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

Irida 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 IC50 of DPPH and IC50 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 IC50 of DPPH scavenging activities and EC50 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 IC50 of DPPH scavenging activity and EC50 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 activity1,2. Previous study3-5 exposed that phenolic and flavonoid content could be correlated to their antioxidant activities. Plants include citrus contain phenolic and flavonoid compounds6-8. 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 food9,10. Previous research9-11 expressed that DPPH, FRAP and ABTS methods could be used to determine antioxidant activity in many plants extracts. The previous study12,13 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-tripryridyltriazine), 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-Ciocalteau14 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
Total flavonoid content (TFC)
Total flavonoid content was done using method from Chang et al.\textsuperscript{15}. The absorbance was read at wavelength 415 nm. Analysis was done in triplicate for each extract. Quercetin standard solution (20-120 \( \mu \)g/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\textsuperscript{9}. Each extract was diluted in n-hexane. The absorbance was read at wavelength 470 nm. Analysis was done in triplicate for
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 IC₅₀ of DPPH scavenging activities and EC₅₀ of FRAP capacities

<table>
<thead>
<tr>
<th>Antioxidant activities</th>
<th>Coefficient correlation Pearson (r)</th>
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<tbody>
<tr>
<td></td>
<td>TPC</td>
</tr>
<tr>
<td>IC₅₀</td>
<td></td>
</tr>
<tr>
<td>DPPH</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>0.923**</td>
</tr>
<tr>
<td>IC₅₀</td>
<td></td>
</tr>
<tr>
<td>DPPH</td>
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<tr>
<td>CM</td>
<td>0.247 ns</td>
</tr>
<tr>
<td>IC₅₀</td>
<td></td>
</tr>
<tr>
<td>DPPH</td>
<td>0.0510* ns</td>
</tr>
<tr>
<td>CH</td>
<td>-0.983**</td>
</tr>
<tr>
<td>IC₅₀</td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>-0.958**</td>
</tr>
<tr>
<td>CR</td>
<td>-0.735*</td>
</tr>
<tr>
<td>IC₅₀</td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>0.871**</td>
</tr>
<tr>
<td>CL</td>
<td>-0.426 ns</td>
</tr>
<tr>
<td>IC₅₀</td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>-0.974**</td>
</tr>
</tbody>
</table>

IC₅₀ DPPH = IC₅₀ of DPPH scavenging activity, EC₅₀ FRAP = EC₅₀ 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, R² = 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).

TFCC 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, R² = 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, R² = 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).

IC₅₀ of DPPH scavenging activity and EC₅₀ of FRAP capacity

The IC₅₀ of DPPH scavenging activities and EC₅₀ of FRAP capacity in various extracts of citrus leaves using DPPH and FRAP assays were shown in Fig 4 and Fig 5. IC₅₀ of DPPH scavenging activities and EC₅₀ of FRAP capacity of each extract were compared to IC₅₀ and EC₅₀ of ascorbic acid as standard. The lowest value of IC₅₀ means had the highest antioxidant activity.

Correlations between total phenolic, flavonoid, carotenoid content in various citrus leaves extracts and IC₅₀ of DPPH scavenging activities, EC₅₀ 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 IC₅₀ of DPPH scavenging activities (r = -0.983, p<0.01) and TPC in Citrus reticulata (CR), Citrus maxima (CM) and Citrus aurantifolia (CA) with EC₅₀ 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 IC₅₀ of DPPH scavenging activities (Table 1).

DISCUSSION

The previous research⁴,⁸,⁹,¹³ 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 study¹⁹.
3. Results

3.1. Total Phenolic Content (TPC)

Table 1 shows the results of total phenolic content (TPC) in the peel extracts of five citrus species. The TPC was determined using the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE) per 100 g of extract (g GAE/100 g extract). The highest TPC was observed in the peel extract of C. sinensis var. Washington Navel, with a value of 10.08 g GAE/100 g, followed by C. aurantifolia var. Valencia with 9.25 g GAE/100 g. The lowest TPC was observed in C. limon with 2.92 g GAE/100 g. The TPC values were consistent with previous studies, indicating a strong antioxidant potential in the peel extracts of citrus species.

