RJC

RASĀYAN ***J. Chem.***
Vol. 11 | No. 4 |1628 - 1634| October - December | 2018
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

COMPARISON PROFILE OF DIFFERENT EXTRACTS OF *Averrhoa Bilimbi* L. IN ANTIOXIDANT PROPERTIES AND PHYTOCHEMICAL CONTENT

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ABSTRACT

The negative effects of free radicals can be reduced by antioxidants. Antioxidants inhibit the oxidation reaction due to free radicals. Antioxidants can be found in a variety of plants, such as Bilimbi (*Averrhoa Bilimbi* L.). The objectives of this research were to study antioxidant activity of leaves, fruit and twigs of Bilimbi by DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6- sulphonic acid)] and determine its phytochemical content. Antioxidant activities, total phenolic content (TPC) and total flavonoid content (TFC) were performed by the UV-Visible spectrophotometric method. Correlation of TPC and TFC with their IC_{50} of DPPH and IC_{50} of ABTS and also a correlation of two methods were conducted by Pearson's method. All parts extracts of Bilimbi were generally classified as a very strong antioxidant by DPPH and ABTS methods. Waste products of Bilimbi (leaves and twigs) were a potential antioxidant. The major contributors in antioxidant activities of leaves, fruit and twigs extracts of Bilimbi by DPPH and ABTS methods were phenolic compounds. DPPH and ABTS methods gave linear results in antioxidant activities of leaves and twig extracts of Bilimbi.

Keywords: antioxidant, Bilimbi, leaves, fruits, twigs, DPPH, ABTS

INTRODUCTION

Bilimbi (*Averrhoa Bilimbi* L.) belongs to Oxalidaceae family contained an alkaloid, carbohydrate, phenols, flavonoid, saponin, tannin, triterpenoid, steroid.[1-3]Vegetables and fruits contain many phenolic and flavonoid compounds which are suggested to prevent development in degenerative diseases such as cancer and heart diseases.[4,5] Many degenerative diseases such as hypercholesterolemia and cardiovascular are closely related with the excessive of free radical.[6]

Free radicals are either atoms or molecules containing unpaired electrons in their outer orbitals so that they are very reactive. The accumulation of free radicals in the body triggers the formation of oxidative stress which is a condition when harmful oxygen invades biological molecules such as lipids, proteins, and DNA. The human body actually has a series of defense systems against free radical attacks such as superoxide dismutase. The body must obtain intake of antioxidants from outside to prevent the occurrence of bad things that are triggered by free radicals.[7]

Many previous types of research published that plants contained flavonoid and phenolic compounds had antioxidant,[8-15]anti-cancer, [16]antimicrobial,[17]and anti-inflammatory activities.[18] Navarro and Ona [19]studied regarding the comparison between three different drying methods (sun drying, freeze drying and cabinet drying) of Bilimbi flowers, then the total phenolic content, flavonoid content and its antioxidant activity were determined. Comparison of three parts of Bilimbi using three polarities solvent have not been reported yet. Leaves and twigs were the waste products of Bilimbi, which might have similar antioxidant potential with their fruit.

The aims of this study were to compare antioxidant activity by DPPH and ABTS methods, determine phytochemical content of different parts of Bilimbi, then analyze the correlation of their phytochemical content and antioxidant activities.

http://dx.doi.org/10.31788/RJC.2018.1143091

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Chemicals

Gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (MO, USA). Other chemicals used were analytical grade.

Sample Preparation

Parts of Bilimbi which were used in this study: leaves namely as LV, fruits as FR, and twigs as TW then selected, washed, dried and ground into powder. Bilimbi was collected from Subang, West Java-Indonesia and identified in Herbarium Bandungense- School of Life Science and Technology- Bandung Institute of Technology, exposed as Bilimbi (*Averrhoa Bilimbi* L.).

Preparation of Extraction

Extraction was done triplicate by reflux for each solvent. Sample 300 g was extracted using different polarity solvent: n-hexane, ethyl acetate and ethanol, consecutively. There were n-hexane leaves extract (LV1), n-hexane fruits extract (FR1), n-hexane twigs extract (TW1), ethyl acetate leaves extract (LV2), ethyl acetate fruits extract (FR2), ethyl acetate twigs extract (TW2), ethanol leaves extract (LV3), ethanol fruits extract (FR3), and ethanol twigs extract (TW3).

