Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(4); 611-618

ISSN: 0975-4873

Research Article

Antioxidant Activities Evaluation of Citrus Leaves Extracts from West Java-Indonesia Using DPPH and FRAP Assays

Irda Fidrianny*, Andi Amaliah, Sukrasno

Pharmaceutical Biology Research Group, School of Pharmacy, Bandung Institute of Technology, Indonesia

Available Online: 31st March, 2016

ABSTRACT

The aim of this research were to measure antioxidant activity from different polarities leaves extracts of five citrus 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 different polarities extracts of citrus leaves with their IC_{50} of DPPH and IC_{50} of FRAP antioxidant activities. Extraction was performed by reflux using different polarity solvents. The extracts were evaporated using rotary evaporator. Antioxidant activities using DPPH and FRAP assays, determination of total phenolic, flavonoid and carotenoid content were performed by UV-visible spectrophotometry and its correlation with IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities were analyzed by Pearson's method. All of different polarities leaves extracts of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* (except n-hexane extract of *C. hystrix*, n-hexane extract of *C. aurantifolia* and ethanolic extract of *C. maxima*) were very strong antioxidant, using DPPH assays. Phenolic compounds in *C. aurantifolia* leaves extracts were the major contributor in IC_{50} of DPPH scavenging activity and EC_{50} of FRAP capacity. Leaves extract of *C. hystrix* and *C. aurantifolia* had linear result in DPPH and FRAP assays.

Keywords: Antioxidant, DPPH, FRAP, citrus leaves, phenolic, flavonoid, carotenoid

INTRODUCTION

Antioxidant can prevent oxidative stress which can cause many diseases. Phenolic compounds are commonly found in plants, and they have been reported to have multiple biological effects, included antibacterial and antioxidant activity[1,2]. Previous study[3-5] exposed that phenolic and flavonoid content could be correlated to their antioxidant activities. Plants include citrus contain phenolic and flavonoid compounds[6-8]. DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power) and ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) can be used to predict antioxidant activity of vegetables, fruits and food[3,9]. Previous research[9-11] expressed that DPPH, FRAP and ABTS methods could be used to determine antioxidant activity in many plants extracts. The previous study[12,13] stated that citrus had antioxidant activities by using ABTS, DPPH, and FRAP assays. The aim of this research were to measure antioxidant activities of different polarities leaves extracts (n-hexane, ethyl acetate and ethanol) of five leaves in West Java-Indonesia using DPPH and FRAP assays, and correlations of total phenolic, flavonoid and carotenoid content with their antioxidant activities.

MATERIALS AND METHODS

Materials

DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), TPTZ (TPTZ (2-4-6-tripyridyltriazine), gallic acid, quercetin, beta carotene was purchased from Sigma-Aldrich (MO, USA), citrus leaves, ethanol. All other reagents were analytical grades.

Preparation of sample

Citrus leaves were: *Citrus reticulata* namely as CR and *Citrus hystrix* as CH were collected from Garut- West Java, *Citrus limon* as CL and *Citrus aurantifolia* as CA from Ciwidey-West Java, *Citrus maxima* as CM from Bandung-West Java, were thoroughly washed with tap water, wet sortation, cut, dried and grinded into powder.

Extraction

Three hundred gram of powdered sample was extracted by reflux using different polarity solvents. Extraction using n-hexane was repeated three times. The remaining residue was then extracted three times by using ethyl acetate. Finally the remaining residue was extracted three times using ethanol. So totally there were fifteen extracts: five of n-hexane extracts (namely CR1, CM1, CL1, CH1 and CA1), five of ethyl acetate extracts (CR2, CM2, CL2, CH2 and CA2) and five of ethanolic extracts (CR3, CM3, CL3, CH3 and CA3).

Total phenolic content (TPC)

Total phenolic content was performed using Folin-Ciolcalteu[14] with minor modification. The absorbance was read at wavelength 765 nm. Analysis was done in triplicate for each extract. Gallic acid standard solution (45-165 µg/ml) was used to obtain a calibration curve. Total phenolic content was reported as percentage of total gallic acid equivalent per 100 g extract (g GAE /100 g).

