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Research Article

ANTIOXIDANT ACTIVITIES OF ARABICA GREEN COFFEE FROM THREE REGIONS USING ABTS AND DPPH ASSAYS

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ABSTRACT

Objectives: The aim of this research were to determine antioxidant activity from various extracts of arabica green coffee from three different regions using two methods of antioxidant testing which were 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and correlation of total phenolic, flavonoid, and carotenoid content in various extracts of arabica coffee with their IC50 of ABTS and IC50 of DPPH antioxidant activities.

Methods: Extraction was conducted by reflux using various polarity solvents. The extracts were evaporated using rotary evaporator. Antioxidant activities using ABTS and DPPH assays, determination of total phenolic, flavonoid, and carotenoid content were performed by UV-visible spectrophotometry and its correlation with IC50 of ABTS and IC50 of DPPH scavenging activities were analyzed by Pearson's method.

Results: The lowest IC50 of ABTS scavenging activity 3.47 µg/ml was given by n-hexane extract of Toraja arabica coffee, while ethanolic extract of Lintong arabica coffee gave the lowest IC50 of DPPH scavenging activity 0.7 µg/ml. Ethanolic extract of Madailing arabica coffee gave the highest phenolic content, and its ethyl acetate extract had the highest total flavonoid. There were negative and high correlation between phenolic content and carotenoid content in Lintong arabica coffee extract with their IC50 of DPPH.

Conclusions: All of the arabica green coffee extracts from three regions except LIN1 by ABTS method were categorized as a very strong antioxidant and except TOR1 for DPPH method. Phenolic and carotenoid compounds in Lintong arabica coffee extracts were the major contributor in IC50 of DPPH scavenging activities. Arabica coffee extracts from Lintong showed the linear result in ABTS and DPPH assays.

Keywords: Antioxidant, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 2,2-diphenyl-1-picrylhydrazyl, Arabica green coffee, Phenolic, Flavonoid, Carotenoid.

INTRODUCTION

Antioxidant consumption can prevent the excessive of oxidative stress which can cause many diseases. The phenolic compound can be found in plants, and they have been reported to have multiple biological effects, including antioxidant and antibacterial activity [1-4]. Previous research [5-7] demonstrated that phenolic and flavonoid content could be correlated to their antioxidant activities. Plants included coffee contained phenolic and flavonoid compounds [8-10].

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) can be used to predict the antioxidant activity of fruits, vegetables, and food [4,11,12]. Previous studies [9-12] reported that DPPH, FRAP, and ABTS methods could be used to determine antioxidant activity in many plants extracts. Coffee had antioxidant activities using ABTS and DPPH assays [13,14]. Coffee bean contained many compound such as flavonoid, chlorogenic acid, and caffeic acid which were phenolic compounds that can act as antioxidant [8,14].

The objective of this research were to determine antioxidant activities of various polarities extracts (n-hexane, ethyl acetate, and ethanol) of arabica green coffee from three different regions in Indonesia using antioxidant ABTS and DPPH assays, and correlations of total phenolic, flavonoid, and carotenoid content with their antioxidant activities.

METHODS

Materials

ABTS diammonium salt, DPPH, gallic acid, quercetin, beta carotene were purchased from Sigma-Aldrich (MO, USA), arabica coffee from three regions, ethanol. All other reagents were analytical grades.

Preparation of sample

Arabica green coffee were collected from three different regions were: Toraja namely as TOR, Lintong as sample LIN and Mandailing as sample MAN, were thoroughly washed with tap water, wet sortation, cut, dried, and grinded into powder.

Extraction

About 300 g of powdered samples were extracted by reflux using different polarity solvents. 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, totally there were nine extracts: Three of n-hexane extracts (namely TOR1, LIN11, and MAN1), three of ethyl acetate extracts (TOR2, LIN2, and MAN2), and three of ethanolic extracts (TOR3, LIN3, and MAN3).

