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Research Article

ANTIOXIDANT ACTIVITIES OF DIFFERENT POLARITY EXTRACTS FROM THREE ORGANS OF MAKRUT LIME (*CITRUS HYSTRIX* DC) AND CORRELATION WITH TOTAL FLAVONOID, PHENOLIC, CAROTENOID CONTENT

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ABSTRACT

Objectives: The objectives of this research were to study antioxidant activities from various extracts of three organs of makrut lime (*Citrus hystrix*) using two methods of antioxidant assays, which were 2,2-diphenyl-1-picrylhydrazyl (DPPH) and cupric ion reducing antioxidant capacity (CUPRAC); and correlation of total flavonoid, phenolic, and carotenoid content in various extracts of three organs of makrut lime with IC_{50} of DPPH antioxidant activities and EC_{50} of CUPRAC capacities.

Methods: 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, flavonoid and carotenoid content performed by ultraviolet-visible and their correlation with IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities were analyzed by Pearson's method.

Results: Ethyl acetate stem extract of makrut lime (ST2) had the lowest IC_{50} of DPPH scavenging activity 0.6 µg/ml and the lowest EC_{50} of CUPRAC capacity 123 µg/ml. N-hexane stem extract of makrut lime (ST1) had the highest total flavonoid content (8.7 g QE/100 g), ethyl acetate stem extract (ST2) contained the highest total phenolic content (TPC) (8.3 g gallic acid equivalent /100 g and total carotenoid content (1.8 g BE/100 g).

Conclusions: There was negatively high correlation between TPC in peel and stem extracts of makrut lime with their IC_{50} of DPPH scavenging activity. EC_{50} of CUPRAC capacity of leaves, peel and stem extracts of makrut lime had negative and high correlation with their total flavonoid and carotenoid content. IC_{50} of DPPH scavenging activities in leaves, peel and stem extracts of makrut lime had no linear result with EC_{50} of CUPRAC capacities.

Keywords: Antioxidant, 2,2-diphenyl-1-picrylhydrazyl, Cupric ion reducing antioxidant capacity, Organs, Makrut lime, Flavonoid, Phenolic, Carotenoid.

INTRODUCTION

Many degenerative diseases have related with oxidative stress. Antioxidant has the 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 reported that phenolic content in plants could be correlated to their antioxidant activities. Plants contained phenolic, and polyphenol compounds can act as antioxidant [3-6].

Some of antioxidant methods such as cupric ion reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were widely used to predict antioxidant capacity of fresh fruits, beverages, and food [2,7-11]. Previous studies [2,12,13] revealed that DPPH and CUPRAC methods could be used to determine antioxidant activity in many plants extracts. The previous studies [14,15] exhibited antioxidant capacities of some plants including Citrus spp.

The objectives of this research were to study antioxidant activities of various different polarities extracts (n-hexane, ethyl acetate and ethanol) from three organs (leaves, peel and stem) of makrut lime (*Citrus hystrix*) using DPPH and CUPRAC assays; and correlation of their antioxidant capacities with total phenolic, flavonoid and carotenoid content in each extract.

METHODS

Materials

DPPH, neocuproine, gallic acid, quercetin, beta-carotene was purchased from Sigma-Aldrich (MO, USA), cupric chloride, ethanol, ethyl acetate, n-hexane. All other reagents were analytical grades.

Preparation of sample

Three organs of makrut lime (*C. hystrix* DC) were collected from Boyolali-Center of Java that were: Leaf namely as sample LE, peel as sample PE and stem as sample ST, 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. Hence, there were three n-hexane extracts (namely LE1, PE1, ST1), three ethyl acetate extracts (LE2, PE2, ST2) and three ethanolic extracts (LE3, PE3, ST3).

IC_{50} of DPPH scavenging activity

Preparation of DPPH solution was adopted from Blois [16] with minor modification. Various concentration of each extract was pipetted into DPPH 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 ultraviolet-visible (UV-Vis) Hewlett Packard 8435. Methanol was used as a blank. DPPH 50 µg/ml was as control. 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 [17]. IC_{50} of DPPH scavenging activity of each extract can be calculated using its calibration curve.