*Author for Correspondence

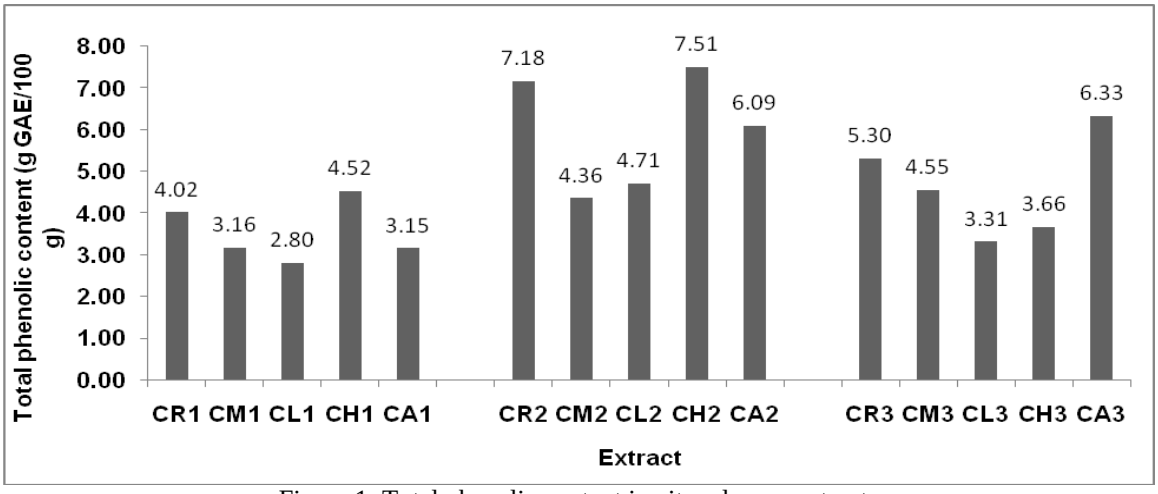

Figure 1: Total phenolic content in citrus leaves extracts

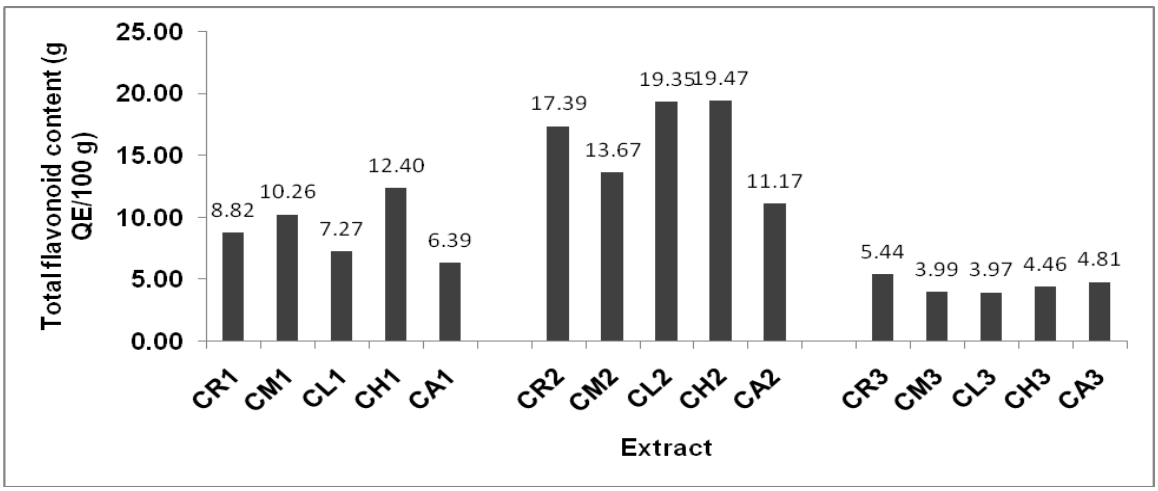

Figure 2: Total flavonoid content in citrus leaves extracts

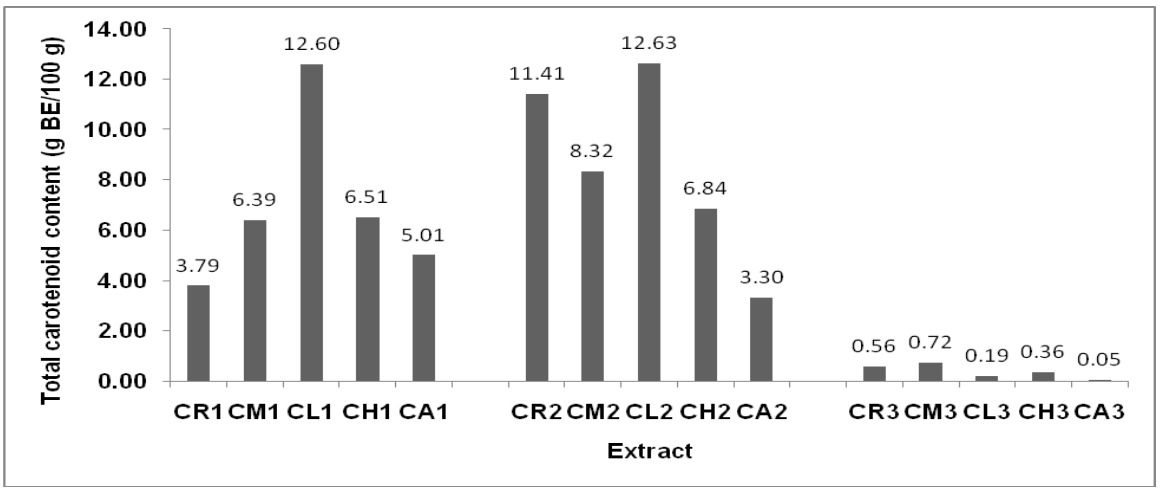

Figure 3: Total carotenoid content in citrus leaves extracts

Total flavonoid content (TFC)

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

Total carotenoid content (TCC)

Total carotenoid content was measured using modified method which was adapted from[9]. Each extract