ABTS scavenging activity

Preparation of ABTS solution was adopted from Li *et al*. [15] method with minor modification. ABTS diammonium salt solution 7.6 mM in aquadest and potassium per-sulfate solution 2.5 mM in aquadest were prepared. Each solution was left in the dark room for 12 hours. The two solutions were mixed with 30 minutes incubation, left the mixture in the refrigerator for 24 hrs, and then diluted in ethanol. Various concentrations of each extract were pipetted into ABTS solution 50 µg/ml (volume 1:1) to initiate the reaction for obtaining a calibration curve. The absorbance was read at wavelength 734 nm using a spectrophotometer UV-Vis Beckman Coulter DU 720. Ethanol (95%) was used as a blank, ABTS solution 50 µg/ml as control and ascorbic acid as standard. Analysis was done in triplicate for standard and each extract. Antioxidant capacity of each extract by ABTS method was determined by calculating the percentage of antioxidant activity

using reduction of ABTS absorbance [16]. IC50 of ABTS scavenging activity of each extract can be calculated using its calibration curve.

DPPH scavenging activity

Preparation of DPPH solution was adopted from Blois [17] with minor modification. Various concentrations 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 using the spectrophotometer UV-Vis Beckman Coulter DU 720. Methanol was used as a blank. DPPH solution 50 µg/ml was used as a control. Ascorbic acid was used as a standard. Analysis was done in triplicate for standard and each extract. Antioxidant activity of each extract by DPPH method was determined by calculating the percentage of antioxidant activity using reduction of DPPH absorbance [16]. IC50 of DPPH scavenging activity of each extract can be calculated using its calibration curve.

Total flavonoid content (TFC)

TFC was measured using modified 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 (36-120 mg/µl) was used to obtain a calibration curve. The TFC was exposed as a percentage of total quercetin equivalent per 100 g extract (g QE/100 g).

Total phenolic content (TPC)

TPC were adapted from Pourmorad [7] using the modified Folin–Ciocalteu method. The absorbance was read at wavelength 765 nm. Analysis was done in triplicate for each extract. Standard solution of gallic acid (40-165 µg/ml) was used to obtain a calibration curve. TPC was reported as a percentage of total gallic acid equivalent per 100 g extract (g GAE/100 g).

Total carotenoid content (TCC)

TCC was measured using the method from Thaipong *et al*. [12] with minor modification. 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 (15-55 µg/ml) was used to obtain a calibration curve. The TCC was demonstrated as a percentage of total beta carotene equivalent per 100 g extract (g BE/100 g).

Statistical analysis

Each sample analysis was performed in triplicate. All of the presented results 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 the correlation between two antioxidant activity methods were performed using the Pearson's method.

RESULTS

ABTS and DPPH scavenging activity

The IC50 of ABTS and IC50 of DPPH scavenging activities in various extracts from three different regions of arabica coffee using ABTS and DPPH assays were shown in Figs. 1 and 2. IC50 of ABTS and IC50 of DPPH scavenging activities of each extract were compared to IC50 ascorbic acid as standard. The lowest value of IC50 means had the highest antioxidant activity.

TFC in various extracts of arabica coffee

TFC among the various extracts were reported in term of quercetin equivalent using the standard curve equation y = 0.0069 x - 0.019, R^2 = 0.9983. The TFC in various extracts from three different regions of arabica coffee had a different result in the range of 3.04-7.41 g QE/100 g (Fig. 3). Ethyl acetate extract of Mandailing arabica coffee (MAN2) had the highest TFC (7.41 g QE/100 g), and the lowest was given by its n-hexane extract (MAN1).