Total Phenolic Content (TPC)

Gallic acid 40-120 µg/ml was used as a standard. Folin-Ciocalteu reagent (which diluted 1:10 with distilled water) was used to observe total phenolic content. Folin-Ciocalteu reagent 5 ml was put into gallic acid 0.5 ml, then added by 4 ml sodium carbonate 1 M. The mixture was allowed to stand 15 min at room temperature, the absorbance was read at wavelength 765 nm. The sample was performed by the same procedure. TPC in each sample was presented as gallic acid equivalent (GAE) per 100 g extract (g GAE /100 g).[20]

Total Flavonoid Content (TFC)

Quercetin 36-104 µg/ml was used as a standard. TFC was calculated using Chang's method with minor modification. Quercetin solution 0.5 ml was diluted by adding methanol 1.5 ml, aluminium (III) chloride 10% 0.1 ml, sodium acetate 1M 0.1 ml and 2.8 ml distilled water. The same procedure was carried out for the sample. Absorbance was observed at wavelength 415 nm after incubation 30 min. TFC was calculated using a calibration curve of quercetin and expressed by quercetin equivalent (QE) per 100 g extract (g QE/100 g).[21]

DPPH Assay

Various concentrations were prepared for each extract and ascorbic acid as standard. DPPH 50 µg/ml as control and methanol was used as a blank. Modification of Blois's method[25]was conducted in this research. Two ml DPPH 50 µg/ml was added by 2 ml extract, then absorbance was investigated at wavelength 515 nm by UV-Vis spectrophotometer after incubation 30 min. A calibration curve was used to determine IC_{50} (inhibitory concentration 50%) of DPPH scavenging activity which expressed its antioxidant activity.

ABTS Assay

Preparation of ABTS solution has been modified in the previous research.[22]Each ABTS diammonium salt solution 7.6 mM and potassium persulfate solution 2.5 mM was prepared in distilled water, then left 12 hours in dark room. ABTS and potassium persulfate were mixed and incubated 30 min in room temperature, the mixture was left in the refrigerator for 24 hours, then diluted in ethanol.Each extract was prepared in various concentration. Extract 1 ml was put into 1 ml ABTS solution 50 µg/ml. Ascorbic acid was used as standard, ethanol (95%) as a blank, and ABTS solution 50 µg/ml as a control. The absorbance was evaluated at wavelength 734 nm. Antioxidant activity by ABTS method (IC_{50} of ABTS) was determined using its calibration curve.

Statistical Analysis

Each experiment was done at least triplicate. The results were stated as means ± standard deviation. Statistical analysis was observed using oneway ANOVA-post hoc Tukey ($p<0.05$) by SPSS 16 for Windows. Meanwhile Pearson's method was performed to analyze the correlation between TFC, TPC and their antioxidant activities and also between two antioxidant testing methods.

RESULTS AND DISCUSSION

Different polarities solvents such as n-hexane (nonpolar), ethyl acetate (semi-polar) and ethanol (polar) consecutively were used to separate most nonpolar compound, semi-polar compound and polar compound.

The thick extract (100% concentrated extract) can't be inserted into pycnometer, so it presented by 1%, 5% or other concentration. In the present research, the density of each extract of Bilimbi parts showed similarity density around 0.66 - 0.89 g/ml for all extracts and be determined as density 1% extract.The similarity density among extracts was important to point, because the extract with higher density may give higher activity and phytochemical content than lower density extract.

Based on the result of this study it can be seen ethanol extract of Bilimbi parts had higher TPC than the other solvent. The properties of most of the phenolic compounds were polar which soluble in ethanol. Meanwhile, ethyl acetate extract of Bilimbi parts generally contained higher TFC than the other solvents. It is in line with a solubility of most of the flavonoid compounds were semipolar and soluble in ethyl acetate.