was diluted in n-hexane. The absorbance was read at wavelength 470 nm. Analysis was done in triplicate for

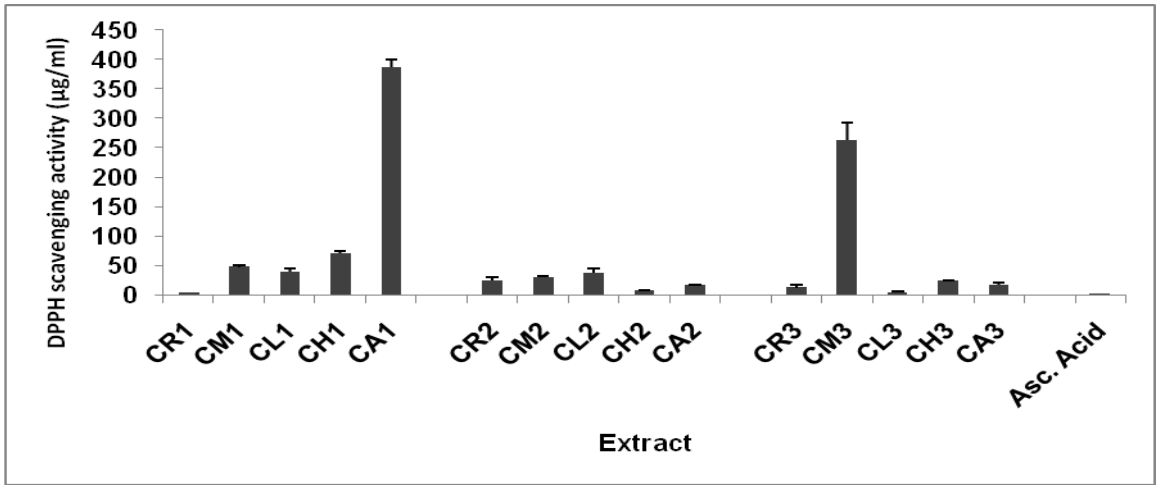

Figure 4: IC_{50} of DPPH scavenging activities in citrus leaves extracts

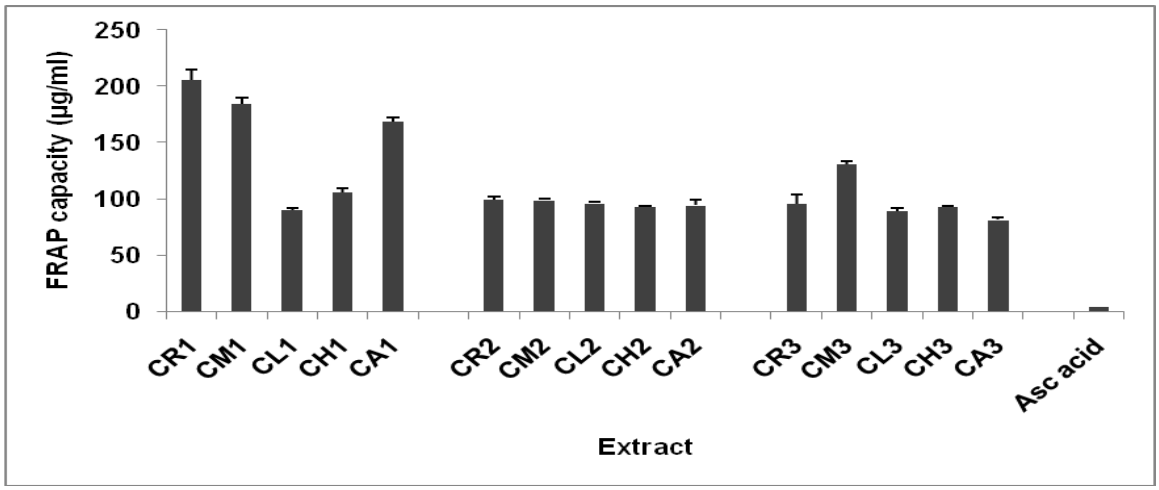

Figure 5: EC_{50} of FRAP capacities in citrus leaves extracts

each extract. Beta carotene standard solution (10-70 µg/ml) was used to obtain a calibration curve. The total carotenoid content was reported as percentage of total beta carotene equivalent per 100 g extract (g BE/100 g).