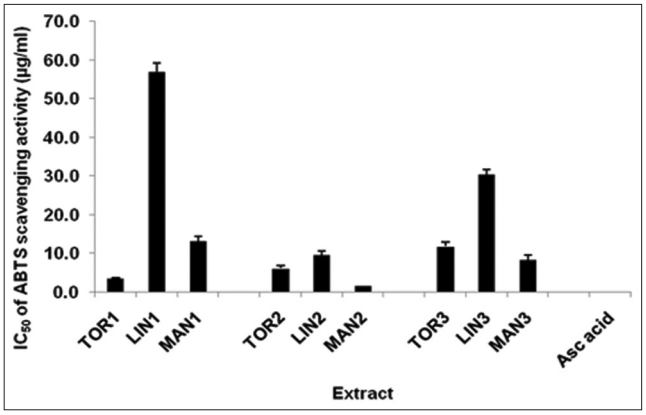

Fig. 1: IC50 of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) scavenging activities in various extracts of arabica coffee from three regions, n=3

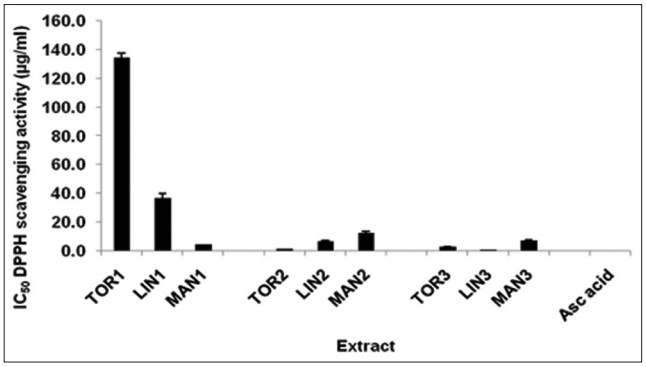

Fig. 2: IC_{50} of 2,2-diphenyl-1-picrylhydrazyl scavenging activities in various extracts of arabica coffee from three regions, n=3

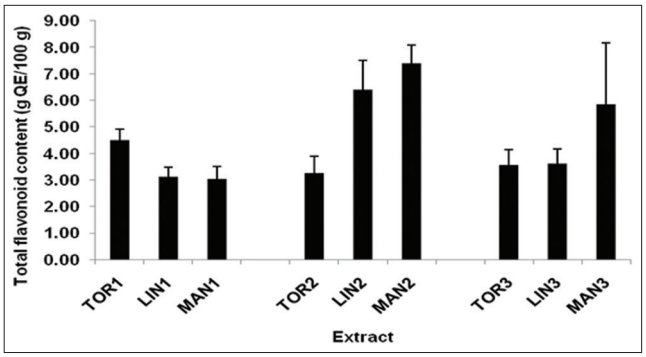

Fig. 3: Total flavonoid content in various extracts of arabica coffee, n=3

TPC in various extracts of arabica coffee

TPC among the various extracts were revealed in term of GAE using the standard curve equation y = 0.0048 x + 0.00257, R^2 = 0.9989. The TPC in various extracts from three different regions of arabica coffee expressed result ranged from 0.75 to 70.55 g GAE/100 g. The highest phenolic content (70.55 g GAE/100 g) was given by ethanolic extract of Mandailing arabica coffee (MAN3) and the lowest for its n-hexane extract (MAN1) 0.75 g GAE/100 g (Fig. 4).

TCC in various extracts of arabica coffee

TCC among the various extracts were reported 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 from three different regions of arabica coffee showed different result ranged from 0.01 to 0.28 g BE/100 g (Fig. 5). Ethanolic extract of arabica coffee from Lintong

(LIN3) gave the highest carotenoid content (0.28 g BE/100 g), while the lowest carotenoid (0.01 g BE/100 g) for n-hexane extract of arabica coffee from Toraja (TOR1).

