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Research Article

IN VITRO ANTIOXIDANT ACTIVITIES OF VARIOUS LEAVES EXTRACTS FROM FIVE VARIETIES OF RAMBUTAN *(NEPHELIUM LAPPACEUM)* AND IT'S CORRELATION WITH TOTAL FLAVONOID, PHENOLIC, CAROTENOID CONTENT

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ABSTRACT

Objectives: The objectives of this research were to study antioxidant activity from different polarities extracts of rambutan (*Nephelium lappaceum*) leaves using two methods of antioxidant testing which were 2-2-diphenyl-1-picrylhydrazyl (DPPH) and 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and correlation of total flavonoid, phenolic and carotenoid content in various extracts of rambutan leaves with DPPH and ABTS antioxidant activities.

Methods: Extraction was performed by reflux using different polarity solvents. The extracts were evaporated using rotavapor. Antioxidant activities using DPPH and ABTS assays, determination of total phenolic, flavonoid and carotenoid content were conducted by spectrophotometry ultra violet-visible and its correlation with DPPH and ABTS antioxidant capacities were analyzed by Pearson's method.

Results: RPH3 (ethanolic leaves extract of rapiah rambutan) had the highest DPPH scavenging capacity with inhibitory concentration 50 (IC_{50}) 14.666 µg/mL and the highest ABTS scavenging activity with IC_{50} 12.826 µg/mL. RPH3 had the highest phenolic content (29.46 g gallic acid equivalents/100 g), NON2 (ethyl acetate leaves extract of non-consumption rambutan) contained the highest total flavonoid (9.59 g quercetin equivalents/100 g), and NON1 (n-hexane leaves extract of non-consumption rambutan) had the highest carotenoid 10.99 g beta-carotene equivalent/100 g.

Conclusions: There were positively and high correlation between total phenolic content in all of the leaves extracts with their antioxidant activity using DPPH and ABTS assays. DPPH scavenging activities in all of the samples gave linear result with ABTS scavenging capacities.

Keywords: Antioxidants, 2-2-diphenyl-1-picrylhydrazyl, 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid), Rambutan leaves, Flavonoid, Phenolic, Carotenoid.

INTRODUCTION

The risk of many diseases that related to oxidative stress can be reduced by antioxidant. Phenolic compounds are commonly found in plants, and they have multiple biological effects, including antibacterial, anti-inflammatory and antioxidant activity [1-4]. Many studies [5-9] exhibited that phenolic content and flavonoid content in plants could be correlated to their antioxidant activities.

Some of antioxidant methods such as 2-2-diphenyl-1-picrylhydrazyl (DPPH) and 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) were used to predict antioxidant capacity of vegetables, fruits, beverages, and food [1,10,11]. In previous study [1,12-14] revealed that DPPH and ABTS methods could be used to determine antioxidant activity in many plants extracts. Study by Thitilertdecha *et al*. [4] and Tachakittirungrod *et al*. [14] demonstrated that rambutan (*Nephelium lappaceum*) had antioxidant capacities using DPPH, ABTS and ferric reducing antioxidant power assays.

The objective of this research were to study antioxidant activities of different polarity extracts (n-hexane, ethyl acetate and ethanol) of leaves form five varieties of rambutan (rapiah rambutan, rajah rambutan, binjai rambutan, lebak bulus rambutan and non-consumption rambutan) using antioxidant testing DPPH and ABTS assays and correlations of their antioxidant capacities with total flavonoid, phenolic, and carotenoid content in each extracts.

METHODS

Materials

DPPH, ABTS diammonium salt, gallic acid, quercetin, beta carotene was purchased from Sigma-Aldrich (MO, USA), ferric chloride, methanol, ethanol, acetone. All other reagents were analytical grades.

Preparation of sample

Leaves from five varieties of rambutan (*N. lappaceum*) were collected from Subang-West Java that were: Rapiah rambutan namely as RPH, rajah rambutan as sample RJH, binjai rambutan as sample BNJ, lebak bulus rambutan as sample LBB and non-consumption rambutan as sample NON, were thoroughly washed with tap water, wet sortation, cut, dried and grinded into powder.

Extraction

A volume of 300 g of powdered samples were extracted by reflux using increasing gradient polarity solvents. The n-hexane extract was repeated 3 times. The remaining residue was then extracted 3 times with ethyl acetate. Finally, the remaining residue was extracted 3 times with ethanol. So there were five n-hexane extracts (namely RPH1, RJH1, BNJ1, LBB1 and NON1), five ethyl acetate extracts (RPH2, RJH2, BNJ2, LBB2 and NON2) and five ethanolic extracts (RPH3, RJH3, BNJ3, LBB3 and NON3).

