. Sample that has IC_{50} or EC_{50} less than 50 µg/ml is a very strong antioxidant, 50-100 µg/ml is a strong antioxidant, 101-150 µg/ml is a medium antioxidant, while IC_{50} or EC_{50} greater than150 µg/ml is a weak antioxidant [12].

In the DPPH method, IC_{50} of various peel extracts from five Citrus ranged from 11 to 106.4 µg/ml. Ethyl acetate extract of *C. sinensis* peels (MA2) had the lowest IC_{50} of DPPH radical scavenging activity 11 µg/ml, followed by MA1 11.4 µg/ml, and PU1 12.3 µg/ml, while ascorbic acid standard gave IC_{50} of DPPH scavenging activity 2 µg/ml. Based on the value of IC_{50} of DPPH scavenging activity it could be concluded that MA2, MA1, and PU1 could be categorized as strong antioxidant. The current study showed that IC_{50} of DPPH scavenging activities of ethanolic peel extract of *C. sinensis* and *C. limon* from Bandung using reflux extraction were 35.5 and 45.3 µg/ml, while the previous study [17] ethanolic peel extract of *C. sinensis* from three locations Kintamani, Jember and Banyuwangi were 2.25, 8.84, 17.94 µg/ml, respectively. Study by Ghasemi [3] revealed that methanolic extract of *C. sinensis* var. Sungin, *C. sinensis* var. Valencia, *C. sinensis* var Navel and *C. limon* using percolation extraction were 1.7, 2.1, 1.1 and 1.4 mg/ml. N-hexane extract of *C. sinensis* peels (MA1) had the lowest EC_{50} of CUPRAC capacity (22.9 µg/ml) while ascorbic acid standard gave EC_{50} of CUPRAC capacity 5.7 µg/ml. It showed that potency of ascorbic acid was around four times as much as the potency of MA1 using CUPRAC assays.

Total phenolic content in various peel extracts from five Citrus: The total phenolic content among the various extracts expressed in term of gallic acid equivalent using the standard curve equation y = 0.006x - 0.055, R^2 = 0.998. The total phenolic content in various peel extracts from five Citrus showed different result ranged from 48.42 to 431.25 mg GAE/100 g. Ethyl acetate extract of *C. sinensis* peels (MA2)had the highest phenolic content (431.25 mg GAE/100 g) (Fig 3).