Correlations between total phenolic, flavonoid, and carotenoid content in various extracts of arabica coffee and IC50 of ABTS, IC50 of DPPH scavenging activities

Pearson's correlation coefficient between TPC in various extracts of arabica coffee and their antioxidant activities exposed that TPC in Toraja extracts (TOR) had negatively high correlation with their IC50 of ABTS scavenging activities (r=−0.940, p<0.01), while TPC in Lintong extracts (LIN) had negative and high significant correlation with IC50 of DPPH scavenging activities (r=−0.832, p<0.01). TFC in Lintong and Mandailing extracts had highly negative correlation with their IC50 of ABTS scavenging activities (r=−0.829, p<0.01; r=−0.735, p<0.05, respectively) and TFC in Toraja extracts had negative and significant correlation with their IC50 of DPPH scavenging activities (r=−0.740, p<0.05). Only TCC in Lintong extract which had negative and high correlation with their IC50 DPPH scavenging activities (r=−0.926, p<0.01) (Table 1).

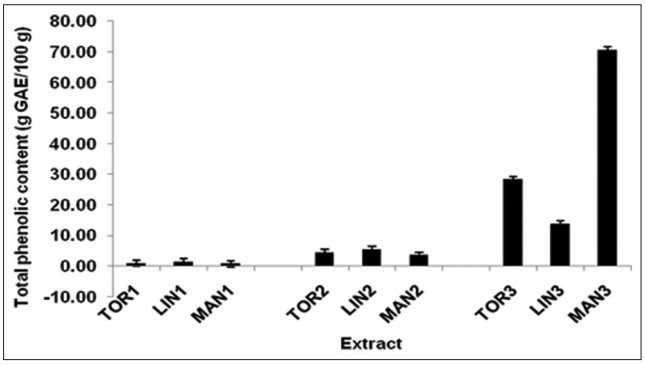

Fig. 4: Total phenolic content in various extracts of arabica coffee, n=3

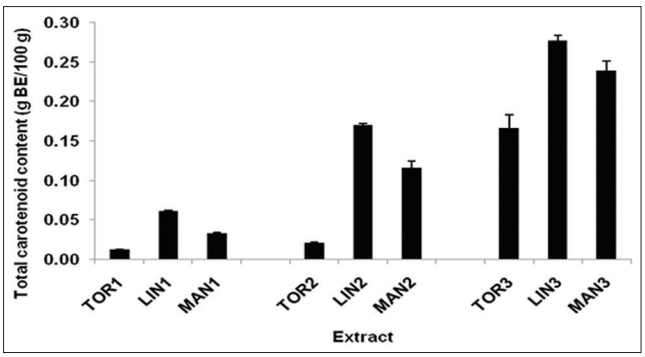

Fig. 5: Total carotenoid content in various extracts of arabica coffee, n=3

DISCUSSION

The previous research [19-21] expressed that coffee had antioxidant capacity. Antioxidant activity of green coffee and roasted coffee had been studied [19,20], while Naidu *et al*. [21] compared the antioxidant activity of robusta coffee and arabica coffee using DPPH method. There was no research regarding the antioxidant activity of various extracts (which were n-hexane, ethyl acetate, and ethanol) of arabica coffee from three regions using ABTS and DPPH assays.

ABTS free radicals dissolve in ethanol have characteristic absorption at l 734 nm and DPPH free radicals dissolve in methanol give absorption at wavelength 516 nm. Colors of ABTS and DPPH would be changed when the free radicals were scavenged by antioxidant [15]. ABTS would be changed from turquoise to white color while DPPH changed from purple to yellow.

The IC50 of ABTS scavenging activities and IC50 of DPPH scavenging activities in various extracts of arabica coffee from three different regions were shown in Figs. 1 and 2. The IC50 of ABTS scavenging activities and IC50 of DPPH scavenging activities in various extracts were compared to IC50 of the ascorbic acid standard. The lowest value of IC50 means had the highest antioxidant activity. Sample which had IC50 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 IC50 >150 µg/ml [17].