Determination of DPPH scavenging capacity

Preparation of DPPH solution was adopted from Molyneux [15] and Blois [16] with minor modification. Each extracts 50 µg/mL was pipetted into DPPH solution concentration 50 µg/mL (1:1) to initiate the reaction. After 30 minutes incubation, the absorbance was read at wavelength 516 nm using spectrophotometer ultraviolet-visible (UV-Vis) Hewlett Packard 8435. Methanol was used as a blank. DPPH solution 50 µg/mL and methanol (1:1) 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 [15,17].

Determination of ABTS capacity

Preparation of ABTS radical solution was adopted from Li *et al*. [18] and Pellegrini *et al*. [19] method with minor modification. ABTS diammonium salt solution 7.6 mM in water and potassium persulfate solution 2.5 mM in water were prepared. Each solution allowing to stand in the dark room for 12 hrs. The two solutions were mixed with 30-60 minutes incubation, then diluted in ethanol. Each extracts 50 µg/mL was pipetted into ABTS solution 50 µg/mL (1:1) to initiate the reaction. The absorbance was read at wavelength 734 nm without incubation time using spectrophotometer UV-Vis Hewlett Packard 8435. Ethanol (95%) was used as a blank. ABTS solution 50 µg/mL and ethanol (1:1) was used as a standard. Analysis was done in triplicate for standard and each extract. Antioxidant capacity of each extract was determined based on the reduction of ABTS absorbance by calculating percentage of antioxidant activity [17].

Determination of total phenolic content

Total phenolic content were measured using the modified Folin-Ciolcalteu method adapted from Pourmorad *et al*. [20]. The absorbance was read at wavelength 765 nm. Analysis was done in triplicate for each extract. Standard solutions of gallic acid with concentration 45-150 µg/mL were used to obtain a standard curve. The total phenolic content was reported as a percentage of total gallic acid equivalents per 100 g extract (g GAE/100 g).

Determination of total flavonoid content

Total flavonoid content was measured using adapted method from Chang *et al.* [21]. The absorbance was read at wavelength 415 nm. Analysis was done in triplicate for each extract. Standard solutions of quercetin with concentration 20-160 µg/mL were used to obtain a standard curve. The total flavonoid content was reported as a percentage of total quercetin equivalents per 100 g extract (g QE/100 g).

Determination of total carotenoid content

Total carotenoid content was measured using the modified carotene method adapted from Thaipong *et al.* [1]. Each extract were diluted in n-hexane. The absorbance was read at wavelength 470 nm. Analysis was done in triplicate for each extracts. Standard solutions of beta carotene with concentration 5-40 µg/mL were used to obtain a standard curve. The total carotenoid content 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 16.0 for Windows. Correlations between the total phenolic, flavonoid and total carotenoid content and antioxidant capacities were made using the Pearson's procedure ($p<0.01$).

RESULTS

Antioxidant capacities of various leaves extracts from five varieties of rambutan using DPPH and ABTS assays

The antioxidant capacities using DPPH and ABTS assays of various leaves extracts from five varieties of rambutan were shown in Tables 1-3. In the DPPH method, free radical scavenging capacities of various leaves extracts from five varieties of rambutan ranged from 3.69% to 94.63%. BNJ3 (ethanolic leaves extract of binjai rambutan) had the highest DPPH radical scavenging capacity (94.63%), while LBB1 (n-hexane leaves extract of lebak bulus rambutan) had the lowest DPPH antioxidant capacity (3.69%).

In ABTS method, radical scavenging activities in the range of 4.81-91.17%. RPH3 (ethanolic leaves extract of rapiah rambutan) had the highest ABTS capacity (91.17%), while the lowest capacity (4.81%) was given by BNJ1 leaves extract.