EC_{50} of CUPRAC capacity

Preparation of CUPRACsolution was adopted from Apak [12]. The CUPRAC solution was prepared in ammonium acetate buffer pH 7.

The various concentration of each extract was pipetted into CUPRAC 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. CUPRAC 50 µg/ml was as control. 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 the percentage of antioxidant capacity [12]. EC_{50} of CUPRAC capacity of each extract can be calculated using its calibration curve.

Total flavonoid content (TFC)

TFC was measured using adapted method from Chang *et al*. [18]. The absorbance was read at wavelength 415 nm. Analysis was done in triplicate for each extract. Standard solution of quercetin with concentration 36-120 µg/ml were used to obtain a standard curve. The TFC was reported as percentage of total quercetin equivalents per 100 g extract (g QE/100 g).

Total phenolic content (TPC)

TPC were measured using the modified Folin–Ciolcalteu method adapted from Pourmorad [19]. The absorbance was read at wavelength 765 nm. Analysis was done in triplicate for each extract. Standard solution of gallic acid with concentration 40-165 µg/ml were used to obtain a standard curve. The TPC was reported as a percentage of total gallic acid equivalents per 100 g extract (g gallic acid equivalent [GAE]/100 g).

Total carotenoid content (TCC)

TCC was measured using the modified carotene method adapted from Thaipong *et al.* [2]. Each extract was diluted in n-hexane. The absorbance was read at wavelength 470 nm. Analysis was done in triplicate for each extract. Standard solution of beta carotene with concentration 15-55 µg/ml were used to obtain a standard curve. The TCC was reported as a percentage of total beta-carotene equivalents per 100 g extract (g BE/100 g).

Statistical analysis

Each sample analysis was performed in triplicate. All results presented are means (±standard deviation) of at least three independent experiments. Statistical analysis (ANOVA with a statistical significance level set at $p<0.05$ with *post-hoc* Tukey procedure) was carried out with SPSS 16 for windows. Correlations between the total phenolic, flavonoid and TCC and antioxidant capacities were made using the Pearson procedure ($p<0.01$).

RESULTS

IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities

The IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities in various extracts from three organs of makrut lime using DPPH and CUPRAC assays were shown in Fig. 1 and 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. The lowest IC_{50} and or EC_{50} means had the highest antioxidant capacity.

TFC in various organs extracts of makrut lime

TFC in various extracts were demonstrated in term of quercetin equivalent using the standard curve equation $y = 0.006x - 0.019$, $R^2 = 0.998$. TFC in various makrut lime organs extracts showed different result ranged from 0.9 to 8.7 g QE/100 g (Fig. 3). N-hexane stem extract of makrut lime (ST1) had the highest TFC (8.7 g QE/100 g) and the lowest (0.9 g QE/100 g) was given by ethanolic stem extract (ST3).

TPC in various organs extracts of makrut lime

TPC in various extracts were revealed in term of gallic acid equivalent using the standard curve equation $y = 0.004x + 0.025$, $R^2 = 0.998$. TPC in various organs extracts of makrut lime showed different result ranged from 2.8 to 8.3 g GAE/100 g. Ethyl acetate stem extract of makrut lime (ST2) had the highest phenolic content (8.3 g GAE/100 g) (Fig. 4).

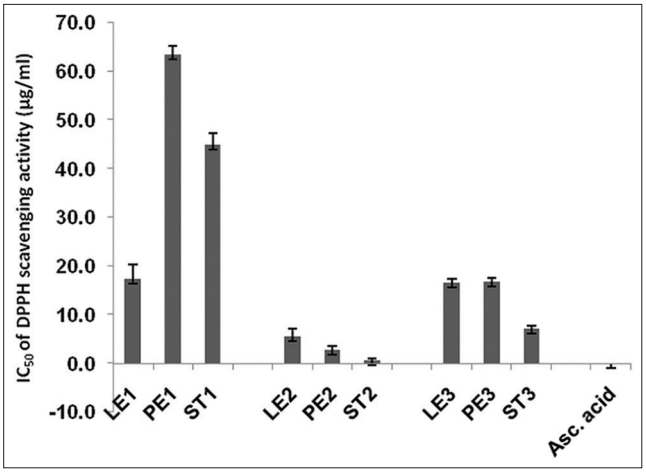

Fig. 1: IC_{50} of 2,2-diphenyl-1-picrylhydrazyl scavenging activities in various organs extracts of makrut lime, n=3