In the present study exposed that IC50 of DPPH scavenging activities of various extracts of arabica green coffee from three different regions ranged from 0.70 to 134.56 µg/ml and IC50 of ABTS 3.47 to 56.84 µg/ml, respectively. Based on the value of IC50 of DPPH and IC50 of ABTS scavenging activities, it can be concluded that all of the extracts of arabica coffee from Toraja, Lintong, and Mandailing (except IC50 DPPH of n-hexane Toraja coffee extract and IC50 of ABTS of n-hexane Lintong coffee extract) can be classified as very strong antioxidant. The ethanolic extract of arabica coffee from Lintong had the lowest IC50 of DPPH (LIN3) 0.70 µg/ml, while IC50 of DPPH of ascorbic acid was 0.11 µg/ml. It exposed that potency of ascorbic acid was around six times potency of LIN3 using DPPH method. The lowest IC50 of ABTS scavenging activities 3.47 µg/ml was given by n-hexane extract of arabica coffee from Toraja (TOR1) while ascorbic acid standard showed IC50 of ABTS scavenging activity 0.09 µg/ml.

Brezova [14] studied regarding antioxidant activity of water extract of eleven of ground coffee and nine of instant coffee, demonstrated that antioxidant in the range of 0.15-0.21 mmol TEAC/g and 0.62-0.72 mmol TEAC/g, respectively, by DPPH assay and 0.19-0.25 mmol TEAC/g and 0.59-0.82 mmol TEAC/g, respectively, by ABTS assay. In the present study reported that IC50 of DPPH scavenging activity of ethanolic extract of arabica green coffee from Toraja, Lintong, and Mandailing were 2.67, 0.70, 7.21 µg/ml, respectively, while their IC50 of ABTS scavenging activity were 11.77, 30.34, 8.21 µg/ml, respectively. Previous research exhibited that antioxidant activity of ethanol extract of arabica green coffee and roasted coffee which was roasted in 3 minutes: Light coffee

Table 1: Pearson's correlation coefficient of total phenolic, flavonoid, carotenoid content in various extracts of arabica coffee with their IC_{50} of ABTS scavenging activities and IC_{50} of DPPH scavenging activities

Antioxidant activities	Pearson's correlation coefficient (r)					
	TFC	TPC	TCC	IC_{50} ABTS TOR	IC_{50} ABTS LIN	IC_{50} ABTS MAN
IC_{50} ABTS TOR	−0.380ns	−0.940**	0.928**			
IC_{50} ABTS LIN	−0.829**	−0.383ns	−0.560ns			
IC_{50} ABTS MAN	−0.735*	0.048ns	−0.314ns			
IC_{50} DPPH TOR	−0.740*	−0.582ns	−0.530ns	−0.721*		
IC_{50} DPPH LIN	−0.452ns	−0.832**	−0.926**		0.814**	
IC_{50} DPPH MAN	0.806**	−0.141ns	0.229ns			−0.941**

IC_{50} ABTS: IC_{50} ABTS scavenging activity, IC_{50} DPPH: IC_{50} DPPH scavenging activity, TOR: Arabica coffee from Toraja, LIN: Arabica coffee from Lintong, MAN: Arabica coffee from Mandailing, ns: Not significant, *significant at p<0.05, **significant at p<0.01