Inhibitory concentration 50 (IC_{50}) of DPPH and ABTS scavenging activities

The IC_{50} of DPPH and ABTS scavenging activities in various leaves extracts from five varieties of rambutan using DPPH and ABTS assays

Table 1: DPPH and ABTS scavenging activities of n-hexane leaves extracts

Sample	DPPH scavenging activities (%)	ABTS scavenging activities (%)
RPH1	8.39±0.00[a]	11.04±0.56[a]
RJH1	3.71±0.00[b]	11.76±0.23[a]
BNJ1	4.16±0.48[b]	4.81±0.36[b]
LBB1	3.69±0.08[b]	11.58±0.14[a]
NON1	11.11±0.24[c]	23.04±0.00[c]
Ascorbic acid	95.78±0.00	99.78±0.00
p value	<0.05	<0.05

[a-c]Means within a column with the different letter were significantly different (p<0.05), DPPH: 2-2-diphenyl-1-picrylhydrazyl, ABTS: 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)

Table 2: DPPH and ABTS scavenging activities of ethyl acetate leaves extracts

Sample	DPPH scavenging activities (%)	ABTS scavenging activities (%)
RPH2	53.68±0.08[a]	64.73±0.45[a]
RJH2	43.53±0.08[b]	64.32±0.96[ab]
BNJ2	33.57±0.64[c]	52.44±0.44[c]
LBB2	19.22±0.00[d]	26.71±1.23[d]
NON2	68.78±0.00[e]	62.38±0.23[b]
Ascorbic acid	95.78±0.00	99.78±0.00
p value	<0.05	<0.05

[a-e]Means within a column with the different letter were significantly different (p<0.05), DPPH: 2-2-diphenyl-1-picrylhydrazyl, ABTS: 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)

Table 3: DPPH and ABTS scavenging activities of ethanolic leaves extracts

Sample	DPPH scavenging activities (%)	ABTS scavenging activities (%)
RPH3	93.18±0.14[a]	91.17±0.22[a]
RJH3	93.04±0.00[a]	89.79±0.46[b]
BNJ3	94.63±0.00[b]	86.44±0.32[c]
LBB3	92.21±0.08[c]	81.14±0.38[d]
NON3	91.70±0.00[d]	83.98±0.45[e]
Ascorbic acid	95.78±0.00	99.78±0.00
p value	<0.05	<0.05

[a-e]Means within a column with the different letter were significantly different (p<0.05), DPPH: 2-2-diphenyl-1-picrylhydrazyl, ABTS: 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)

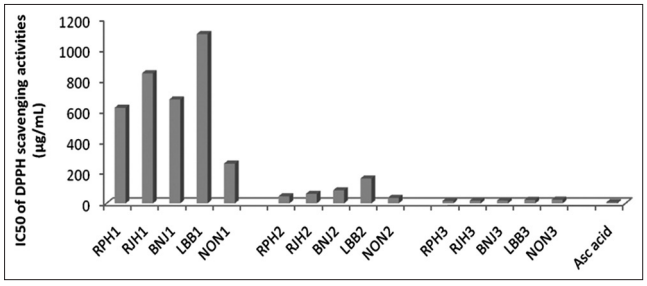

Fig. 1: Inhibitory concentration 50 of 2-2-diphenyl-1-picrylhydrazyl scavenging activities in various leaves extracts from five varieties of rambutan

were shown in Figs. 1 and 2. IC_{50} of DPPH and ABTS scavenging activities were compared to IC_{50} of ascorbic acid as standard. The lowest IC_{50} means had the highest antioxidant capacity.

Total phenolic in various leaves extracts from five varieties of rambutan

The total phenolic content among the various extracts were expressed in term of gallic acid equivalent using the standard curve equation y=0.0055x+0.0308, R^2=0.9996. The total phenolic content in various leaves extracts from five varieties of rambutan showed a different result ranged from 0.84 to 29.46 g GAE/100 g. RPH3 (ethanolic leaves extract of rapiah rambutan) had the highest phenolic content (29.46 g GAE/100 g) (Fig. 3).

Total flavonoid in various leaves extracts from five varieties of rambutan

The total flavonoid content among the various extracts were expressed in term of quercetin equivalent using the standard curve equation y=0.0072x−0.0196, R^2=0.9998. The total flavonoid content in various leaves extracts from five varieties of rambutan showed a different result in the range of 1.83-9.59 g QE/100 g (Fig. 4). NON2 (ethyl acetate leaves extract of non-consumption rambutan) had the highest total flavonoid content (9.59 g QE/100 g) and the lowest (1.83 g QE/100 g) for NON3 leaves extract.

Total carotenoid in various leaves extracts from five varieties of rambutan

The total carotenoid content among the various extracts were expressed in term of beta carotene equivalent using the standard curve equation y=0.0175x+0.0045, R^2=0.9996. The total carotenoid content in various leaves extracts from five varieties of rambutan showed a different result in the range of 0.003-10.99 g BE/100 g (Fig. 5). The highest carotenoid content (10.99 g BE/100 g) for NON1 leaves extract, while the lowest carotenoid (0.003 g BE/100 g) for RJH3 leaves extract.