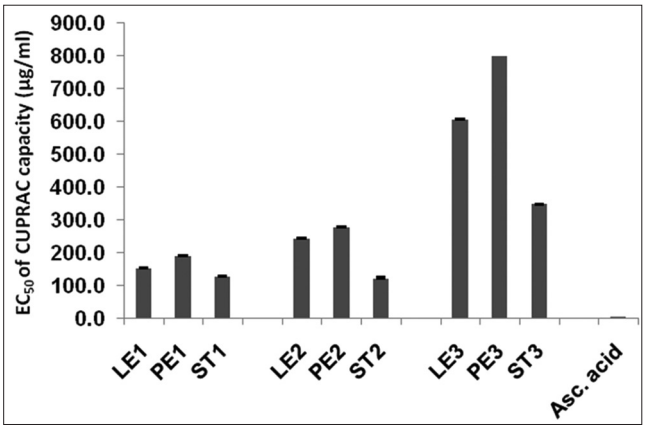

Fig. 2: EC_{50} of cupric ion reducing antioxidant capacity capacities in various organs extracts of makrut lime, n=3

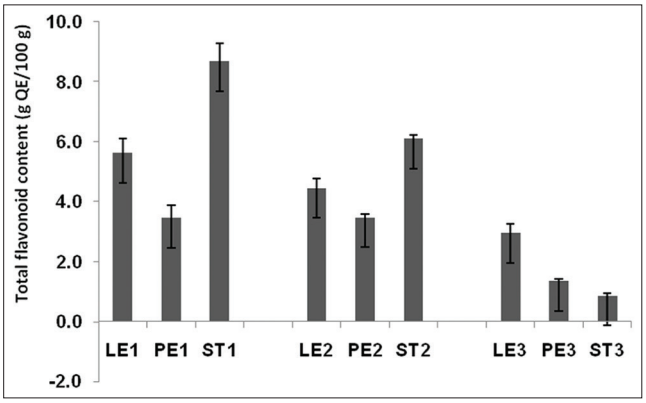

Fig. 3: Total flavonoid content in various organs extracts of makrut lime, n=3

TCC in various organs extracts of makrut lime

TCC in various extracts were expressed in term of beta-carotene equivalent using the standard curve equation $y = 0.015x + 0.002$, $R^2 = 0.9996$. TCC in various organs extracts of makrut lime showed the different result in the range of 0.2-1.8 g BE/100 g (Fig. 5). The highest carotenoid content (1.8 g BE/100 g) for ethyl acetate stem extract (ST2), while the lowest carotenoid (0.2 g BE/100 g) were given by ethanolic leaves, peel and stem extract (LE3, PE3 and ST3).

