Food Research 4 (1) : 127 - 134 (February 2020)
Journal homepage: http://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Antioxidant activities of methanolic extract and its fractions of *Baccaurea racemosa* and *Macaranga subpeltata* leaves

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Article history:
Received: 2 July 2019
Received in revised form: 8 August 2019
Accepted: 12 August 2019
Available Online: 26 August 2019

Keywords:
Macaranga subpeltata,
Baccaurea racemosa,
Antioxidant activity,
Flavonoid,
Phenolic compound

DOI:
https://doi.org/10.26656/fr.2017.4(1).144

Abstract

Oxidative stress, the excessive presence of reactive oxygen species (ROS), is suggested as a basal cause of aging as well as various degenerative and chronic diseases in human. Antioxidants are believed to play a very vital role in the body defense system against ROS. Plant-based antioxidants with their prominence have gained tremendous worldwide interest nowadays. *Baccaurea racemosa* and *Macaranga subpeltata* are among ethnomedical used plants for liver diseases medication which have potential source as natural antioxidants. The study aimed to evaluate the antioxidant activities of the methanolic crude extract (CE) and their fractions of the plant's leaves. Maceration was performed to obtain CE, which then subjected to fractionation using *n*-hexane, dichloromethane, chloroform, ethyl acetate, and ethanol to obtain fractions of hexane fraction (HF), dichloromethane (DF), chloroform (CF), ethyl acetate (EAF), and ethanol fractions (EF), respectively. The CE and all fractions included water fraction (WF) and residue (R) were examined for its total phenolic contents, total flavonoid contents, and antioxidant activities using various *in vitro* assay. In general, EAF demonstrated as the best solvent for the extracting phenolic compounds with higher antioxidant activity. The CE and its fractions of *M. subpeltata* contained higher of TPC and TFC, also demonstrated higher antioxidant capacity, than that *B. racemosa*. The phenolics compounds were responsible for the antiradical properties. The EAF of *M. subpeltata* was scavenging radicals better than that of L-(+)ascorbic acid as a positive control. The high antioxidant activities and phenolics contents make both the plant extracts to be developed as a food supplement.

1. Introduction

Reactive oxygen species (ROS) is indispensable in many biological processes, mainly during cell differentiation, defense mechanism, immunity, etc. (Baunthiyal *et al*., 2017), therefore ROS is produced in normal metabolic reaction (endogenous ROS) and is maintained at physiological levels by several endogenous antioxidant systems. In addition, exogenous ROS may be generated from environmental pollutants, excessive alcohol consumption, radiations exposure, viral and bacterial infections, and others (Cacciapuoti, 2016). However, a condition in which ROS are excessively generated, oxidative stress is going to be inevitable causing multiple cellular compartments damage, cell injury or cell damage. The oxidative stress, triggered by the imbalance between oxidants and antioxidants, eventually leads to many degenerative and chronic diseases in human (Gangwar *et al*., 2014; Matschke *et al*., 2019).

Any molecules capable of retarding or preventing the oxidation of other molecules are considered as an antioxidant (Ali *et al*., 2013). Oxidation produces free radical reactions that highly reactive species which contains one or more unpaired electrons in their outermost shell (Mamta *et al*., 2014). Antioxidants supplied in a diet are needed to counter the undesirable effects of oxidative stress by various mechanisms such as scavenging the active free radicals, depressing the active

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species formation, sequestering metal ion, inducing biosynthesis and enhancing the activity of endogenous antioxidant, repairing damaged molecules and cleaning (Baunthiyal *et al*., 2017).

In recent years, natural antioxidant has gained tremendous interest in preventing and treating various health problems. Ethnomedicinal studies play a pivotal role and have significant attention worldwide contributing as an open door to discover and develop new drugs or other plant-derived antioxidants (Katiyar *et al*., 2012). Phytochemicals especially polyphenols such as flavonoids are believed to have antioxidant properties. Many studies revealed that total antioxidant activity has a direct relation to the total phenolic content (Zhang *et al*., 2015). Polyphenol may act as reducing agents, by quenching singlet oxygen, donating hydrogen, chelating compound, and/or by trapping the free radicals (Pandey and Rizvi, 2012), whereas antioxidant properties of flavonoids undergo several ways, namely scavenging radicals, preventing metal transition, and interacting with other antioxidants (Apak *et al*., 2007).