Correlations between DPPH, ABTS scavenging activities and total phenolic, flavonoid, carotenoid content in various leaves extracts from five varieties of rambutan

Pearson's correlation coefficient was positively high if $0.68 \leq r \geq 0.97$ [1]. Pearson's correlation coefficient between total phenolic content from various extracts of five varieties of rambutan and their antioxidant activities demonstrated that total phenolic all of sample had positively high correlation with DPPH scavenging activities (RPH, r=0.958; RJH, r=0.975; BNJ, r=1.000; LBB, r=0.998; NON, r=0.972, p<0.01) and ABTS scavenging activities (RPH, r=0.902; RJH, r=0.884; BNJ, r=0.952; LBB, r=0.994; NON, r=0.987, p<0.01) (Table 4).

All of the samples exposed that total carotenoid had a negative correlation with their DPPH and ABTS scavenging activities, and almost all of total flavonoid of sample had a negative correlation also with their DPPH and ABTS.

DISCUSSION

Study by Thitilertdecha *et al*. [4,9], Tachakittirungrod *et al*. [14], exposed that rambutan had antioxidant capacity. There were no study regarding antioxidant activity of three different polarities extracts (that were n-hexane, ethyl acetate, and ethanol) of leaves form five varieties of rambutan using DPPH and ABTS assays.

Both of ABTS and DPPH are stable free radicals that dissolve in methanol or ethanol, and their colors show the characteristic absorption at wavelength 734 nm or 516 nm, respectively. Colors ABTS and DPPH would be changed when the free radicals were scavenged by antioxidant [22].

In the present study, 50 µg/mL of ethanolic leaves extract from five varieties of rambutan (rapiah rambutan, rajah rambutan, binjai rambutan, lebak bulus rambutan and non-comsumption rambutan) which were reacted with 50 µg/mL DPPH solution gave DPPH scavenging capacity 93.18%, 93.04%, 94.63%, 92.21%, 91.70%, respectively.

IC_{50} of ABTS scavenging activity is concentration of sample or standard that can exhibit 50% of ABTS activity, while IC_{50} of DPPH scavenging capacity is concentration of sample or standard that can inhibit 50% of DPPH scavenging capacity. The lowest IC_{50} means had the highest antioxidant capacity. IC_{50} were used to determine antioxidant capacity of

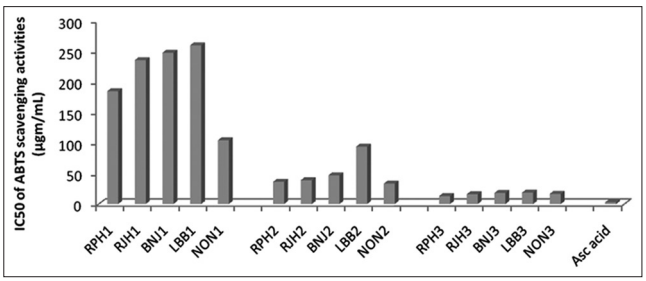

Fig. 2: Inhibitory concentration 50 of 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) scavenging activities in various leaves extracts from five varieties of rambutan