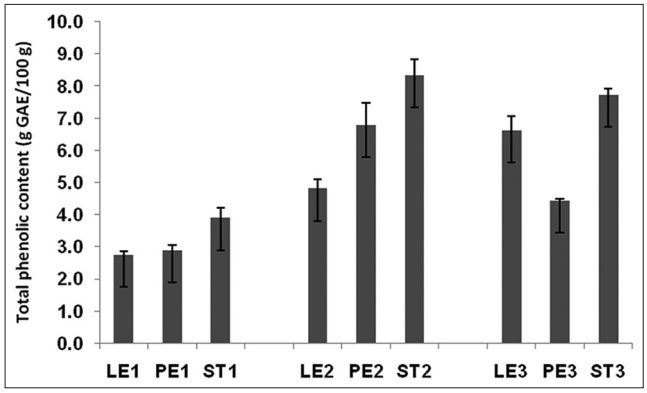

Fig. 4: Total phenolic content in various organs extracts of makrut lime, n=3

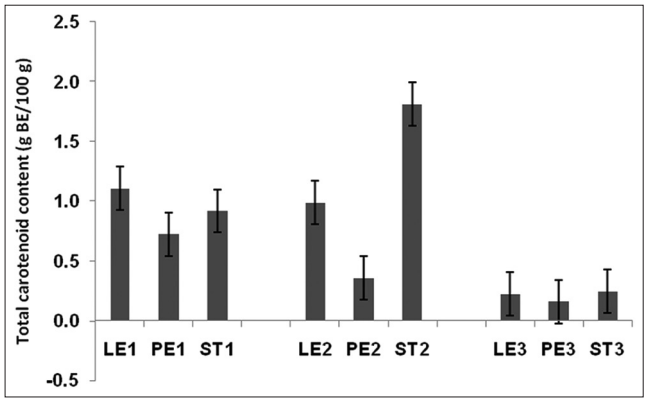

Fig. 5: Total carotenoid content in various organs extracts of makrut lime, n=3

Correlations between IC_{50} of 2,2 -diphenyl-1- picrylhydrazyl scavenging activities, EC_{50} of cupric ion reducing antioxidant capacity capacities and total flavonoid content, total phenolic content and total carotenoid content in various organs extracts of makrut lime

Pearson's correlation coefficient between TFC from various extracts of three organs of makrut lime and their antioxidant activities revealed that TFC had negatively high correlation with EC_{50} of CUPRAC capacities (LE, r=−0.931; PE, r=−0.968; ST, r=−0.936, p<0.01). TPC in sample PE and ST had negative and high correlation with their IC_{50} of DPPH scavenging activities (r=−0.89; r=−0.986, p<0.01). TCC in all of sample (LE, PE and ST) had negatively high correlation with their EC_{50} of CUPRAC capacities (r=−0.996, r=−0.845, r=−0.83, p<0.01).

DISCUSSION

Previous study [5,20], reported that makrut lime (*C. hystrix*) had antioxidant capacity. There were no study regarding antioxidant activity of three different polarities extracts (which were n-hexane, ethyl acetate and ethanol) of three organs (leaves, peel and stem) of makrut lime using DPPH and CUPRAC assays.

The DPPH is stable free radicals which dissolve in methanol or ethanol, and its colors show characteristic absorption at wavelength 515-520 nm, respectively. Colors of DPPH would be changed when the free radicals were scavenged by antioxidant [12,21]. 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 assay 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 half minimum IC_{50} 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 have IC_{50} or EC_{50} <50 µg/ml is very strong antioxidant, 50-100 µg/ml is strong antioxidant,101-150 µg/ml is medium antioxidant, while IC_{50} or EC_{50} >150 µg/ml is weak antioxidant [16].

IC_{50} of DPPH method of various organs extracts of makrut lime ranged from 0.6 to 63.4 µg/ml. Ethyl acetate extract of *C. hystrix* stem (ST2) had the lowest IC_{50} of DPPH radical scavenging activity 0.6 µg/ml. Based on the value of IC_{50} of DPPH scavenging activity it could be concluded that all of organs extracts could be categorized as very strong antioxidant, except n-hexane extract of *C. hystrix* peel as strong antioxidant (63.4 µg/ml). The current study showed that IC_{50} of DPPH scavenging activities of ethanolic extract of leaves, peel and stem of *C. hystrix* from Boyolali were 16.6, 16.7 and 7.1 µg/ml. It was contrast with the previous study Prasad [5] reported that methanolic and ethanolic leaves extracts of *C. hystrix* had IC_{50} of DPPH scavenging activities 805 and 740 µg/ml, respectively. IC_{50} of DPPH scavenging activities of methanolic and ethanolic leaves extracts of *Citrus aurantifolia*, *Citrus maxima*, *Citrus reticulata*, *Citrus medica* were 967 and 736 µg/ml, 867 and 730 µg/ml, 902 and 1070 µg/ml, 916 and 1753 µg/ml [5]. In research by Fidrianny *et al.* [14] expressed that ethanolic peel extract of *Citrus sinensis* from three locations Kintamani, Jember and Banyuwangi were 2.25, 8.84, 17.94 µg/ml, respectively. Ghasemi *et al.* [6] revealed that methanolic peel extract of *C. sinensis* var. Sungin, *C. sinensis* var. Valencia, *C. sinensis* var Navel and *Citrus limon* using percolation extraction were 1.7, 2.1, 1.1 and 1.4 mg/ml. Ethyl acetate stem extract of *C. hystrix* (ST2) had the lowest EC_{50} of CUPRAC capacity (123 µg/ml) while ascorbic acid standard gave EC_{50} of CUPRAC capacity 5.3 µg/ml. It showed that potency of ascorbic acid was around 25 times as much as the potency of ST2 using CUPRAC assays.