The previous study on antioxidant activities of selected medicinal plant utilized for liver diseases medication by ethnic traditional healers in Indonesia showed that methanolic extract of *M. subpeltata* and *B. racemosa* leaves had a potential antioxidant property even though its IC_{50} is still inferior to the L-(+)-ascorbic acid as reference standard (Widodo *et al*., 2019). However, there are scarce reports about the pharmacological properties particularly antioxidant activity of these plants. Fractionation of methanolic extract using solvents with different polarity could facilitate the grouping of compounds according to its polarity and hence offering different antioxidants ranging from weak to very strong activities. Several studies revealed that fractionation of methanolic extract of plants could get higher antioxidant activities. Rohman *et al*. (2006) reported that ethyl acetate fraction had the highest antioxidant activities and total phenolics contents of Mengkudu (*Morinda citrifolia*) fruit than initial methanolic extract and other fractions. The similar results were also obtained by Rohman *et al.* (2010), Samirana *et al.* (2017) and Permatasari *et al*. (2019). Therefore, it is needed to evaluate whether the fractions of the methanolic crude extract of these plants have antioxidant activity to provide basic data on its uses in traditional medicine and/or to explore some new natural antioxidants. The objective of this study was to evaluate the antioxidant activities, phenolics contents, flavonoid contents and their correlations of methanolic crude extract and their fractions of plant's leaves of *B. racemosa* and *M. subpeltata.*

2. Materials and methods

2.1 Materials

Plant samples of *Baccaurea racemosa* (Reinw. ex Blume) Müll.Arg. and *Macaranga subpeltata* K. Schum. & Lauterb. were collected from Central Java and East Java, respectively. All chemicals used in this study were pro-analytical grade. The solvents and Folin-Ciocalteau's phenol Reagent (FCR), Acetic acid, $AlCl_3$, $NaCO_3$, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, L-(+)-ascorbic acid were obtained from E. Merck (Darmstadt, Germany). 2,2'-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ), Trolox, Gallic acid, Quercetin, and Rutin were purchased from Sigma (Aldrich, USA).

2.2 Preparation of methanolic extract

Preparation of methanolic extract was performed according to the previous study (Widodo *et al*., 2019). Fractionations using various solvents were described in Figure 1. The extract and fraction were air-dried using the oven at 40^{o}C until dried (constant weight). The extracts were dissolved and diluted using methanol prior to assay (10 mg dissolved up to a volume of 1 mL).

2.3 Determination of total phenolic content

The total phenolic content (TPC) was performed using the method described by Cicco *et al*. (2009) with a minor adjustment. The final condition of the test solution was: 40 µg extract, 4% methanol, 10% FCR, and 5% $CaCO_3$ in the total volume reaction of 1.0 mL. A-40 µL of plant extract was completely mixed with 360 µL of dH_2O and 100 µL of FCR and left for 2 mins. The reaction was then added and homogeneously mixed with 500 µL of 10% $CaCO_3$ and left for 20 mins at 40^{o}C in the incubator afterward. The absorbance at 732 nm of 150 µL test solution was measured. Gallic acid used as standard reference was prepared at series methanolic dilutions of 0, 5, 10, 15, 20, and 25 µg/mL to generate linear regression for determining phenolics content of the extract by plotting the absorbance of the samples. TPC is expressed as mg gallic acid equivalent (GAE)/g of the extract.