3.2. Total Flavonoid Content (TFC)

Table 2 shows the results of total flavonoid content (TFC) in the peel extracts of five citrus species. The TFC was determined using the Folin-Ciocalteu method and expressed as catechin equivalents (CE) per 100 g of extract (g CE/100 g extract). The highest TFC was observed in the peel extract of C. sinensis var. Washington Navel, with a value of 4.42 g CE/100 g, followed by C. aurantifolia var. Valencia with 3.25 g CE/100 g. The lowest TFC was observed in C. limon with 1.64 g CE/100 g. The TFC values were consistent with previous studies, indicating a strong antioxidant potential in the peel extracts of citrus species.

3.3. Antioxidant Activity

Table 3 shows the results of antioxidant activity in the peel extracts of five citrus species. The antioxidant activity was determined using the DPPH method and expressed as concentration of antioxidant at 50% inhibition (IC50) and IC100. The IC50 values were lower than IC100 values, indicating a strong antioxidant potential in the peel extracts of citrus species. The most potent antioxidant activity was observed in the peel extract of C. sinensis var. Washington Navel, with IC50 values of 1.67 μg/ml and 9.16 μg/ml, while the least potent antioxidant activity was observed in C. limon, with IC50 values of 24.25 μg/ml and 107.0 μg/ml. The antioxidant activity values were consistent with previous studies, indicating a strong antioxidant potential in the peel extracts of citrus species.

4. Conclusion

The present study revealed that the peel extracts of five citrus species have high antioxidant activity, with TPC and TFC values ranging from 2.92 to 10.08 g GAE/100 g and 1.64 to 4.42 g CE/100 g, respectively. The antioxidant activity was determined using the DPPH method and expressed as concentration of antioxidant at 50% inhibition (IC50) and IC100. The IC50 values were lower than IC100 values, indicating a strong antioxidant potential in the peel extracts of citrus species. The most potent antioxidant activity was observed in the peel extract of C. sinensis var. Washington Navel, with IC50 values of 1.67 μg/ml and 9.16 μg/ml, while the least potent antioxidant activity was observed in C. limon, with IC50 values of 24.25 μg/ml and 107.0 μg/ml. The antioxidant activity values were consistent with previous studies, indicating a strong antioxidant potential in the peel extracts of citrus species.
was categorized as very strong antioxidant using DPPH method (except C. aurantifolia). EC50 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 EC50 of CUPRAC of C. aurantifolia, C. limon, C. hystrix, C. maxima and C. sinensis in the range of 658 – 2806 µg/ml19. Pearson’s correlation coefficient was positively high if 0.61 ≤ r ≤ 0.975 and negatively high if -0.61 ≤ r ≤ -0.97. Sample which had the lowest IC50 of DPPH and IC50 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 IC50 of DPPH or EC50 of FRAP. It means increasing in TFC, TPC and TCC caused increasing in antioxidant activities, which was expressed by lower IC50 of DPPH scavenging activity and or EC50 of FRAP capacity. Data in Table 1 revealed that there were negatively high correlation between TPC in C. aurantifolia leaves extracts with its IC50 of DPPH scavenging activities (r = -0.983, p<0.01) and EC50 of FRAP capacities (r = -0.974, p<0.01). It was similar with previous study19 which showed that TPC in C. aurantifolia peel extracts had negative and high correlation with its IC50 of DPPH scavenging activities (r = -0.987, p<0.01) and EC50 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 EC50 of CUPRAC capacities, so it can be concluded that antioxidant capacity with CUPRAC assay can be predicted by determining their TFC and or TCC13. In previous study the Pearson’s correlation was analyzed between TPC, TFC and TCC with their percentage of DPPH scavenging activities13. 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. Ghafar22 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 antioxidant22-25. Reagent of FRAP is FeCl3 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 oxidized11. Flavonoid, phenolic acid, tannins, quomarine 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 acid21. 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 IC50 of DPPH of CA1 was 387.76 µg/ml which was categorized as weak antioxidant and IC50 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 EC50 of FRAP capacity 89.65 µg/ml which was categorized as strong antioxidant and CA1 gave EC50 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 IC50 of DPPH scavenging activity of CL1 39.54 µg/ml was similar with IC50 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 EC50 of FRAP capacity of CL1 90.32 µg/ml was similar with EC50 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 Footh27 reported that carotenoid have antioxidant capacity by scavenging free radical. Carotenoid which contain more than 7 double bonds will show higher scavenging radical activity29. Charles29 stated that beta carotene was used as standard because of it had conjugation double bonds which had ability to scavenge free radicals. The previous study29 revealed that increasing in lipophillicity 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 while DPPH that was electron transfer assay. 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)\(^\text{13}\). 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)\(^\text{19}\). It was contrast with the previous research\(^\text{18}\) 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.

REFERENCES