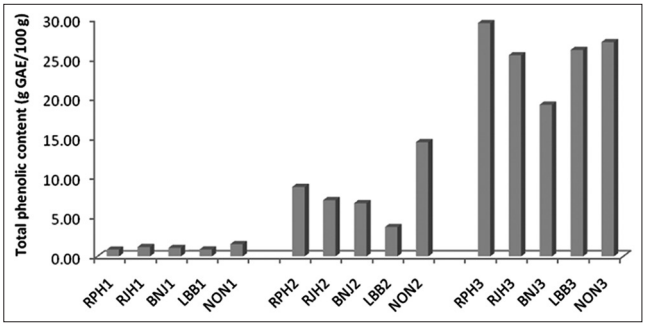

Fig. 3: Total phenolic content in various rambutan leaves extracts

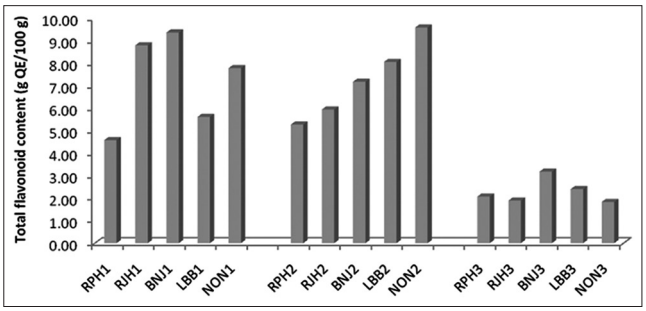

Fig. 4: Total flavonoid content in various rambutan leaves extracts

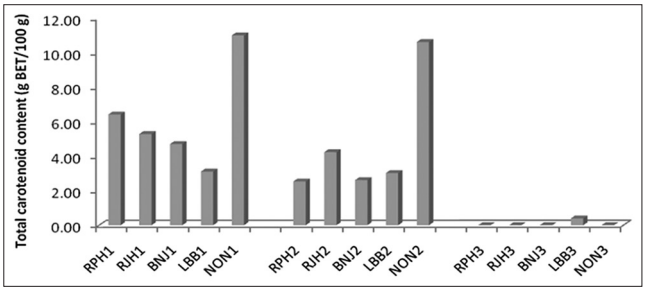

Fig. 5: Total carotenoid content in various rambutan leaves extracts

Table 4: Pearson's correlation coefficient of DPPH, ABTS scavenging activities and total phenolic, flavonoid, carotenoid of leaves extract from five varieties of rambutan

	Total phenolic	Total flavonoid	Total carotenoid	ABTS RPH	ABTS RJH	ABTS BNJ	ABTS LBB	ABTS NON
DPPH RPH	0.958**	−0.718*	−0.996**	0.988**				
DPPH RJH	0.975**	−0.999**	−0.963**		0.966**			
DPPH BNJ	1.000**	−0.999**	−0.990**			0.957**		
DPPH LBB	0.998**	−0.819**	−0.991**				0.999**	
DPPH NON	0.972**	−0.547^{ns}	−0.740**					0.997**
ABTS RPH	0.902**	−0.602^{ns}	−0.997**					
ABTS RJH	0.884**	−0.956**	−0.861**					
ABTS BNJ	0.952**	−0.965**	−0.987**					
ABTS LBB	0.994**	−0.793*	−0.984**					
ABTS NON	0.987**	−0.610^{ns}	−0.790*					

DPPH: DPPH scavenging activity, ABTS: ABTS scavenging activity, RPH: Leaves extract of RPH, RJH: Leaves extract of RJH, BNJ: Leaves extract of BNJ, LBB: Leaves extract of LBB, NON: Leaves extract of NON, ns: Not significant, *Significant at p<0.05, **Significant at p<0.01, DPPH: 2-2-diphenyl-1-picrylhydrazyl, ABTS: 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)

the sample was compared to standard. Classification by Blois stated that sample which had IC_{50}<50 µg/mL, it was very strong antioxidant, 50-100 µg/mL strong antioxidant, 101-150 µg/mL medium antioxidant, while weak antioxidant with IC_{50} or effective concentration >150 µg/mL [16].

In the DPPH method, IC_{50} of various extracts from five varieties of rambutan ranged from 14.666 to 1100.656 µg/mL. RPH3 (ethanolic leaves extract of rapiah rambutan) had the lowest IC_{50} of DPPH radical scavenging capacity 14.666 µg/mL, while ascorbic acid standard gave IC_{50} of DPPH scavenging capacity 6.470 µg/mL. Based on the value of IC_{50} of DPPH scavenging capacity it can be concluded that ethanolic extract of all of the sample can be categorized as very strong antioxidant. It showed that the potency of RPH3 was a half of potency of ascorbic acid using DPPH method. Study by Thitilertdecha *et al*. [4] expressed that IC_{50} of DPPH scavenging activity of methanol peels extract of rambutan was 4.94 µg/mL that was lower than ascorbic acid as standard. Ethanol 80 % peels extract which was extracted in 120 minutes at 50°C had IC_{50} of DPPH scavenging activity 8.87 µg/mL [8], while Thitilertdecha *et al*. [9] demonstrated that methanolic peels extract of rambutan had strong antioxidant activity.

