Original Article

DETERMINATION OF ANTIOXIDANT CAPACITY IN *PLUCHEA INDICA* LESS LEAVES EXTRACT AND ITS FRACTIONS

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ABSTRACT

Objective: This study was conducted to determine the DPPH free radical scavenging activity of pluchea extract (PE) and its fraction (ethyl acetate (EAF), n-butanol (BF) and aqueous (AF)) and comparing with the its activity of control antioxidant, such as green tea (GE) and rosemary extracts (RE), α-tocopherol succinate (ATS) and BHT.

Methods: The antioxidant activity was evaluated by the decrease in absorbance as the result of DPPH˙ color change from purple to yellow at 517 nm by using a stopped-flow spectrometer UV-Vis. AE (antiradical efficiency) was a parameter more exactly used to measure the antioxidant power than EC_{50} (efficient concentration to reduce 50% of DPPH concentration). This parameter was determined by EC_{50} and TEC_{50} (the time needed to reach the steady state).

Results: PE and its fraction had free radical-scavenging activity compared with control antioxidant. Based on EC_{50}, the orders of ability to scavenging DPPH radicals was determined as the EAF~ GE>PE ~ ATS ~ RE ~ AF>BHT ~ BF. The results were not exactly because BHT and ATS were potentially as a synthetic antioxidant. Therefore AE parameter was used to determine scavenging activity. The classification order of scavenging activity based on AE was BHT>GE ~ ATS>EAF~RE~ BF~ PE~ AF.

Conclusion: The PE and its fractions had DPPH free radical scavenging activity. Based on EC_{50} and AE parameters, the EAF showed the strongest DPPH free radical scavenging activity compared with PE and the other fractions.

Keywords: Antioxidant capacity, 2,2-diphenyl-1-picrylhydryzyl (DPPH), *Pluchea* leaves extract, Fractions

INTRODUCTION

Pluchea indica Less, belonging to the family of *Asteraceae*, has been used as traditional medicine and food in Indonesia and Malaysia [1, 2]. Its leaf contains phytochemicals, such as phenol hydroquinone, tannin, alkaloid and sterol [3-5], flavonoid (quercetin, myricetin and kaemferol) [4-7], saponin and cardiac glycoside [4, 5].

The phytochemicals have an antioxidant activity to inhibit peroxidation reaction in food or the living body of humans, animals and plants [8, 9]. Phenolic compounds have multifunctional and can act as reducing agents, free radical terminators, metal chelators and singlet oxygen quenchers [10]. All phenolic classes have the structural requirements of free radical scavengers and the potential as strong antioxidants [11, 12].

Antioxidant activity is a complex process that can occur through several mechanisms. Due to the complexity test must be carried out to evaluate the antioxidant activity of pure compounds or extracts. One of the tests proposed for assessment of antioxidant activity (AOA) is DPPH˙ free-radical colorimetry, whose color changes from purple to yellow in the presence of antioxidants [13]. The kinetics of decoloration reactions directly was related to the types and the different concentrations of antioxidants. The more rapidly the absorbance decreases, the more potentially the antioxidant activity donates hydrogen atom. The rapid reduction of DPPH˙ radical by antioxidants is allowed the evaluation of antioxidant power of different antioxidants [14, 15]. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers [16].

EC_{50} parameter is efficient concentration to reduce 50% of DPPH concentration that is used to determine antioxidant power of substances without involving reaction time between DPPH free radical and antioxidant compounds. AE (antiradical efficiency) is a parameter more exactly used to measure the antioxidant power than EC_{50} because this parameter involves reaction time to determine antioxidant capacity. AE is calculated by formulation:

$$AE = \frac{1}{(EC50 \times TEC50)}$$ (Equation 1)

This study was aimed to determine the DPPH free radical scavenging activity of pluchea extract (PE) and its fraction (ethyl acetate (EAF), n-butanol (BF) and aqueous (AF)) and comparing with the its activity of control antioxidant, such as green tea (GE) and rosemary extracts (RE), α-tocopherol succinate (ATS) and BHT.

MATERIALS AND METHODS

Chemicals and reagents

Pluchea leaves were collected from a pluchea garden in Dramaga, Bogor, Indonesia. Dried green tea was supplied by a tea factory in Singapore (Lim Lam Thye PTE, LTD). Dried rosemary was purchased in Cold Storage Supermarket in Holland Avenue Singapore. Methanol, n-butanol, petroleum ether and ethyl acetate were purchased from Merck Company (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), BHT (butylated hydroxytoluene) and α-tocopherol succinate were purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK).

Sampling

Pluchea indica less leaves were harvested from 1-6 age level [17]. The leaves were dried at room temperature and ground to a fine powder (40 mesh). The moisture content was determined to be 10.38%. And then dried leaves powder was mixed before extraction.