DPPH scavenging activity

Preparation of DPPH solution was adopted from Blois[16] with minor modification. Various concentration of each extract were pipetted into DPPH solution 50 µg/ml (volume 1:1) to initiate the reaction for obtaining a calibration curve. The absorbance was measured after 30 minutes incubation at wavelength 515 nm by using UV-Vis Spectrophotometer Hawlett Packard 8435. Methanol was used as a blank. DPPH solution 50 µg/ml was used as control. Ascorbic acid was used as standard. Analysis was done in triplicate for standard and each extract. Antioxidant activity of each extract by DPPH method was determined by calculating percentage of antioxidant activity using reduction of DPPH absorbance[17]. IC_{50} of DPPH scavenging activity of each extract can be calculated using its calibration curve.

FRAP capacity

Preparation of FRAP solution was adopted from Benzi [18]. The FRAP solution was prepared in acetate buffer pH 3.6. Each extract 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 UV-Vis Spectrophotometer Hawlett Packard 8435. Acetate buffer was used as a blank and FRAP solution 50 µg/mL and methanol (1:1) 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 Fe (II)-TPTZ absorbance by calculating percentage of antioxidant capacity[16].

Statistical Analysis

Each sample analysis was performed in triplicate. All results presented are means (± standard deviation) of at least three independent experiments. Statistical analysis using ANOVA with a statistical significance level set at $p < 0.05$ and post-hoc Tukey procedure was carried out with SPSS 16 for Windows. Correlation between the total phenolic, flavonoid, carotenoid content and antioxidant activities, and correlation between two antioxidant activity methods were performed using the Pearson's method.

RESULTS

TPC in citrus leaves extracts

Table 1: Pearson's correlation coefficient of total phenolic, flavonoid, carotenoid content in various citrus leaves extracts with their IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities

Antioxidant activities	Coefficient correlation Pearson (r)							
	TPC	TFC	TCC	EC_{50} FRAP CR	EC_{50} FRAP CM	EC_{50} FRAP CL	EC_{50} FRAP CH	EC_{50} FRAP CA
IC_{50} DPPH CR	0.923**	0.705*	0.692*	-0.766**				
IC_{50} DPPH CM	0.406 ns	-0.914**	-0.975**		-0.740*			
IC_{50} DPPH CL	0.247 ns	0.611*	0.971**			0.472 ns		
IC_{50} DPPH CH	-0.510 ns	-0.185 ns	0.229 ns				0.800**	
IC_{50} DPPH CA	-0.983**	-0.28 ns	0.761*					0.988**
EC_{50} FRAP CR	-0.958**	-0.210 ns	-0.196 ns					
EC_{50} FRAP CM	-0.735*	-0.214 ns	-0.104 ns					
EC_{50} FRAP CL	0.871**	0.856**	0.512 ns					
EC_{50} FRAP CH	-0.426 ns	-0.24 ns	0.132 ns					
EC_{50} FRAP CA	-0.974**	-0.14 ns	0.842**					

IC_{50} DPPH = IC_{50} of DPPH scavenging activity, EC_{50} FRAP = EC_{50} of FRAP capacity, CR = *Citrus reticulata*, CM = *Citrus maxima*, CL = *Citrus limon*, CH = *Citrus hystrix*, CA = *Citrus aurantifolia*, ns = not significant, * = significant at $p < 0.05$, ** = significant at $p < 0.01$

TPC among the various extracts were reported in term of gallic acid equivalent using the standard curve equation y = 0.003 x + 0.082, R^2 = 0.991. The TPC in various extracts of citrus leaves showed different result ranged from 2.80 to 7.51 g GAE/100 g. The highest phenolic content (7.51 g GAE/100 g) was given by ethyl acetate extract of *Citrus hystrix* leaves (CH2) (Fig 1) and the lowest given by n-hexane extract of *Citrus limon* (CL1).