2.4 Determination of total flavonoid content

The total flavonoid content (TFC) was done using a slight modification of the method described by Li *et al.* (2013). The samples were diluted with methanol to get a concentration of 1,250 µg/mL (w/v). The mixture reaction consisted of 100 µL sample, 250 µL acetate buffer (pH 3.8), 150 µL of 0.1 M $AlCl_3$ solution (replaced with methanol 150 µL for the blank), 350 µL ultra-pure dH_2O, and adjusted with methanol to gain a

eISSN: 2550-2166

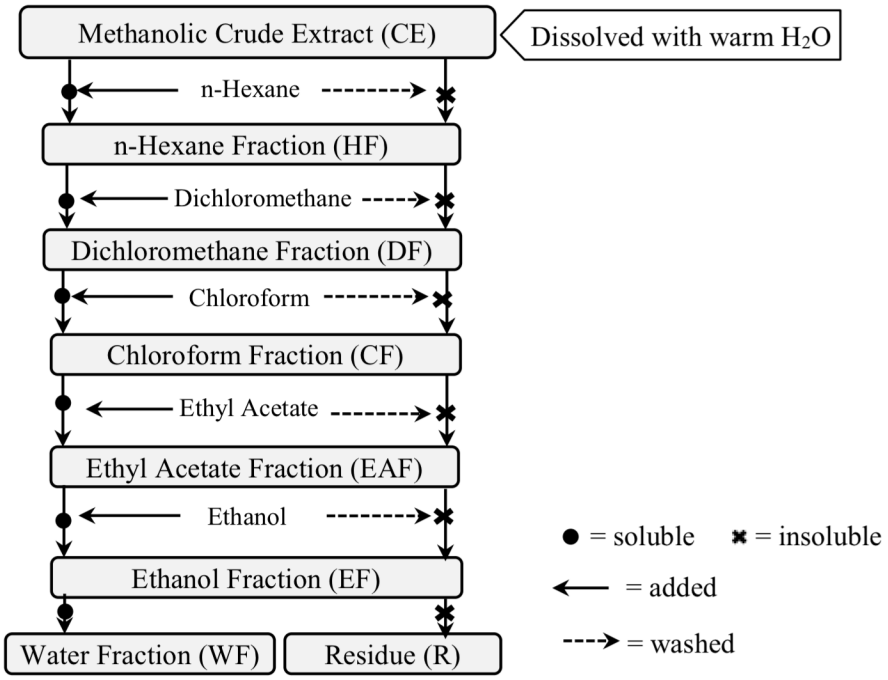

Figure 1. Scheme of the fractionation of methanolic crude extract of *M. subpeltata* and *B. racemosa* leaves.

volume of 1,250 µL. The test solution was placed into the incubator (35°C for 30 mins). A sample volume of 150 µL was pipetted into the microplate prior to absorbance measurement at 398 nm. Rutin was used as a standard reference via generating linear regression using a series of concentrations of 0 - 90 µg/mL (methanolic dilution). The TFC is expressed as gram rutin equivalents per g extract (mg RE/g).

2.5 Determination of DPPH Radical Scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) was dissolved using methanol to get a concentration of 0.4 µM. Plant methanolic extract and its fractions in a volume of the 825 µL (with the series final concentrations of 0-150 µg/mL) and 175 µL DPPH (with final concentration 70 µM) were thoroughly mixed. The control was prepared with the same final concentration of sample without addition of DPPH solution. The sample and control reaction tubes were placed in the dark (in the incubator: at 37°C for 30 mins). The solution in a volume of 150 µL was put into the 96 wells microplate and observed the absorbance using a spectrophotometer at a wavelength of 515 nm. L-(+)-ascorbic acid was used as a comparative standard compound. IC_{50} was calculated using formula $[(A_o - A_1)/A_o]$ x 100, where A_o: the absorbance of the control and A_1: absorbance of the extract or standard).