Extraction and fractionation

Extraction and fractionation of pluchea leaves were determined according to the method of [18] with slight modifications. The dried powder (100g) of pluchea leaves was macerated with 400 ml petroleum ether at room temperature for 24 h with constant stirring. The resulting extracts were filtered, and the residue was dried at ambient temperature and lyophilized. The dried residue

(10g) was extracted by soxhlet extraction with 150 ml methanol. The PE was obtained by concentrating the supernatant using a rotary vacuum evaporator at 40°C (Laborota 4000, Heindolph, Germany) and freeze dried. The same process was repeated for dried green tea and rosemary to obtain GE and RE used as an antioxidant control. PE was dissolved in distilled water and then partitioned sequentially in two different solvents, namely EAF and BF, respectively (1:1 v/v), to fractionate the semipolar and polar compounds in the PE. And then the resulting solvent fractions were concentrated by a rotary vacuum evaporator at 40 °C and the AF was concentrated by a rotary evaporator and freeze-dried. The PE and its fractions were stored in the dark at 4 °C before analysis.

DPPH free radical-scavenging method

The antioxidant activity of PE, EAF, BF and AF were measured in terms of hydrogen atom donating or radical scavenging ability, using DPPH free radical [19]. A methanolic solution (150 μl) of each sample of various concentrations (0.05–0.25 mg/ml) was placed in a quartz cuvette, and 75 μl of (1 x 10^{-4}) mmol/l methanolic solution of DPPH· was added. The mixture was measured for absorbance at 517 nm by using a stopped-flow spectrometer UV-Vis (MOS-200/M Spectrometer, Belgium). The decrease in absorbance was determined continuously with data detecting at one-second interval until a reaction reached a plateau. Methanol was used to zero a spectrophotometer (the negative control). The absorbance of the DPPH· radical without antioxidant (i.e. the positive control) was measured daily. The calibration curve was presented as a mean value from the triplicate measurement. The DPPH· concentration in the reaction medium was calculated from the following equation as determined by the linear regression:

$$A517\ nm = 10.111\ x\ [DPPH] - 0.011$$ ..... (Equation 2)

Where A was absorbance; [DPPH·] was DPPH concentration expressed in mg/liter; with r = 0.9995. The percentage of inhibition of the DPPH· by the samples was calculated according to [20].

$$Inhibition\ (\%) = \frac{(Ac(0) - Ac(t))}{Ac(0)}\ x\ 100\%$$ .... (Equation 3)

Where AC(0) was the absorbance of the control at t =0 seconds and AC(t) was the absorbance of the antioxidant at t, which varied with the different concentrations.

Statistical analysis

All experiments were performed in triplicate. Data were presented as a mean value±standard deviation. EC_{50} and TEC_{50} values were estimated by linear regression. Statistical analyses were performed with the Excel and SPSS software package.

RESULTS AND DISCUSSION

Petroleum ether was non-polar solvent used to reduce lipid, wax, essential oil, and chlorophyll of pluchea leaves. Pluchea leaves contain essential oil [21, 22]. DPPH free radical scavenging activity of antioxidant tested was showed at fig. 1.

The results showed that the different types of antioxidants in samples and their concentrations caused the different absorbance of the DPPH· radical. Absorbance decreases a result of a color change from purple to yellow as the DPPH radical because antioxidant constituents donate a hydrogen atom to form the stable DPPH-H [13, 19, 23]. Verzelloni *et al.* (2007) said that all antioxidant samples don't have the same phenolic composition and all phenolics don't have the same antioxidant capacity. Hence, it is the quality and isn't the quantity of polyphenols that determines the antioxidant capacity of food.

Based on IC_{50} (inhibition concentration to reduce 50% of DPPH concentration), fig. 2 explained that GE and EAF had the same antioxidant activity for the similar concentration used. They were substantially more active than the other antioxidants at the same concentration. For GE and EAF, with 200 mg/ml concentrations, the bleaching ability of the DPPH solution was higher than 50%, but PE, BF, AF, ATS, and RE only had the bleaching ability lower than 50%.
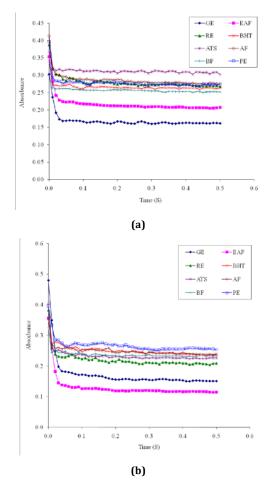

(a)

(b)