TFC in citrus leaves extracts

TFC among the various extracts were demonstrated in term of quercetin equivalent using the standard curve equation y = 0.007 x - 0.029, R^2 = 0.998. The TFC in various extracts of citrus leaves showed different result ranged from 3.97 to 19.47 g QE/100 g (Fig 2). Ethyl acetate extract of *Citrus hystrix* (CH2) had the highest total flavonoid content (19.47 g QE/100 g).

TCC in citrus leaves extracts

TCC among the various extracts were expressed in term of beta carotene equivalent using the standard curve equation y = 0.0121x - 0.0084, R^2 = 0.9998. The TCC in various extracts of citrus leaves gave different result in the range of 0.05 – 12.60 g BE/100 g (Fig 3). The highest carotenoid content (12.60 g BE/100 g) was given by n-hexane extract of *Citrus limon* (CL1), while the lowest carotenoid (0.05 g BE/100 g) for ethanolic extract of *Citrus aurantifolia* (CA3).

IC_{50} of DPPH scavenging activity and EC_{50} of FRAP capacity

The IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacity in various extracts of citrus leaves using DPPH and FRAP assays were shown in Fig 4 and Fig 5. IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacity of each extract were compared to IC_{50} and EC_{50} of ascorbic acid as standard. The lowest value of IC_{50} means had the highest antioxidant activity.

Correlations between total phenolic, flavonoid, carotenoid content in various citrus leaves extracts and IC_{50} of DPPH scavenging activities, EC_{50} of FRAP capacities

Pearson's correlation coefficient between TPC in various extracts of citrus leaves and their antioxidant activities revealed that TPC in *Citrus aurantifolia* (CA) had negative and significant correlation with IC_{50} of DPPH scavenging activities (r = -0.983, $p<0.01$) and TPC in *Citrus reticulata* (CR), *Citrus maxima* (CM) and *Citrus aurantifolia* (CA) with EC_{50} of FRAP capacities (r = -0.958, $p<0.01$; r = -0.735, $p<0.05$; r = -0.974, $p<0.01$, respectively). Only TFC and TCC in *Citrus maxima* leaves extracts had negatively high correlation with their IC_{50} of DPPH scavenging activities (Table 1).

DISCUSSION

The previous research[4,8,9,13] reported that citrus had antioxidant capacity. There were no study regarding antioxidant activity of various extracts (which were n-hexane, ethyl acetate and ethanol) of five citrus leaves from West Java- Indonesia using DPPH and FRAP assays. The present research demonstrated that TPC in ethanolic leaves extract of *Citrus reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* from West Java-Indonesia were 5.30, 4.55, 3.31, 3.66, 6.33 g GAE/100 g respectively. It was different with the previous study[19]