Previous study stated that TPC in methanol extract of Bilimbi fruits from Sri Lanka was 56.1 mg GAE/100 g,[23] meanwhile ethanol extract of Bilimbi fruits from Bangladesh gave TPC 38.78 mg GAE/g.[24] The other research presented that methanol fruit extract of Bilimbi from Bangladesh, which was extracted by maceration exhibited TPC 65.16 mg GAE/g extract.[1]Study by Asna and Noriham[3]exposed that TPC in water fruits extract of Bilimbi from Malaysia were 41 mg GAE/g for *A. Bilimbi* L. and 53.01 mg GAE/g for *A. Bilimbi* cv. It was similar to the present research which expressed that TPC in ethanol fruits extract of Bilimbi was 3.22 g/100 g extract, but TPC in ethanol leaves extract (11.43 g GAE/100 g) and twigs extract of Bilimbi (7.90 g GAE/100 g) higher than its ethanol fruits extract (Table-1). The ethyl acetate Bilimbi leaves extract showed the highest TPC (11.48 g GAE/100 g), but it was not significantly different to TPC in ethanol leaves extract.The previous study by Chowdhury et al.[2]demonstrated that TPC in 70% methanol fruits extract of Bilimbi was 106.16 mg GAE/g. Research by Navarro and Ona[19] revealed that TPC in a water extract of Bilimbi flower higher than rose flower.

Table- 1: Total Phenolic Content in Bilimbi Parts

Sample	Total Phenolic Content (g GAE/100 g)		
	n-Hexane Extract	Ethyl Acetate Extract	Ethanol Extract
Leaves	2.95 ± 0.07^{a}	11.48 ± 0.79^{a}	11.43 ± 0.23^{a}
Fruits	1.12 ± 0.05^{b}	1.45 ± 0.06^{b}	3.22 ± 0.05^{b}
Twigs	3.77 ± 0.15^{c}	6.07 ± 0.16^{c}	7.90 ± 0.26^{c}

a-c = means with different superscript letter in the same column are significantly different ($p<0.05$)

In the present study found that ethyl acetate Bilimbi leaves extract showed the highest TFC (6.51g QE/100g extract), followed by ethyl acetate twigs extract of Bilimbi (4.12g QE/100 g extract)(Table-2). TFC in ethanol fruit extract of Bilimbi was 0.26 g QE/100 g which was lower than TFC in its ethanol leaves extract (2.21 g QE/100 g). It was different from the previous study which presented that TFC in water fruit extract of Bilimbi were 23.32 mg QE/g for *A. Bilimbi* L. and 19.62 mg QE/g for *A. Bilimbi* cv.[3] Study by Rahman et al.[24] exposed that ethanol Bilimbi fruits extract had TFC 1.67 mg QE/g, while water flower extract of Bilimbi gave higher TFC than rose flower.[19] The previous research presented that TFC

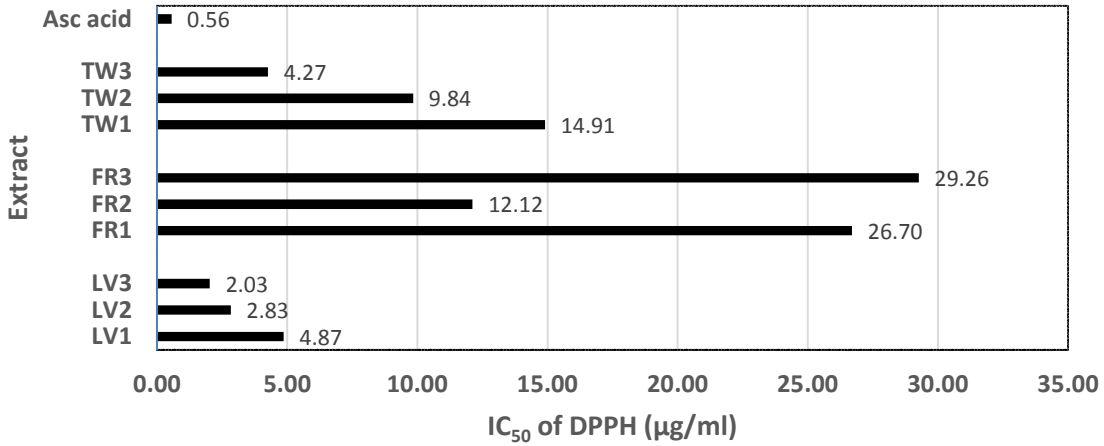
in 70% methanol fruit extract of Bilimbi was 276 mg QE/g,[2]meanwhile the other research showed that TFC in methanol Bilimbi fruits extract 29.7 mg CE/g.[23]