2.6 Determination of Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC value of the extract was determined using the assay described by Dong *et al.* (2015). The weighed ABTS of 38.4 mg was dissolved completely with 10 mL of 2.5 mM $K_2S_2O_8$ then kept in the dark for 12-16 hrs at room temperature. Before applying TEAC assay, the ATBS·+ solution should be diluted using methanol to obtain 0.70±0.02 absorbance at 743 nm. The sample reaction was done in the total volume of 2.0 mL, which contained the mixture of 50 µL of the sample and 1950 µL ATBS·+ solution (for control reaction, replaced by diluted 2.5 mM $K_2S_2O_8$ using methanol in the same volume as the dilution factor of ATBS·+ solution). A linear regression of Trolox as standard reference was generated by making a series concentrations of 0 – 45 µM. The TEAC value was expressed as the Molar Trolox Equivalent per gram extract (M TE/g)

2.7 Determination of Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP value reflects the reducing ability of the plant extracts, which estimated using a slight modifications assay of Benzie and Strain (1996). Firstly, 20 mM $FeCl_3 \cdot 6H_2O$ solution, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and acetate buffer (300 mM; pH 3.6) were made to produce the FRAP reagent by mixing those solutions by volume ratio of 1:1:10 respectively. A linear regression was generated using $FeSO_4 \cdot 7H_2O$ in the final concentration 100 – 1,000 µM/mL applied in 2.0 mL final volume reaction. The contained 150 mL extract or $FeSO_4 \cdot 7H_2O$ mixed with 1,350 µL of FRAP reagent. The mixture was then incubated for 30 min at 37°C, prior to measuring the absorbance at 595 nm. Quercetin, L-(+)-ascorbic acid were used as the standard reference.

2.8 Data analysis

All data represented from the average of triplicate analyses and recorded as mean±SD. The data were

subjected to variance (ANOVA) and correlation analysis using SPSS version 22.

3. Result and discussion

B. racemosa and *M. subpeltata* are among 381 medicinal plants which have been used for liver diseases medication by Indonesia ethnic traditional healers. The activity as an antioxidant considered to be one of the major mechanism underlying their healing utilization (Widodo, Rohman and Sismindari, 2019). The previous study showed that leaves methanolic extract of these two plants had high potential antioxidant activity. The fractionation using different polarity solvent to the crude methanolic extract (CE) showed that they had the same tone in the yield percentage except for those in water fraction and the residue (Table 1).

Table 1. The yield of fraction crude methanolic extract leaves, Total Flavonoid Content and Total Phenolic Content of *B. racemosa* and *M. subpeltata*

Extracts/ Fractions	Yield (%)	TFC (mg RE/g)	TPC (mg GAE/g)
B. racemosa			
CE	17.26	21.39±3.64^{a}	237.58±16.71^{f}
HF	4.3	13.82±2.00^{a}	20.28±0.11^{a}
DF	0.72	53.25±9.71^{bc}	93.53±7.21^{b}
CF	0.18	58.96±1.61^{c}	136.23±8.00^{c}
EAF	2.07	179.86±3.57^{h}	412.50±18.43^{h}
EF	2.76	44.26±1.74^{b}	423.42±18.70^{h}
WF	0.74	18.09±0.81^{a}	284.83±10.4^{g}
R	6.4	17.21±1.14^{a}	70.76±6.63^{b}
M. subpeltata			
CE	14.39	74.70±0.36^{d}	204.25±2.22^{e}
HF	3.12	52.76±1.12^{bc}	77.58±4.03^{b}
DF	1.39	105.15±13.56^{e}	177.91±8.24^{d}
CF	0.12	131.06±0.49^{f}	172.62±8.19^{d}
EAF	2.61	203.28±9.10^{i}	619.69±31.49^{i}
EF	0.53	149.33±6.02^{g}	432.75±12.66^{h}
WF	6.24	52.42±2.42^{bc}	254.20±9.58^{f}
R	0.32	123.72±3.29^{f}	284.08±10.25^{g}

Values followed by the same alphabet in a column did not differ significantly at p=0.05 according to Duncan's Multiple Range Test (DMRT).