regarding citrus peel extracts, which showed that TPC in *C. aurantifolia*, *C. limon*, *C. hystrix*, *C. maxima* and *C. sinensis* from West Java-Indonesia were 83, 73, 188, 167 and 198 mg GAE/100 g, respectively. Total phenolic content can be correlated with antioxidant activity[20]. Cinnamic acid had higher antioxidant capacity than phenyl acetic acid and benzoic acid[21]. Ghasemi[22] reported that TPC in methanolic peel extract of *C. sinensis* var. Washington Navel, *C. sinensis* var. Sungin, *C. sinensis* var. Valencia were 16, 15.4, 13.3 g GAE/100 g extract, respectively. The previous study revealed that TPC in ethanolic peel extract of *C. hystrix* was 4.4 g GAE/100 g extract[13] and methanolic peel extract of *C. limon* was 13.1 g GAE/100 g extract[12]. Previous study by Hayat[22] expressed that TPC in methanolic peel extract of *C.sinensi*s and *C. reticulata* by ultrasound-assisted extraction method were 6.64, 5.87 g GAE/100 g extract, respectively. It was similar with the present study which reported that TPC in ethanolic leaves extract of *C. reticulata* was 5.30 g GAE/100 g and the previous research[11] stated that TPC in ethanolic peel extract of *C. sinensis* from Kintamani, Jember and Banyuwangi which extracted by reflux were 10.08, 8.85, 9.54 g GAE/100 g extract, respectively. It was contrast with previous study which exhibited that TPC in methanolic peel extract of *C. reticulata* using microwave - assisted extraction was 17.5 mg GAE/100 g extract[22] and TPC in fruit juice of *C. hystrix and C. aurantifolia* and C. *sinensis* were 490, 211, 135 mg GAE/100 ml juice, respectively[23]. Previous research[19] stated that TFC in peel extracts of *C. aurantifolia*, *C. limon*, *C. hystrix*, *C. maxima* and *C. sinensis* were 47, 49, 69, 26, 46 mg QE/100 g, respectively, while the present study reported that TFC in leaves extracts of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* were 5.44, 3.99, 3.97, 4.46 and 4.81 g QE/100 g, respectively. Ghafar[23] 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. TFC in ethanolic leaves extract of *C. hystrix* was 3.0 g QE/100 g which higher than its peel and stem extracts 1.3 and 0.9 g QE/100 g[13], while TFC in methanolic peel extract of *C. sinensis* var. Washington Navel, *C. sinensis* var. Sungin, *C. sinensis* var. Valencia were 2.3, 0.21, 0.72 g QE/100 g extract, respectively[12]. Study by Fidrianny[11] 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. The previous research[13] expressed that TCC in all of ethanolic peel, leaves and stem extracts of *C. hystrix* from Boyolali-Central Java- Indonesia was 0.2 g BE/100 g extract. TCC in peel extract from five citrus from West Java –Indonesia demonstrated that TCC of *C. aurantifolia*, *C. limon*, *C. hystrix*, *C. maxima* and *C. sinensis* were 7.0, 5.4, 15.6, 6.3 and 20.9 mg BE/100 g, respectively[19]. The present study found that TCC in leaves extract of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* from West Java-Indonesia were 0.56, 072, 0.19, 0.36 and 0.05 g BE/100 g, respectively. It was contrast with the previous study which 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[11]. The IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities in various leaves extracts from five citrus using DPPH and FRAP assays were shown in Fig 4 and Fig 5. The IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities in various extracts compared to IC_{50} or EC_{50} of ascorbic acid standard. The lowest IC_{50} means showed the highest antioxidant activity. Sample which had IC_{50} lower than 50 µg/ml was a very strong antioxidant, 50-100 µg/ml was a strong antioxidant, 101-150 µg/ml was a medium antioxidant, while a weak antioxidant with IC_{50} greater than 150 µg/ml[16]. In the present study revealed that IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities of various leaves extracts of five citrus in the range of 2.93 – 387.76 µg/ml and 81.54 to 205.56 µg/ml, respectively. Based on value of IC_{50} of DPPH scavenging capacity and EC_{50} of FRAP capacity it can be concluded that all of leaves extracts of five citrus *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia*, except n-hexane leaves extract of *C. hystrix* (CH1), *C. aurantifolia* (CA1) and ethanolic extract of *C. maxima (*CM3) can be classified as very strong antioxidant. The lowest IC_{50} of DPPH was given by n-hexane leaves extract of *C. reticulata* (CR1) 2.93 µg/ml, while IC_{50} of DPPH of ascorbic acid was 2.36 µg/ml. It exposed that potency of CR1 was similar with ascorbic acid using DPPH method. Ethanolic leaves extract of *C. aurantifolia* (CA3) showed the lowest EC_{50} of FRAP capacity (81.54 µg/ml) while ascorbic acid standard had EC_{50} of FRAP capacity 4.41 µg/ml. It exposed that antioxidant potency of ascorbic acid was around twenty times of potency of CA3 using FRAP assay. Ghasemi[12] expressed that methanolic peel extract of *C. sinensis* var. Sungin, *C. sinensis* var. Valencia, *C. sinensis* var Navel and *C.limon* using percolation extraction had IC_{50} of DPPH 1.7, 2.1, 1.1 and 1.4 mg/ml, respectively. The previous study showed that IC_{50} of DPPH scavenging activities of ethanolic extract of leaves, peel and stem of *C. hystrix* from Boyolali, Central Java-Indonesia were 16.6, 16.7 and 7.1 µg/ml. In the present research revealed that IC_{50} of DPPH scavenging activities of ethanolic leaves extract of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* were 12.76, 263.49, 4.42, 23.27 and 17.38 µg/ml. It can be seen that all of ethanolic leaves extracts can be classified as very strong antioxidant using DPPH method (except *C. maxima*). It was contrast with the previous study which exposed that methanolic and ethanolic leaves extracts of *C. hystrix*, *C. aurantifolia*, *C. maxima*, *C reticulata*, *C. medica* had IC_{50} of DPPH scavenging activities 805 and 740 µg/ml, 967 and 736 µg/ml, 867 and 730 µg/ml, 902 and 1070 µg/ml, 916 and 1753 µg/ml [24]. In research by Fidrianny[11] expressed that ethanolic peel extract of *C. sinensis* from three locations Kintamani, Jember and Banyuwangi were 2.25, 8.84, 17.94 µg/ml, respectively. While the ethanolic peel extracts of *C. aurantifolia*, *C. limon*, *C. hystrix*, *C. maxima* and *C. sinensis* had IC_{50} of DPPH scavenging activities 106.36, 45.28, 21.22, 32.83 and 35.54 µg/ml, respectively[19]. It can be concluded that all of peel extracts