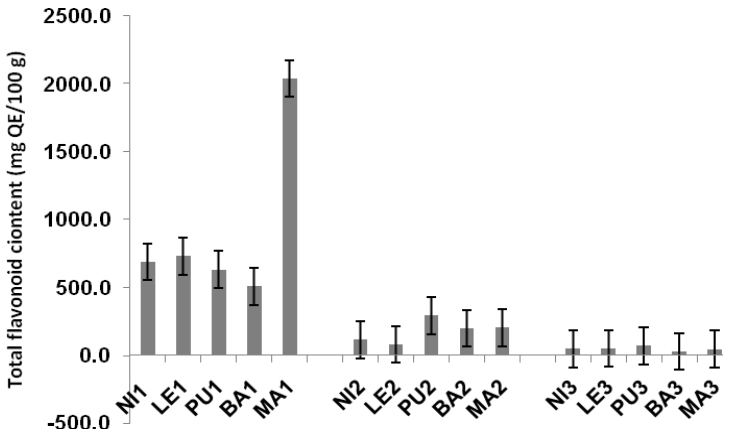


n=3

Fig 3: Total phenolic content in various Citrus peel extracts

The total phenolic content can be contributed in antioxidant capacity [4]. Phenolic acid might contributed in antioxidant capacity. Phenyl acetic acid and benzoic acid had lower antioxidant capacity than cinnamic acid [19]. The previous study [3] revealed that TPC in methanolic peel extract of *C. sinensis* var. Washington Navel, *C. sinensis* var. Sungin, *C. sinensis* var. Valencia were 160.3, 153.8, 132.9 mg GAE/g extract, respectively. It was contrast with the study which exposed that TPC in ethanolic peel extract of *C. sinensis* was 197.6 mg GAE/100 g. The present study demonstrated that TPC in ethanolic extract of *C. limon* was 73.4 mg GAE/100 g extract, while research by Ghasemi [3] found that TPC in methanolic extract of *C. limon* was 131 mg GAE/g extract. Research by Londono [16] stated that TPC in methanolic peel extract of *C. latifolia*, *C. sinensi*s and *C. reticulata* by ultrasound-assisted extraction method were 74.8, 66.36, 58.68 mg GAE/g extract, respectively. TPC in dry material (19.595 mg GAE/g peel dry matter) were higher than wet material and time of extraction had no influence on TPC. Previous study [17] expressed that ethanolic peel extract of *C. sinensis* from three different growth locations Kintamani, Jember and Banyuwangi had TPC 10.08, 8.85, 9.54 g GAE/100 g extract, respectively. Hayat [1] revealed that TPC in methanolic peel extract of *C. reticulata* by microwave - assisted extraction method (175.22 µg GAE/g extract) was higher than ultrasound extraction (162.93 µg GAE/g extract) and rotary extraction (79.80 µg GAE/g extract). *C. reticulata* contained p-hydroxybenzoic acid, vanillic acid, p-coumaric acid and ferulic acid which could act as antioxidant [1]. TPC in fruit juice of *C. hystrix*, *C. aurantifolia*, *C. microcarpa* and C. *sinensis* were 490.74, 211.70, 105, 135.3 mg GAE/100 ml juice, respectively [10].

Total flavonoid content in various peel extracts from five Citrus: The total flavonoid content among the various extracts expressed in term of quercetin equivalent using the standard curve equation y = 0.007x - 0.027, R^2 = 0.995. The total flavonoid content in various peel extracts from five Citrus showed different results within the range of 26 to 2037.1 mg QE/100 g (Fig 4). N-hexane extract of *C.sinensis* peels (MA1) had the highest total flavonoid content (2037.1 mg QE/100 g) and ethanolic extract of *C. maxima* peels (BA3) had the lowest (26 mg QE/100 g).



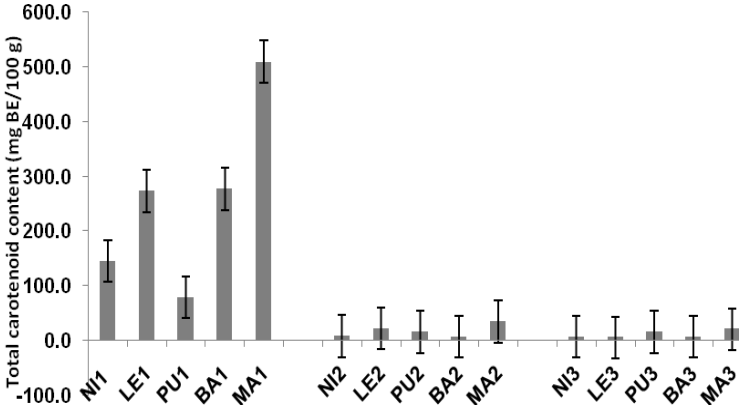
n=3

Fig 4: Total flavonoid content in various Citrus peel extracts

In the present study TFC in ethanolic extract of *C. sinensis* was 45.5 mg QE/100 g, while in previous research by Ghasemi [3] revealed that TFC in methanolic extract of *C. sinensis* var. Washington Navel, *C. sinensis* var. Sungin, *C. sinensis* var. Valencia were 23.2, 2.1, 7.2 mg QE/g extract, respectively. TFC in methanolic extract of *C. limon* was 16.2 mg QE/g extract, while in the present study TFC in ethanolic extract of *C. limon* was 48.6 mg QE/100 g. Ghafar [10] demonstrated that TFC in fruit juice of *C. hystrix*, *C. aurantifolia*, *C. microcarpa* and C. *sinensis* were 22.25, 10.67, 8.77, 2.99 mg QE/100 ml juice. Study by Fidrianny [17 exhibited that ethanolic peel extract of *C. sinensis* from Kintamani, Jember and Banyuwangi had TFC 1.22, 1.50, 0.93 g QE/100 g extract, respectively.

Total carotenoid content in various peel extracts from five Citrus: The TCC among the various extracts expressed in term of beta carotene equivalent using the standard curve equation y = 0.012 x - 0.008, R^2 = 0.998. The total carotenoid content in various peel extracts from five Citrus showed different result in the range of 5.4 to 509.5 mg BE/100 g (Fig 5). N-hexane extract of *C. sinensis* peels (MA1) had the highest carotenoid content (509.5 mg BE/100 g), while ethanolic extract of *C. limon* peels (LE3) had the lowest carotenoid content (5.4 mg BE/100 g).