Antioxidant activity might be related with phenolic content, included phenolic acid [22]. Phenylacetic acid and benzoic acid had lower antioxidant capacity than cinnamic acid [23]. The present research exposed that TPC in ethanolic extract of leaves, peel, and stem of *C. hystrix* were 6.6, 4.4, 7.7 g GAE/100 g. It was contrast with the previous study [5] which 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 present study revealed that TPC in ethanolic peel extract of *C. hystrix* was 4.4 g GAE/100 g extract, while research by Ghasemi *et al.* [6] found that TPC in methanolic peel extract of *C. limon* was 13.1 g GAE/100 g extract. The previous study [24] demonstrated that TPC in methanolic peel extract of *Citrus aurantifolia*, *C. sinensis* and *C. reticulata* by ultrasound-assisted extraction method were 7.48, 6.64, 5.87 g GAE/100 g extract, respectively, and no relation between TPC and time of extraction. Previous research [14] 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. It was not similar with study by Hayat *et al.* [24] which revealed that TPC in methanolic peel extract of *C. reticulata* by microwave - assisted extraction method (17.5 mg GAE/100 g extract) was greater than ultrasound extraction (16.2 mg GAE/g extract) and rotary extraction (7.98 mg GAE/100 g extract). *C. reticulata* contained p-hydroxybenzoic acid, vanillic acid, p-coumaric acid and ferulic acid which could act as antioxidant [24]. While research by Ghafar *et al.* [20] expressed that TPC in fruit juice of *C. hystrix*, *C. aurantifolia*, *Citrus microcarpa* and C. *sinensis* were 490.74, 211.70, 105, 135.3 mg GAE/100 ml juice, respectively.

TFC in ethanolic extract of leaves, peel and stem of *C. hystrix* were 3.0, 1.3, 0.9 g QE/100 g, while in previous research by Ghasemi *et al.* [6] revealed that TFC in methanolic peel extract of *C. sinensis* var. Washington Navel, *C. sinensis* var. Sungin, *C. sinensis* var. Valencia were 2.3, 0.21,

Table 1: Pearson's correlation coefficient of IC_{50} of DPPH scavenging activities, EC_{50} of CUPRAC capacities and TFC, TPC, TCC in various organs extracts of makrut lime

	Pearson's correlation coefficient (*r*)					
	TFC	**TPC**	**TCC**	**EC_{50} CUPRAC LE**	**EC_{50} CUPRAC PE**	**EC_{50} CUPRAC ST**
IC_{50} DPPH LE	−0.014	−0.08	−0.316	0.259		
IC_{50} DPPH PE	0.278	−0.89**	0.838**		−0.421	
IC_{50} DPPH ST	0.65**	−0.986**	−0.214			−0.364
EC_{50} CUPRAC LE	−0.931**	0.921**	−0.996**			
EC_{50} CUPRAC PE	−0.968**	0.016	−0.845**			
EC_{50} CUPRAC ST	−0.936**	0.366	−0.83**			

TFC: Total flavonoid content, TPC: Total phenolic content, TCC: Total carotenoid content, DPPH: 2,2-diphenyl-1-picrylhydrazyl, CUPRAC: Cupric ion reducing antioxidant capacity

0.72 g QE/100 g extract, respectively. Ghafar *et al.* [20] 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 *et al.* [14] 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. TFC in methanolic peel extract of *C. limon* was 1.62 g QE/100 g extract [6], it was similar with the present study which showed that TFC in ethanolic peel extract of *C. hystrix* was 1.3 g QE/100 g.