was categorized as very strong antioxidant using DPPH method (except *C. aurantifolia*). EC_{50} of FRAP capacity of ethanolic leaves extracts of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* in the present study ranged from 81.54 to 131.06 µg/ml, while EC_{50} of CUPRAC of *C. aurantifolia*, *C. limon*, *C. hystrix*, *C. maxima* and *C. sinensis* in the range of 658 – 2806 µg/ml[19]. Pearson's correlation coefficient was positively high if $0.61 \leq r \leq 0.97$ [9] and negatively high if $-0.61 \leq r \leq -0.97$. Sample which had the lowest IC_{50} of DPPH and IC_{50} of ABTS scavenging activity had the highest antioxidant activity. So negatively and high correlation will be given in good correlation between TPC, TFC and TCC with IC_{50} of DPPH or EC_{50} of FRAP. It means increasing in TFC, TPC and TCC caused increasing in antioxidant activities, which was expressed by lower IC_{50} of DPPH scavenging activity and or EC_{50} of FRAP capacity. Data in Table 1 revealed that there were negatively high correlation between TPC in *C. aurantifolia* leaves extracts with its IC_{50} of DPPH scavenging activities ($r = -0.983$, $p<0.01$) and EC_{50} of FRAP capacities ($r = -0.974$, $p<0.01$). It was similar with previous study[19] which showed that TPC in *C. aurantifolia* peel extracts had negative and high correlation with its IC_{50} of DPPH scavenging activities ($r = -0.987$, $p<0.01$) and EC_{50} of CUPRAC capacities ($r = -0.998$, $p<0.01$). Based on the result it can be concluded phenolic compounds were the major contributor in antioxidant activities of leaves and peel extracts of *C. aurantifolia* using DPPH, FRAP and CUPRAC methods. It means antioxidant capacities of *C. aurantifolia* leaves and peel extracts using DPPH, FRAP and CUPRAC methods can be predicted indirectly by determining TPC. There were negatively and high correlation also between TFC, TCC in leaves, peel and stem extracts of *C. hystrix* with EC_{50} of CUPRAC capacities, so it can be concluded that antioxidant capacity with CUPRAC assay can be predicted by determining their TFC and or TCC[13]. In previous study the Pearson's correlation was analyzed between TPC, TFC and TCC with their percentage of DPPH scavenging activities[11]. So the good correlation between TPC, TFC and or TCC with percentage of DPPH scavenging activity or percentage of FRAP capacity when there were positive and high correlation. The previous result showed that TPC in peel extracts of *C. sinensis* from Kintamani, Jember and Banyuwangi had highly positive correlation with their percentage of DPPH scavenging activities. Ghafar[23] exposed 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. 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[25,26]. Reagent of FRAP is $FeCl_3$ that combined with TPTZ in acetate buffer pH 3.6. Fe (III) will be reduced to Fe (II). Complex of Fe (II) - TPTZ shows blue color and gave characteristic absorption at wavelength 593 nm. Intensity of blue color depends on amount of Fe (III) which 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. Sample will act as antioxidant in FRAP assays if sample had reduction potential lower than reduction potential of Fe (III)/Fe (II) which was 0.77 V, so the sample had the reducing power to reduce Fe (III) to Fe (II) and this sample will be oxidized[11]. Flavonoid, phenolic acid, tannins, qoumarine and quinone were included in phenolic groups. Flavonoid which had OH in ortho C 3',4', OH in C3, oxo function in C4, double bond at C2 and C3 have high antioxidant activity. The OH with ortho position in C3'-C4' had the highest influence to antioxidant activity of flavonoid. The flavonoid aglycones would give higher antioxidant activity than flavonoid glycosides. Flavonoid had greater antioxidant activity than phenolic acid[21]. It could be seen in Fig 1 that TPC in n-hexane leaves extract of *C. aurantifolia* (CA1) 3.15 g GAE/100 g was similar with TPC in ethanolic leaves extract of *C. limon* (CL3) 3.31 g GAE/100 g, but IC_{50} of DPPH of CA1 was 387.76 µg/ml which was categorized as weak antioxidant and IC_{50} of DPPH of CL3 was 4.42 µg/ml as very strong antioxidant. It can be predicted that many phenolic compounds in CA1 had low antioxidant and many phenolic compounds in CL3 had high antioxidant. CA1 and CL3 also showed different result with FRAP method. CL3 had EC_{50} of FRAP capacity 89.65 µg/ml which was categorized as strong antioxidant and CA1 gave EC_{50} of FRAP capacity 168.41 µg/ml as weak antioxidant. Based on the result it can predicted that many phenolic compounds in CL3 had potential redox below than 0.77 V potential redox of Fe(III)/Fe(II), so it can be oxidized and at the same time it will reduce Fe(III) to Fe(II) and then Fe(II) will react with TPTZ and gave the blue color of complex Fe(II)-TPTZ. TFC in n-hexane leaves extract of *C. limon* (CL1) 7.27 g QE/100 g was lower than ethyl acetate leaves extract of *C. limon* (CL2) 19.35 g QE/100 g, but IC_{50} of DPPH scavenging activity of CL1 39.54 µg/ml was similar with IC_{50} of DPPH scavenging activity of CL2 37.54 µg/ml. It can be estimated that many flavonoid compounds in CL1 had OH group at C3'-C4', C3, double bond at C2-C3, oxo function at C4 which had high antioxidant activity, while many of flavonoid compounds in CL2 had OH group at C5, C7, or C3' only, or C4' only, or C3 only without oxo function in C4 which had no or low antioxidant activity. The result was similar with FRAP, the EC_{50} of FRAP capacity of CL1 90.32 µg/ml was similar with EC_{50} of FRAP capacity of CL2 96.20 µg/ml. It also can be predicted that many flavonoid compounds in CL1 had potential redox below than potential redox of Fe (III)/Fe(II).