In the previous study [17] showed that TCC of ethanolic peel extract of *C. sinensis* from Kintamani, Jember and Banyuwangi were 0.037, 0.021, 0.022 g BE/100 g extract, respectively. It was similar with the result in the current study which expressed that TCC in ethanolic peel extract of *C. sinensis* from Bandung was 20.9 mg BE/100 g extract.



n=3

Fig 5: Total carotenoid content in various Citrus peel extracts

Correlations between total phenolic, flavonoid, carotenoid content with DPPH scavenging activities, and CUPRAC capacities in various peel extracts from five Citrus: Pearson's correlation coefficient was positively high if $0.61 \leq r \leq 0.97$ [2] and negatively high if $-0.61 \leq r \leq -0.97$. The highest antioxidant activity will be given by sample which had the lowest IC_{50} of DPPH scavenging activity or EC_{50} of CUPRAC capacity. So the good correlation between TPC, TFC and TCC with IC_{50} DPPH or EC_{50} CUPRAC will be shown in highly and negative correlation.

Table 1. Pearson's correlation coefficient of total phenolic, carotenoid of peel extracts from five Citrus and IC_{50} of DPPH scavenging activities, EC_{50} of CUPRAC capacities

	TPC	TFC	TCC	IC_{50} CUPRAC NI	IC_{50} CUPRAC LE	IC_{50} CUPRAC PU	IC_{50} CUPRAC BA	IC_{50} CUPRAC MA
IC_{50} DPPH NI	-0,987**	-0,586ns	-0,512ns	0,996**				
IC_{50} DPPH LE	-0,253ns	-0,668*	-0,679*		0,995**			
IC_{50} DPPH PU	0.981**	-0,513ns	-0,808**			-0,053		
IC_{50} DPPH BA	0.547ns	-0,895**	-0,684*				0,996**	
IC_{50} DPPH MA	-0,459ns	-0,548ns	-0,508ns					0,996**
IC_{50} CUPRAC NI	-0,998**	-0,658*	-0,589ns					
IC_{50} CUPRAC LE	-0,339ns	-0,599ns	-0,611*					
IC_{50} CUPRAC PU	0.142ns	-0,831**	-0,547ns					
IC_{50} CUPRAC BA	0.532ns	-0,887**	-0,671*					
IC_{50} CUPRAC MA	-0,395ns	-0,605*	-0,568ns					

*Note: DPPH = DPPH scavenging activity, CUPRAC = CUPRAC capacity, TPC = total phenolic content, TFC = total flavonoid content, TCC = total carotenoid content, NI = sample NI, LE = sample LE, PU = sample PU, BA = sample BA, , MA = sample MA, ns = not significant, * = significant at $p < 0.05$, ** = significant at $p < 0.01$*

The highest and negative between TPC and IC_{50} of DPPH scavenging activity ($r = -0.987$, $p<0.01$) was given by sample NI. The highest and negative correlation between TPC and EC_{50} of CUPRAC capacity ($r = -0.998$, $p<0.01$) was given also by sample NI (Table 1). It means that increasing in TPC in sample NI would gave increasing in antioxidant activity of sample NI by DPPH and CUPRAC method. Based on this data it could be concluded that antioxidant capacities in *C. aurantifolia* peel extracts with DPPH and CUPRAC assays might be estimated indirectly by determining their TPC. In previous study [17] found that TPC in peel extracts of *C. sinensis* from Kintamani, Jember and Banyuwangi had high correlation with their percentage of DPPH scavenging activities. Ghafar [10] stated that there was no correlation between TPC in fruit juice of *C. aurantifolia* with its percentage of DPPH scavenging activity, but there was high correlation with its percentage of FRAP capacity.