Non-polar solvents such as hexane, dichloromethane, and chloroform usually elute lipoidal material from crude extract (Dai and Mumper, 2010), hence the non-polar fractions of hexane fraction (HF), dichloromethane fraction (DF), and chloroform fraction (CF) contained less phenolic compounds (PC) than that the CE. The color of the HF was darker green (data not shown) than other fractions since hexane can dissolve chlorophyll from the crude extract. Chlorophyll and its derivates exhibit to have low antioxidant activity when measured by DPPH assay, considered that the action of chlorophyll might be involved to metal chelation (Hsu *et al*., 2013) rather than hydrogen donation (Lanfer-Marquez *et al*., 2005). The solubility of phenol compounds into a solvent is determined by activity coefficient as the main thermodynamic factor which governs their tendency to be solubilized, diffused, or transferred into solvents. The stereochemistry of phenols (the polar and the non-polar fragment inside the molecules) and the intermolecular forces (mainly hydrogen bonds) occurred between PCs and the solvents determine their solubility (Galanakis *et al*., 2013).

In the present study, TPC of ethyl acetate fraction (EAF) and ethanol fraction (EF) of *M. subpeltata* and *B. racemosa* were higher than that of other fractions. Polar and semi-polar solvents are suitable for extracting polyphenols in plant matrices, the most convenient solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Do *et al*., 2014). The UNIFAC model for calculating the activity coefficient of PCs indicate that intermediate polarity solvents (alcohols and acetone) are more preferred rather than more polar (e.g., water) or less polar solvents (Galanakis *et al*., 2013). In general, alcohol represented the highest solubility for PCs which had the shortest carbon chain (Boas, 2017). Ethanol dissolved the highest PCs from the CE of *B. racemosa* comparable to that of ethyl acetate. Ethyl acetate also is the solvent of choice for extracting PCs from CE of *M. subpeltata*. The PCs comprise many substances classes of simple phenols, coumarins, lignins, lignans, condensed and hydrolyzable tannins, phenolic acids and flavonoids, which all possess an aromatic ring bearing at least one hydroxyl groups (Khoddami *et al*., 2013).

The solvents usually used to extract flavonoids are methanol, ethanol, acetone, water or mixtures of these solvents (Khoddami *et al*., 2013). The solubility of flavonoid compounds is varied depending on the complex nature of compounds, the selected solvents, and the system such as thermodynamic properties, ability to form hydrogen bonds with the surrounding solvent, solvent polarity, temperature, and pH value. The glycosylated flavonoids (such as in rutin and isoquercitrin) tend to have a lower melting point, higher enthalpy of fusion and lower solubility in polar solvents compared to those of corresponding aglycons. The solubility of flavonoid compounds (FCs) has no direct correlation to their thermodynamic properties in contrast to those of PCs. Moreover, the solubility of FCs is also influenced by a torsion angle θ (OC2C1'C6') that considered to be related to the C2-C3 bonding, in which the C2-C3 single bond instead of double bond would increase a torsion angle θ that contributes to higher solubility in polar solvents (Chebil *et al*., 2007).

Table 2 compiles DPPH radical scavenging assay

expressed by inhibition concentration at 50% of DPPH radicals known as IC_{50}, Trolox Equivalent Antioxidant Capacity (TEAC), and Ferric Reducing Antioxidant Power (FRAP) Crude Extracts and its fractions of *B. racemosa* and *M. subpeltata.* EAF and EF showed prominently radical scavenging activity as showed by the lower IC_{50} value of DPPH radical scavenging activity assay applied to *M. subpeltata* and *B. racemosa,* respectively. The lower concentration of the IC_{50} value, the highest capacity of the sample to scavenge DPPH radical. The EAF of *M. subpeltata* showed the most active in scavenging DPPH radical even compared to the activity of L-(+)-ascorbic acid. Due to its simplicity and high accuracy that makes the DPPH radical scavenging assay becomes the most extensively applied techniques to evaluate antioxidant activity (Nur Alam *et al*., 2012). DPPH scavenging assay posses some other advantages namely DPPH free radical reacts significantly with radical scavengers, DPPH is unaffected by certain side reactions of polyphenols, such as metal ion chelation and enzyme inhibition. In addition, the results of the assay have a high correlation to other antioxidant assays (Babbar *et al*., 2014). Consistent with the result of the present study that DDPH assay correlated to the TPC dan TEAC values indicated that PCs are responsible for the antiradical activity.