(225°C), medium coffee (233°C), and dark coffee (240°C) were 3.2, 3.5, 3.8, and 2.8 mM Trolox, respectively, while antioxidant activity of their water extract were 4.0, 4.8, 4.7, 4.2 mM Trolox, respectively [20]. A study by Richelle [22] revealed that water infusion of robusta green coffee form Indonesia had higher antioxidant activity around two times of water infusion of arabica green coffee from Columbia while their roasted coffee had similar antioxidant activity. Ramalakshmi [8] expressed that DPPH radical scavenging activity of 100 µg/ml methanol extract of arabica coffee was 92.5% which was higher than its acetone extract, chloroform extract, and n-hexane extract. It was similar with b-carotene-linoleic acid method which demonstrated that methanol extract gave the highest antioxidant activity (58.2%) compared to acetone, chloroform, and n-hexane extracts, while using reducing power method (Fe^{3+}-Fe^{2+} reducing system) exposed that methanol extract showed the lowest antioxidant capacity compared to BHA (butylated hydroxyanisole), chlorogenic acid, and ascorbic acid. A previous study [13] exhibited that antioxidant activity of coffee depending on the procedure in brewing the coffee. Filter coffee from different origins had higher antioxidant activity compared to their espresso coffee and Italian coffee by b-carotene-linoleic acid assay. Nicoli *et al*. [19] exposed that 10 minutes was the effective times for roasting arabica coffee which gave the highest antioxidant properties compared to 8, 15, and 20 minutes. Research by Naidu *et al*. [21] stated that 200 µg/ml arabica green coffee which was extracted using isopropanol-water 60:40, 70:30, 80:20 exhibited percentage of DPPH scavenging activity 92%, 87%, and 76%, respectively, while 200 µg/ml robusta green coffee 88%, 82%, and 78%, respectively. The crude coffee extract showed higher percentage hydroxyl scavenging activity (92%) compared to purified coffee extract (80%) by hydroxyl radical scavenging assay using ascorbic acid-iron ethylenediaminetetraacetic acid.

TPC and TFC might be related with antioxidant activity. Previous research [8] demonstrated that methanolic extract of arabica coffee contained TPC 21.90 % GAE, chlorogenic acid 34.16%, and caffeine 8.25%. TPC in water extract of nine of instant coffee ranged from 114 to140 mg GAE/g and eleven of ground coffee 41-58 mg GAE/g. In the present study showed that TPC in ethanolic extract of arabica green coffee from Toraja, Lintong, and Mandailing were 28.31, 13.74, and 70.55 g GAE/100 g. It was similar with the previous study [21] which reported that TPC in arabica coffee which were extracted using isopropanol-water (80:20), (70:30), (60:40) were 23.29, 28.71, 32.19% GAE, while TPC in robusta coffee were 22.89, 26.19, 31.71% GAE. TFC in the present study demonstrated that ethanolic arabica coffee extract of Toraja, Lintong and Mandailing were 3.57, 3.60, 5.86 g QE/100 g and their TCC 0.17, 0.28, 0.24 g BE/100 g, respectively.

Pearson's correlation coefficient was positively high if $0.61 < r < 0.97$ [12] and negatively high if $-0.61 < r < -0.97$. The 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 IC50 of ABTS. 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 IC50 of ABTS scavenging activity.

The data in Table 1 exhibited that the negative and high correlation between TPC and TCC in arabica coffee extracts from Lintong with IC50 of DPPH scavenging activities ($r = -0.832$; $r = -0.926$, $p<0.01$, respectively) and TPC in arabica coffee from Toraja with IC50 of ABTS scavenging activities ($r = -0.940$, $p<0.01$). TFC in arabica coffee extracts from Toraja had negative and high correlation with their IC50 of DPPH ($r = -0.74$, $p<0.05$) and TFC in arabica coffee extracts from Lintong and Mandailing with their IC50 of ABTS ($r = -0.829$, $p<0.01$; $r = -0.735$, $p<0.05$, respectively). Previous research [14] stated that TPC in water extract of ground coffee had positive and high correlation with their antioxidant activity by DPPH method ($r = 0.7289$, $p<0.05$) and ABTS method ($r = 0.9219$, $p<0.01$). A positive correlation was used by Brezova [14] because of their antioxidant activity was stated as TEAC value (mmol/g), so the higher antioxidant activity would be presented by the higher value of TEAC. A positive correlation means that increasing in TPC would increase antioxidant activity (expressed as TEAC value). It was contrast with the present study, which the correlation between TPC, TFC, and TCC and their IC50 DPPH and IC50 ABTS.