Phenolic compound included tannins, flavonoid, phenolic acid and other compounds. Flavonoid which OH in A ring and/or B ring will be included in phenolic groups. Flavonoid had higher antioxidant capacity than phenolic acid [19]. Position OH in ortho C-3',4', OH in C3, oxo function in C4, double bond at C2 and C3 would influence higher antioxidant capacity in flavonoid. Flavonoid which has OH with ortho position in C3'-C4' had the highest antioxidant capacity. The flavonoid glycosides would give lower antioxidant capacity than flavonoid aglycone [19].
Pearson's correlation coefficient between TFC form various extracts of five Citrus and their antioxidant capacities demonstrated that only TFC in sample BA ($r = -0.895$, $p<0.01$) and sample LE ($r = -0.668$, $p<0.05$) had highly negative correlation with IC_{50} of DPPH scavenging activities, while TFC in sample BA ($r = -0.887$, $p<0.01$), sample PU ($r = -0.831$, $p<0.01$) and sample MA ($r = 0.605$, $p<0.05$) had highly negative correlation with EC_{50} of CUPRAC capacities.

In Figure 4 it could be seen that TFC in n-hexane extract of sample *C. maxima* (BA1) 507.9 mg QE/100 g was higher than TFC in ethanolic extract of sample *C. hystrix* (PU3) 68.7 mg QE/100 g, but IC_{50} of DPPH scavenging activities of BA1 (20.7 µg/ml) was similar with PU3 (21.2 µg/ml). Based on the data above it can be predicted that many flavonoids in n-hexane extracts of sample *C. maxima* had OH in C5, C7, or C3' only, or C4' only, or C3 only without oxo function in C4, that had no and low antioxidant capacities. In contrast, almost all of flavonoid in ethanolic extracts of sample *C. hystrix* were flavonoid that had OH in position which can influence high antioxidant capacities.

The correlation between TCC and their antioxidant capacities that peel extracts of *C. limon*, and *C. maxima* had negative and high correlation with IC_{50} of DPPH scavenging activities ($r = -0.679$, $r = -0.684$, $p<0.05$) and EC_{50} of CUPRAC capacities ($r = -0.611$, $r = -0.671$, $p<0.05$), respectively. It means higher in TCC in peel extract of *C. limon*, and *C. maxima* would give higher antioxidant activities by using DPPH and CUPRAC methods.

Carotenoid had antioxidant capacity by scavenging free radical. More double bonds in carotenoid would give higher free radical scavenging capacity [20]. Carotenoid that consisted of above 7 double bonds gave higher free radical scavenging activity than 7 double bonds [21]. revious study [22] demonstrated that increasing in lipophilicity of carotenoid would increase free radical scavenging capacity. Beta carotene was used as standard because it had conjugation double bonds due to its ability to scavenge free radicals [23-24].

In Figure 5 it could be seen that TCC in n-hexane extract of sample NI (NI1) 145.3 mg BE/100 g was higher than ethyl acetate extract of sample NI (NI2) 7.9 mg BE/100 g, but IC_{50} of DPPH scavenging activity of NI1 (18.6 µg/ml) was similar with NI2 (19.4 µg/ml). Based on the data it can be concluded that almost all of carotenoid in NI2 had more than 7 double bonds, while in NI1 only a little of carotenoid had more than 7 double bonds.

The CUPRAC and DPPH methods had different mechanism reaction. Mechanism of DPPH that was electron transfer assays [25] and CUPRAC was redox assays [6]. So the results of the two methods not always linear. The Pearson's correlation coefficient indicated that only four Citrus *C. aurantifolia, C. limon, C. maxima, C. sinensis* had positively high correlation between IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities. It could be seen that antioxidant activities of sample *C. aurantifolia, C. limon, C. maxima, C. sinensis* gave linear result by DPPH and CUPRAC assays.

CONCLUSION

To assess the antioxidant capacity of sample, variety of methods must be used in parallel, because different methods could give different results. All of extracts (except ethanolic extract of *C. aurantifolia*) had IC_{50} of DPPH scavenging activities less than 50 µg/ml that means were very strong antioxidant. The negative high correlation between total phenolic with IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities was given by *C. aurantifolia* peel extracts. Antioxidant capacity using DPPH and CUPRAC assays in *C. aurantifolia* peel extracts might be estimated indirectly by using total phenolic content. Phenolic compounds were the major contributor in antioxidant capacity in *C. aurantifolia* peel extracts. Antioxidant capacities of *C. aurantifolia, C. limon, C. maxima, C. sinensis* gave linear result by DPPH and CUPRAC assays. *C. aurantifolia, C. limon, C. hystrix, C. maxima, C. sinensis* may be exploited as natural antioxidant sources.

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