Table 2. The IC_{50} DPPH scavening activity, Trolox Equivalen Antioxidant Capacity (TEAC), and Ferric Reducing Antioxidant Power (FRAP) Crude Extracts and their fractions of of *B. racemosa* and *M. subpeltata*

Fractions	IC_{50} (µg/mL)	TEAC (mg TE/g)	FRAP (mM Fe^{2+}/10 mg)
B. racemosa			
CE	4.30±0.31	354.88±0.55	900.18±15.41
HF	131.21±2.81	5.31±0.91	12.61±1.40
DF	11.26±0.14	127.52±6.98	231.70±1.40
CF	6.70±0.02	200.64±4.91	404.00±2.68
EAF	3.24±0.01	395.52±3.01	945.14±5.28
EF	2.42±0.08	532.44±6.38	1221.48±41.19
WF	4.25±0.01	311.02±1.74	784.10±3.41
R	11.32±0.38	133.36±1.69	610.84±12.87
M. subpeltata			
CE	4.00±0.15	297.59±0.94	601.46±16.90
HF	19.03±0.83	49.56±0.84	205.36±1.61
DF	7.92±0.04	198.62±1.72	438.51±2.14
CF	10.48±0.03	163.79±1.97	468.13±7.43
EAF	1.98±0.03	703.21±13.99	2007.69±21.55
EF	3.51±0.13	464.77±29.62	1251.06±22.14
WF	5.12±0.25	331.08±3.57	815.26±13.99
R	4.39±0.10	332.95±3.06	719.63±16.89
L-(+)-ascorbic acid	2.10±0.08		2,031.88±51.74

CE = crude methanolic extract; HF = hexane fraction; DF = dichloromethane fraction; CF = chloroform fraction; EAF = ethyl acetate fraction; EF = ethanol fraction; WF = water fraction; R = residue.

The ferric reducing activity power (FRAP) value reflects the antioxidant activity of the samples by measuring their ability to transform Fe^{3+}-TPTZ complex (colorless/slightly brownish) to Fe^{2+}/ferrous form (blue) in acidic solution. The higher the reductive capacity of extracts or compounds, the more intense blue color develops, which can be monitored by absorbance measurement at 595 nm (Benzie and Strain, 1996).

Table 3 exhibits the Pearson correlation (*r*) among total phenolics content (TPC), total flavonoid contents (TFC), IC_{50} of DPPH radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC), and ferric reducing activity power (FRAP). TPC values were moderately correlated with TFC (r=0.680, p<0.001), suggested that 46.24% (r^2) variation in the TPC was due to TFC. Recent studies have revealed that the TPC was associated with the antioxidant activity (Teixeira *et al*., 2017; Li *et al*., 2018). TPC showed a highly significant correlation with FRAP and TEAC values which indicated that the main component of PCs from fractions enabled to transfer their electron to neutralize free radical and to reduce the metal ion. TEAC and FRAP are recognized as electron transfer based methods. TPCs fractions of *M. subpeltata* showed more responsible for the antioxidant activity than *B. racemosa* as shown by higher R^2 value for its correlation with both FRAP and TEAC. Figure 2 reveals the correlations between total phenolics contents (TPC) and Trolox equivalent antioxidant capacity (TEAC) values of *B. racemosa* (A) and *M. subpeltata* (B) along with the correlations between TPC value and FRAP values of *B. racemosa* (C) and *M. subpeltata* (D).

Table 3. Pearson correlation (r) among TPC, TFC, IC_{50} of DPPH radical scavenging activity, TEAC, and FRAP

Variables	TPC	TFC	IC_{50} DPPH	TEAC
TFC	0.680**			
IC_{50} DPPH	-0.464**	-0.338*		
TEAC	0.968**	0.562**	-0.515**	
FRAP	0.949**	0.567**	-0.480**	0.967**

**=Correlation significant at the 0.01 level (2-tailed), *=Correlation significant at the 0.05 level (2-tailed). TPC = total phenolics contents; TFC = Total flavonoid content; DPPH = 2,2'-diphenyl-1-picrylhydrazyl; TEAC = Trolox equivalent antioxidant capacity.