Table- 2: Total Flavonoid Content in Bilimbi Parts

Sample	Total Flavonoid Content (g QE/100 g)		
	n-Hexane Extract	Ethyl Acetate Extract	Ethanol Extract
Leaves	1.68 ± 040^{a}	6.51 ± 0.86^{a}	2.21 ± 0.01^{a}
Fruits	2.00 ± 0.51^{a}	0.44 ± 0.002^{b}	0.26 ± 0.01^{b}
Twigs	3.34 ± 0.09^{b}	4.12 ± 0.34^{c}	0.15 ± 0.02^{b}

a-c = means with different superscript letter in the same column are significantly different ($p<0.05$)

Antioxidant activity of ethanol Bilimbi fruits extracts demonstrated that IC_{50} DPPH was 635.07 µg/ml[24]which was categorized as a weak antioxidant. It was different from the present study which revealed that ethanol Bilimbi fruits extract had IC_{50} DPPH 29.26 µg/ml and classified as a very strong antioxidant (Fig.-1).



Note: TW3 = ethanol twigs extract of Bilimbi
TW2 = ethyl acetate twigs extract of Bilimbi
TW1 = n-hexane twigs extract of Bilimbi
FR3 = ethanol fruits extract of Bilimbi
FR2 = ethyl acetate fruits extract of Bilimbi
FR1 = n-hexane fruits extract of Bilimbi
LV3 = ethanol leaves extract of Bilimbi
LV2 = ethyl acetate leaves extract of Bilimbi
LV1 = n-hexane leaves extract of Bilimbi

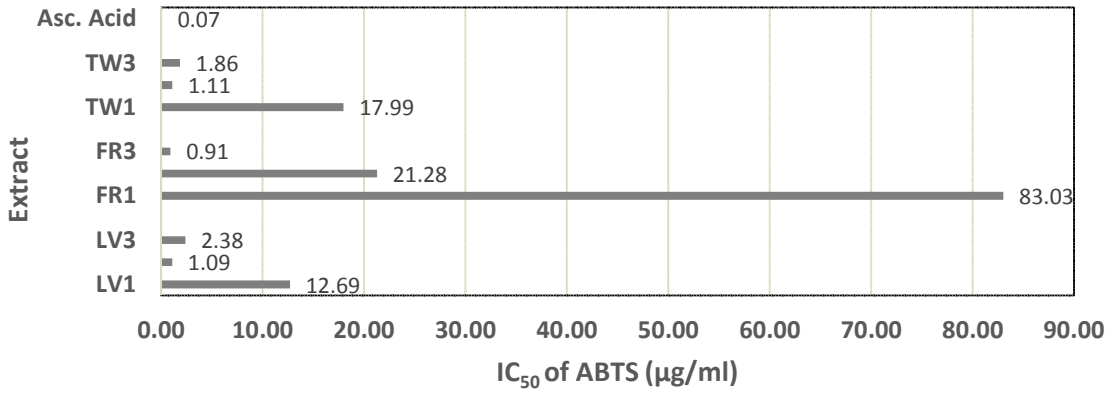
Fig.- 1: Antioxidant Activities of Bilimbi parts by DPPH method

The IC_{50} od DPPH varied from 2.03 to 29.26 µg/ml and the highest antioxidant was given by ethanol Bilimbi leaves extract with IC_{50} DPPH 2.03 µg/ml. All extracts of three parts of Bilimbi (leaves, fruits and twigs) had IC_{50} DPPH < 50 µg/ml, therefore it can be categorized as a very strong antioxidant by DPPH method.[25]The other research stated that IC_{50} DPPH of methanol Bilimbi fruits extract was 30.365 µg/ml.[1] Chowdhury et al.[2]demonstrated that IC_{50} DPPH of 70% methanol Bilimbi fruits extract was 20.35 µg/ml. The previous study stated IC_{50} DPPH of methanol Bilimbi fruits extract in mg ascorbic acid equivalent antioxidant activity (AEAC) and found 2.61 mg AEAC.[23]