Previous research by Foote[27] reported that carotenoid have antioxidant capacity by scavenging free radical. Carotenoid which contain more than 7 double bonds will show higher scavenging radical activity[28]. Charles[29] stated that beta carotene was used as standard because of it had conjugation double bonds which had ability to scavenge free radicals. The previous study[30] revealed that increasing in lipophilicity of carotenoid would increase

scavenging radical activity and will give the lower IC_{50} of DPPH scavenging capacity. TCC in n-hexane leaves extract of *C. hystrix* (CH1) 6.51 g BE/100 g was similar with TCC in ethyl acetate leaves extract of *C. hystrix* (CH2) 6.84 g BE/100 g, but IC_{50} of DPPH scavenging activity of CH2 7.23 µg/ml which was categorized as very strong antioxidant and IC_{50} of DPPH of CH1 70.47 µg/ml. It can be supposed that many carotenoid compounds in CH2 had more than 7 double bonds which had high antioxidant activity and many carotenoid compounds in CH1 contained maximum 7 double bonds which had low antioxidant activity.

DPPH and FRAP had different mechanism reaction. Mechanism of FRAP was redox assay[18] while DPPH that was electron transfer assay[31]. The Pearson's correlation coefficient indicated that IC_{50} of DPPH scavenging activities leaves extracts of *C. hystrix* and *C. aurantifolia* had positive and high correlation with their EC_{50} of FRAP capacities ($r = 0.800$; $r = 0.988$, $p<0.01$, respectively). It could be seen that antioxidant activities of leaves extracts of *C. hystrix* and *C. aurantifolia* by DPPH and FRAP assays gave linear result. In previous study revealed that DPPH and CUPRAC methods showed linear results for antioxidant activities of peel extracts of *C. aurantifolia*, *C. limon*, *C. maxima* and *C. sinensis* ($r = 0.996$; $r = 0.995$; $r = 0.996$; $r = 0.996$, $p<0.01$, respectively)[19], DPPH and FRAP methods exposed linear result for peel extracts of *C. sinensis* from Kintamani, Jember and Banyuwangi ($r = 0.975$; $r = 0.977$; $r = 0.965$, $p<0.01$)[11]. It was contrast with the previous research[13] which reported that DPPH and CUPRAC methods gave no linear results for leaves, peel, stem extracts of *C. hystrix*.

CONCLUSION

Various methods could give different results, so antioxidant activity of sample should be measured by different methods in parallel. All of different polarities leaves extracts of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* (except n-hexane extract of *C. hystrix*, n-hexane extract of *C. aurantifolia* and ethanolic extract of *C. maxima*) were very strong antioxidant, using DPPH assays. TPC in leaves extracts of *C. aurantifolia* had negative and high correlation with IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities. Phenolic compounds in *C. aurantifolia* leaves extracts were the major contributor in IC_{50} of DPPH scavenging activity and EC_{50} of FRAP capacity. There were linear correlation between IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities of leaves extract of *C. hystrix* and *C. aurantifolia*. Leaves of *C. reticulata*, *C. maxima*, *C. limon*, *C hystrix* and *C. aurantifolia* may be exploited as natural antioxidant sources.

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