In the present research expressed that TCC in all of ethanolic peel, leaves and stem extracts of *C. hystrix* from Boyolali was 0.2 g BE/100 g extract. 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 [14].

Pearson's correlation coefficient was positively high if $0.61 \leq r \leq 0.97$ [2] and negatively high if $-0.61 \leq r \leq -0.97$. Sample which had the lowest IC_{50} of DPPH scavenging activity or EC_{50} of CUPRAC capacity gave the highest antioxidant activity. So the good correlation between IC_{50} DPPH and or EC_{50} CUPRAC with TPC, TFC and TCC will be given in negatively and high correlation. 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 CUPRAC capacity.

Based on data in Table 1 demonstrated that there were negatively high correlation between TPC in peel and stem extracts of *C. hystrix* (PE and ST) with IC_{50} of DPPH scavenging activities. Antioxidant capacities with DPPH method in peel and stem extracts of makrut lime 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.

Phenolic compounds included qoumarine, quinone, tannins, flavonoid, phenolic acid and other compounds. Flavonoid which had OH in A ring and or B ring can be included in phenolic groups. Phenolic acid had lower antioxidant capacity than flavonoid [23].

Flavonoid which had OH in ortho C-3', -4', OH in C-3, oxo function in C4, double bond at C2 and C3 would give higher antioxidant activity. The highest influence to antioxidant capacity of flavonoid related with OH in ortho position in C-3'-C-4'. The flavonoid aglycones would give higher antioxidant activity than flavonoid glycosides [23].

TFC in n-hexane leaves extract of makrut lime (LE1) 5.6 g QE/100 g was similar with TFC in ethyl acetate stem extract of makrut lime (ST2) 6.1 g QE/100 g, but IC_{50} of DPPH scavenging activity of ST2 (0.6 µg/ml) was lower than LE1 (17.4 µg/ml). Based on the data above it can predicted that almost all of flavonoids in ST2 were flavonoid that had OH in position which had high antioxidant capacities, while many flavonoid in LE1 had OH, example 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 the present study exposed that total carotenoid in three organs extracts of makrut lime had negative and high correlation with their EC_{50} of CUPRAC capacity. The present study demonstrated that TCC in n-hexane stem extract of makrut lime (ST1) 0.9 µg/ml was similar with TCC in ethyl acetate leaves extract of makrut lime (LE2) 1.0 µg/ml, but EC_{50} of CUPRAC capacity of ST1 (127.1 µg/ml) was lower than EC_{50} of CUPRAC capacity of LE2 (244.6 µg/ml). Sample will act as antioxidant in CUPRAC assay if sample had reduction potential lower than reduction potential of Cu (II)/Cu (I) 0.46 V. So it means many carotenoid in ST1 had reduction potential lower than 0.46 V, while in LE2 contained many carotenoid with reduction potential higher than 0.46 V.

The CUPRAC and DPPH methods had different mechanism reaction. Mechanism of CUPRAC was redox assay [12] while DPPH that was electron transfer assay [25]. The Pearson's correlation coefficient indicated that IC_{50} of DPPH scavenging activities all of organs (leaves, peel stem) of makrut lime (*C. hystrix)* had no correlation with their EC_{50} of CUPRAC capacities. It could be seen that antioxidant activities of leaves, peel and stem of makrut lime by DPPH and CUPRAC assays gave no linear result.

CONCLUSION

Antioxidant capacity of the sample should perform using a variety of methods in parallel because different methods could give different results. All of the extracts in peel, leaves and stem of makrut lime (except n-hexane peel extract) had IC_{50} of DPPH scavenging capacities lower than 50 mg/mL that were very strong antioxidant. Phenolic compounds were the major contributor in DPPH scavenging capacities in peel and stem extracts of makrut lime. Flavonoid and or carotenoid compounds in leaves, peel and stem extracts of makrut lime were the major contributor in CUPRAC capacities. There were no linear correlation between IC_{50} of DPPH scavenging activities and EC_{50} of CUPRAC capacities in leaves, peel and stem extract of makrut lime. Leaves, peel and stem of *C. hystrix* can be exploited as sources of natural antioxidant to alleviate oxidative stress.

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