In the present study, antioxidant activity by DPPH method was demonstrated as IC_{50} of DPPH, meanwhile the other study presented by percentage of DPPH scavenging activity.[3,19] The lower concentration of the sample was not always given a lower percentage of DPPH scavenging activity, and higher concentration was not always given a higher percentage of DPPH scavenging activity. The linear

result will be revealed in some concentration only. Previous research[26] exposed that methanol pineapple peel extract 200 µg/ml had a percentage of DPPH scavenging activity (95.17%), meanwhile100 µg/ml gave higher DPPH scavenging activity (95.74%). The extract contained many compounds, but not all compounds in extract had antioxidant activities and might the other compounds act as an antagonist of antioxidant. In methanol peel extract 100 µg/ml the antagonist antioxidant compounds have not reached their effective minimum concentration yet, so they can give the percentage of DPPH scavenging activity 95.74%. Meanwhile in 200 µg/ml the antagonist antioxidant compounds reached the effective minimum concentration, so they will reduce the ability to scavenge DPPH and showed the percentage of DPPH scavenging activity at 95.17%.

ABTS was the second antioxidant testing method in the present study. IC_{50} of ABTS of all parts extract from Bilimbi (leaves, fruits and twigs) in the range of 0.91-83.03 µg/ml (Fig.-2). In general, those all extracts can be categorized as a very strong antioxidant by ABTS method. The highest antioxidant activity by ABTS method was shown by ethanol Bilimbi fruits extract (IC_{50} ABTS 0.91 µg/ml). Research by Silva and Sirasa[23]expressed antioxidant activities by FRAP method as µmol $FeSO_4$ and found that methanol Bilimbi fruits extracts had 8.64 µmol $FeSO_4$. The previous study revealed that percentage of reducing the power of water Bilimbi flower extract was 66.76%,[19] meanwhile the other research found that methanol Bilimbi fruits extract had antioxidant activity 2 mmol TE/g for *A. Bilimbi* L. and 2.3 mmol TE/g for *A. Bilimbi* cv. by FRAP method.[3]



Note: TW3 = ethanol twigs extract of Bilimbi
TW2 = ethyl acetate twigs extract of Bilimbi
TW1 = n-hexane twigs extract of Bilimbi
FR3 = ethanol fruits extract of Bilimbi
FR2 = ethyl acetate fruits extract of Bilimbi
FR1 = n-hexane fruits extract of Bilimbi
LV3 = ethanol leaves extract of Bilimbi
LV2 = ethyl acetate leaves extract of Bilimbi
LV1 = n-hexane leaves extract of Bilimbi

Fig.- 2: Antioxidant Activities of Bilimbi parts by ABTS method

The highest TPC (11.48 g GAE/100 g) and TFC (6.51 g QE/100 g) was given by ethyl acetate leaves extract of Bilimbi and this extract also had the highest antioxidant activities by DPPH method, which showed the lowest IC_{50} DPPH (2.03 µg/ml). It means phenolic compounds in ethyl acetate leaves extract of Bilimbi had high antioxidant activity. Brewer[27] stated that phenolic compounds which can act as an antioxidant are phenolic acid (gallic acid, caffeic acid, protocatechuic acid, and rosmarinic acid), phenolic diterpenes (carnosol and carnosic acid), flavonoid, and volatile oil (eugenol, thymol, menthol).Benzoic acid has higher antioxidant activity than cinnamic acid.[28]Flavonoid which has keto in C4, double bond in C2 and C3, OH in C3, and di OH in C3'- C4' will give high antioxidant activity, meanwhile substitution di OH in C3' - C4' influence to give higher antioxidant activities.[28] Based on this statement, it can be predicted that flavonoid compounds in ethyl acetate leaves extract of Bilimbi fulfill the above

requirement. TFC in n-hexane twigs extract of Bilimbi TW1 (3.34 g QE/100 g) was higher than TFC in ethanol twigs extract of Bilimbi TW3 (0.15 g QE/100 g), but antioxidant activity of TW3 higher than TW1, which showed by IC_{50} DPPH of TW3 (4.27 µg/ml) lower than IC_{50} DPPH of TW1 (14.91 µg/ml). It can be suggested that most of the flavonoid compounds in TW3 had high antioxidant activities, which has di OH in C3'- C4'.