The TFC showed lower linearity correlation to both DPPH and TEAC assays than that TPC may due to the radical scavenging capability of flavonoids vary depend on the type, structure and the position of hydroxyl groups. In addition flavonoids in plants generally present as glycosides, and many as 3-O-glycosides which found to have lower TEAC and DPPH value than their corresponding aglycones (Csepregi *et al*., 2016).

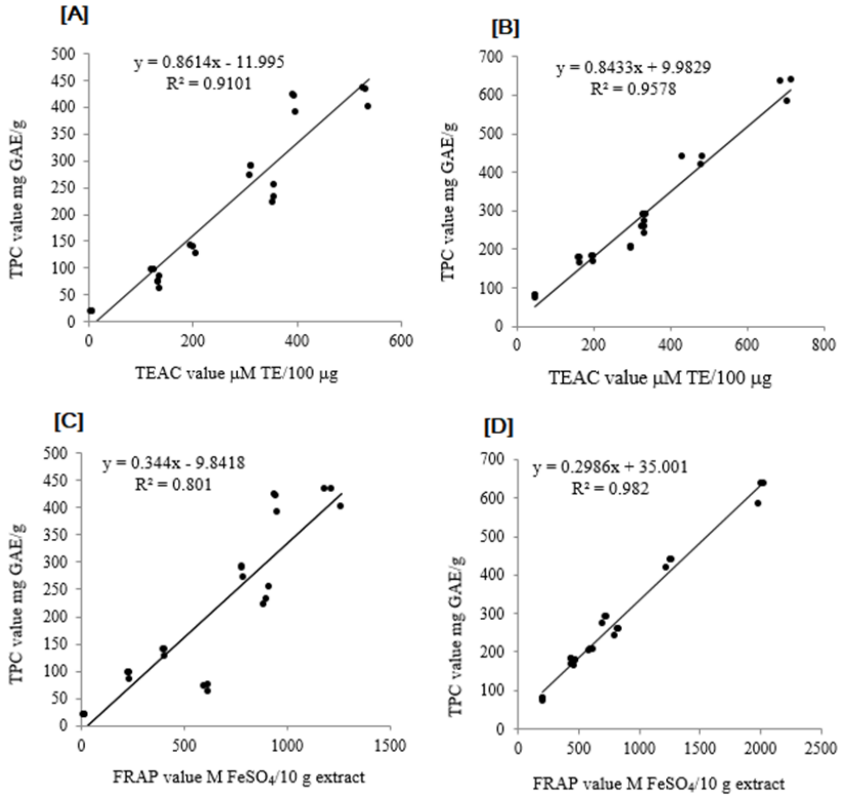

Figure 2. Correlations between total phenolics contents (TPC) and Trolox equivalent antioxidant capacity (TEAC) values of *B. racemosa* (A) and *M. subpeltata* (B); Correlations between TPC value and FRAP values of *B. racemosa* (C) and *M. subpeltata* (D).

4. Conclusion

Two methanolic extracts of leaves of *B. racemosa* and *M. subpeltata* has been fractionated and the fractions obtained were subjected to antioxidant assay. The crude methanolic extract and its fractions of *M. subpeltata* contained higher of total phenolics content and total flavonoid contents, and also demonstrated higher antioxidant capacity, than those of *B. racemosa*. The ethyl acetate fraction of *M. subpeltata* has better DPPH radical scavenging activity than L-(+)-ascorbic acid as a positive control. The high antioxidant activities and phenolics contents make both the plant extracts to be developed as a food supplement.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgement

The publication of this manuscript was supported by the Ministry of Research and Higher Education, Republic of Indonesia for financial support through scheme World Class Research 2019 with contract number of 1973/UN1.DITLIT/DIT-LIT/LT/2019.

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