The phenolic acid flavonoid, tannins, qoumarine, and quinone are included in phenolic groups. Flavonoid compound which have OH in A ring and or B ring will be included in phenolic groups. Flavonoid had higher antioxidant activity than phenolic acid [23]. The flavonoid glycosides would give lower antioxidant activity than flavonoid aglycones [23]. 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 the antioxidant activity of flavonoid. It could be seen in Fig. 4 that TPC in ethyl acetate extract of arabica coffee form Lintong (LIN2) 5.38 g GAE/100 g was smaller than ethanolic arabica coffee extract from Mandailing (MAN3) 70.55 g GAE/100 g, but IC50 of DPPH scavenging activity of LIN2 (6.28 µg/ml) was similar with IC50 of DPPH scavenging activity of MAN3 (7.21 µg/ml). It can be predicted that many phenolic compounds in LIN2 which had influence high antioxidant capacities, whereas in MAN3 only a little phenolic compounds with high antioxidant activities. TFC in ethanolic arabica coffee extract from Toraja (TOR3) 3.57 g QE/100 g was smaller than TFC in n-hexane arabica coffee extract from Toraja (TOR1) 4.50 g QE/100 g, but IC50 of DPPH scavenging activity of TOR3 2.67 µg/ml which was categorized as very strong antioxidant, was smaller than TOR1 134.56 µg/ml which was classified as medium antioxidant. Based on this data, it can be supposed that almost all of flavonoid in TOR3 were flavonoid that had OH in a position which can influence high antioxidant capacities. In contrast, many flavonoids in TOR1 had OH in C5, C7, or C3' only, or C4' only, or C3 only without oxo function in C4, that had no and low influence in antioxidant activities.

Foote [24] stated that carotenoid has antioxidant capacity by scavenging free radical. More double bonds in carotenoid will give higher scavenging free radical activity. Previous research by Beutner *et al*. [25] revealed that carotenoid would give higher scavenging radical activity if contain <7 double bonds. Beta carotene was used as standard because of it has conjugation double bonds which have ability to scavenge free radicals [26]. Decreasing in antioxidant activity which be shown by higher IC50 of DPPH scavenging activity will be given by decreasing in lipophilicity of carotenoid [27]. TCC in n-hexane arabica coffee extract from Lintong (LIN1) 0.06 g BE/100 g was greater than TCC in n-hexane extract of arabica coffee from Mandailing (MAN1) 0.03 g BE/100 g, but IC50 of ABTS dan IC50 of DPPH scavenging activities of MAN1 (13.07 µg/ml, 4,44 µg/ml, respectively) was smaller than LIN1 (56.84 µg/ml, 36.83 µg/ml, respectively), it means antioxidant activity of MAN1 was greater than LIN1. It might be supposed that MAN1 consisted of many carotenoids with more than 7 double bonds which had high antioxidant activities, whereas LIN1 contained many carotenoids with maximum 7 double bonds.

Electron transfer was the principle of both of ABTS and DPPH assays, but in the present study only arabica green coffee extracts from Lintong showed positive and high correlation between IC50 of ABTS scavenging activities and IC50 of DPPH scavenging activities ($r = 0.814$, $p<0.01$). It could be seen that IC50 of ABTS scavenging activities in arabica coffee extracts from Lintong were linear with their IC50 of DPPH scavenging activities.

CONCLUSION

Antioxidant activity of sample should be measured by different methods in parallel because various methods could give different results. All of the extracts of arabica coffee from three regions (Toraja, Lintong, and Mandailing) were very strong antioxidant using ABTS assay (except LIN1), and DPPH assay (except TOR1). TPC and TCC in Lintong extracts had negative and high correlation with their IC50 of DPPH scavenging activities. Phenolic compounds and carotenoid compounds in Lintong arabica coffee extracts were the major contributors in IC50 of DPPH

scavenging activity. There was a linear correlation between IC50 of ABTS scavenging activities, and IC50 of DPPH scavenging activities of Lintong extracts sample. Arabica green coffee from Toraja, Lintong, and Mandailing may be exploited as natural antioxidant sources.

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