The coefficient of Pearson's correlation between TPC, TFC in all Bilimbi parts extracts and its antioxidant activities by DPPH and ABTS methods were determined in the present study. The higher TFC and TPC are often contributed to the higher antioxidant activities, which exposed by lower IC_{50} DPPH and IC_{50} ABTS. Therefore if TPC or TFC contributed to their antioxidant activities, the correlation will be a significantly negative correlation.[29]Thaipong et al.[30]revealed that Pearson's correlation coefficient (r) was significantly negative if $-0.61 \leq r \leq -0.97$ and significantly positive if $0.61 \leq r \leq 0.97$. In Table-3, it can be seen the correlation between TPC and TFC in all extracts of Bilimbi parts and their antioxidant activities by DPPH and ABTS methods. In generally there were significantly negative correlations between TPC in all parts extracts of Bilimbi and their antioxidant activities by DPPH and ABTS methods, and only TFC in Bilimbi leaves extract which had a significant and negative correlation with its antioxidant activity by ABTS method ($r = -0.646$, $p<0.05$). In the previous research showed that antioxidant activity of water fruits extract of Bilimbi by DPPH and FRAP methods had a significant and positive correlation with its TPC.[3]

Table- 3: Correlation of TPC and TFC with its Antioxidant Activities

Antioxidant Parameter	Pearson's Correlation Coefficient (r)	
	TPC	TFC
IC_{50} DPPH LV	-0.934^{**}	-0.298^{ns}
IC_{50} DPPH FR	0.495^{ns}	0.287^{ns}
IC_{50} DPPH TW	-0.982^{**}	0.772^{**}
IC_{50} ABTS LV	-0.990^{**}	-0.646^{*}
IC_{50} ABTS FR	-0.781^{**}	0.967^{**}
IC_{50} ABTS TW	-0.869^{**}	0.292^{ns}

**= significant at $p<0.01$, * = significant at $p<0.05$, ns= not significant

Correlation between the results of two antioxidant testing method which were used in this study also investigated (Table-4). IC_{50} DPPH of leaves and twigs extracts of Bilimbi had a positive correlation with their IC_{50} ABTS.

Table- 4: Correlation Pearson of DPPH and ABTS methods

Antioxidant Parameter	Pearson's Correlation Coefficient (r)		
	IC_{50} ABTS LV	IC_{50} ABTS FR	IC_{50} ABTS TW
IC_{50} DPPH LV	1.000^{**}	-	-
IC_{50} DPPH FR	-	0.148^{ns}	-
IC_{50} DPPH TW	-	-	0.830^{**}

** = significant at $p<0.01$, ns= not significant

CONCLUSION

Leaves and twigs which were Bilimbi waste products had antioxidant activities. In generally antioxidant activities of all extracts of different Bilimbi parts (leaves, fruits and twigs) using DPPH and ABTS assays can be classified as very strong antioxidant. The higher TPC and or TFC did not always show higher antioxidant activities. In generally phenolic compounds in leaves, fruit and twigs of Bilimbi were the major contributors in their antioxidant activities by DPPH and ABTS methods. Bilimbi leaves and twigs extract gave linear result in two antioxidant testing methods. Waste products of Bilimbi (leaves and twigs) have added value to be developed as sources of further natural antioxidant.

ACKNOWLEDGMENT

This work was funded by Research, Community Service and Innovation Program for Research Group from Institute for Research and Community Service – Bandung Institute of Technology. The authors are

very thankful to the authority of the School of Pharmacy- Bandung Institute of Technology for providing all facilities to conduct this research.

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[RJC-3091/2018]