IC_{50} of ABTS scavenging activity of various leaves extracts from five varieties of rambutan in the range of 12.826-259.656 µg/mL. RPH3 (ethanolic leaves extract of rapiah rambutan) had the lowest IC_{50} of ABTS scavenging activity (12.826 mg/mL) while ascorbic acid standard gave IC_{50} of ABTS scavenging capacity 2.690 µg/mL. It exhibited that potency of ascorbic acid was around 5 times of potency of RPH3 using ABTS assays. The previous research [14] revealed that the ethanol extract of fruit peels of rambutan (*N. lappaceum*) had trolox equivalent antioxidant capacity (TEAC) values was 3.07 mM/mg. TEAC assays is the same with ABTS assays. Fruit peels of rambutan can be classified as extremely high antioxidant activity because of its TEAC values above 3.0 mM/mg.

The presence of total phenolic might contributed in antioxidant capacity [10]. Cinnamic acid had higher antioxidant capacity than phenyl acetic acid and benzoic acid [23]. The present study exposed that total phenolic in ethanolic leaves extract of rapiah rambutan, rajah rambutan, binjai rambutan, lebak bulus rambutan and non-consumption rambutan were 29.46, 25.41, 19.16, 26.08, 27.07 g GAE/100 g, respectively. Research by Thitilertdecha *et al*. [4] revealed that total phenolic of methanolic peels extract of rambutan (542.2 mg catechin/g) was higher than water peels extract, ether peels extract, methanol seeds extract, ether seeds extract and water seeds extract Study by Samuagam *et al*. [8] exposed that ethanolic 80% peels extract had total phenolic content 53.94 mg GAE/g extract. In contrast with the previous study [9] demonstrated that methanol peels extract of rambutan contained total phenolic 542 mg/g extract.

The data in Table 4 exposed that there were positively high correlation between total phenolic content in all of the leaves extracts samples (RPH, RJH, BNJ, LBB, and NON) with DPPH and ABTS scavenging activities. Antioxidant capacities in leaves extract of five varieties of rambutan can be predicted indirectly by determining total phenolic content.

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

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

Total flavonoid content in NON3 (ethanolic leaves extract of non-consumption rambutan) was lower (1.83 g QE/100 g) than total flavonoid in NON2 (9.59 g QE/100 g), but antioxidant activity of NON3 was higher than NON2, which was IC_{50} DPPH scavenging activity of NON3 (23.47 µg/mL) was lower than NON2 (35.41 µg/mL). Based on the data above it can predicted that almost all of flavonoids in NON3 were flavonoid that had OH in position which can influence antioxidant capacities, while many flavonoid in NON2 had OH in other position, 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 leaves extract of five varieties of rambutan had a negative correlation with their DPPH and ABTS scavenging activity. Carotenoid had antioxidant capacity by scavenging free radical. More double bonds in carotenoid would give higher scavenging free radical capacity [24]. Carotenoid that consisted of more than seven double bonds would give higher scavenging radical free capacity [25]. Kobayashi and Sakamoto [26] stated that increasing in lipophilicity of carotenoid would increase scavenging radical capacity. Beta carotene was used as standard because of it had conjugation double bonds doe to its ability to scavenge free radicals [27]. NON1 leaves extracts had the highest carotenoid (10.99 g BE/100 g) and the lowest was given RJH3 (0.003 g BE/100 g), but antioxidant capacity of RJH3 was higher than NON1, which was IC_{50} of DPPH scavenging activity of RJH3 (17.17 µg/mL) was lower than NON1 (257.97 µg/mL). Based on this data, it could be seen that many carotenoids in RJH3 had more than seven double bonds, which had high antioxidant capacity. In contrast, many carotenoid in NON1 had lower than seven double bonds.

ABTS and DPPH methods had the same mechanism reaction, which was electron transfer assays [28]. The results of this study showed that DPPH scavenging activities in all of extracts sample were linear with their ABTS scavenging capacities.

CONCLUSION

Antioxidant capacity of the sample should perform using a variety of methods in parallel, because different methods could give different

results. Ethanolic leaves extracts of rambutan had IC_{50} of DPPH scavenging capacities <50 µg/mL that were very strong antioxidant. The positively and high correlation between total phenolic with DPPH and ABTS scavenging activities were given by all of the leaves extracts sample. Phenolic compounds were the major contributor in DPPH and ABTS scavenging capacities in leaves extract of five varieties of rambutan. There was linear correlation between DPPH and ABTS result in all of the leaves extract samples. Rapiah rambutan, rajah rambutan, binjai rambutan, lebak bulus rambutan and non-consumption rambutan may be exploited as a source of beneficial compounds for human health to alleviate oxidative stress.

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