Fig. 1: Absorbance reduction of 75 μl of 1 x 10^{-4} mol/l DPPH· in methanol plotted versus time from each tested sample (GE, RE, PE, EAF, AF, BF, BHT, and ATS) at concentration: a. 0.05 mg/ml, b. 0.20 mg/ml
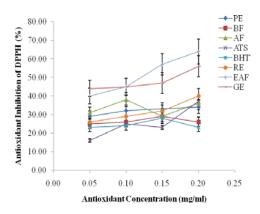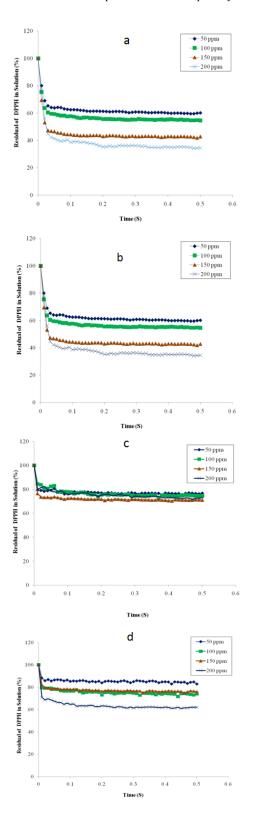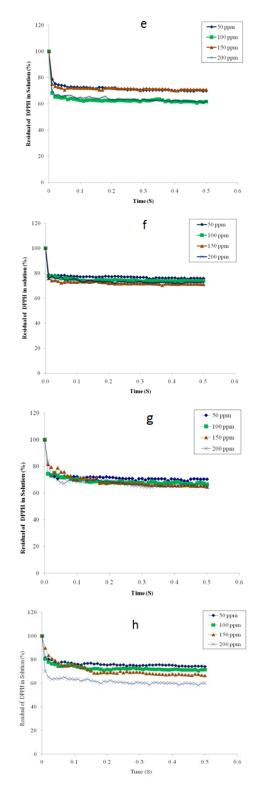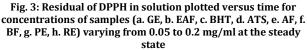

Fig. 2: Antioxidant inhibition of DPPH plotted versus antioxidant concentration of PE, EAF, AF, BF, GE, RE, BHT, and ATS at 200 mg/ml concentration

GE has high antioxidant activity because green tea contains high polyphenolic compounds including catechins, flavonols, flavanones, phenolic acids, glycosides and the aglycones of plant pigments [25] and microelements contributed as an antioxidant and free radical-scavenging properties. The active components of green tea can be extracted by solvent organic. Li *et al.* (2008) reported that the ethanolic extract possesses significantly higher DPPH free radical scavenging activity than the aqueous extract and α-tocopherol.

The higher inhibitor capacity of EAF than that of PE and other fractions (AF and BF) was caused by the different polarity of solvent used to extract antioxidant compounds. The different antiradical activity of PE and its fractions was related to intrinsic compounds. The different structure of phytochemical constituents determines scavenging activity. This argument is supported by [3, 6, 17, 27-31]. Widyawati *et al.* (2011) also informed that PE, BF and AF also contain phytochemical compounds the same as EAF, but AF hasn't founded sterol because it compound has different polarity.

Fig. 3: Residual of DPPH in solution plotted versus time for concentrations of samples (a. GE, b. EAF, c. BHT, d. ATS, e. AF, f. BF, g. PE, h. RE) varying from 0.05 to 0.2 mg/ml at the steady state

The ethyl acetate-soluble components were the most effective electron donor compared with PE and the other fractions. Based on IC_{50} at fig. 2, the orders of ability to scavenging DPPH radicals was determined as the EAF~ GE>PE ~ ATS ~ RE ~ AF>BHT ~ BF. Nevertheless, the order of antioxidant activity from PE, ATS, RE, and AF could not be determined. Data analysis showed hesitancy

because BHT and ATS are a potentially synthetic antioxidant that can scavenge free radical.

Fraction of the residual DPPH· solution plotted versus time

The absorbance of the DPPH solution is decreased depending on the intrinsic antioxidant activity (AOA) of the antioxidant compounds as well as on the speed of the reaction between DPPH· and the antioxidant compounds [32].

In the case of rapid kinetic behavior, practically all samples at high concentrations were reacted within a very short time, and a steady state was reached almost immediately. The reaction kinetics depended on the concentration of the different samples. Samples with the highest concentration showed that the steady state was apparently reached at 0.06 seconds for GE, 0.19 seconds for EAF, 0.28 seconds for RE, 0.09 seconds for BHT, 0.12 seconds for ATS, 0.30 seconds for AF, 0.31 seconds for BF, and 0.11 seconds for PE.

Data on the time course of absorbance (reaction kinetics) (fig. 1) enabled assessment of the extent of AOA for each sample. Therefore it was necessary to convert data on the reaction kinetics into new plots displaying the concentration of DPPH· in solution as a function of time. The concentration of DPPH· (%) was calculated by equation 2 and 3. Fig. 3 was time-dependent plots of residual DPPH· for various concentrations of samples. From the different fig., the decrease in absorbance was a function of the concentration.

At the higher concentration, the greater was the decrease in absorbance. Using data from fig. 3, new plots can be constructed to display fractions of DPPH· residue in the solution at steady state condition as a function of sample concentration (mg antioxidant/g DPPH·) as showed in fig. 4.

At this stage, it is used a concept of efficient concentration (EC_{50}) defined as the amount of antioxidant concentration needed to reduce 50% of the initial DPPH· concentration, $C_{DPPH·}$ (t=0) by a factor of two (at steady state). The parameter EC_{50} is a direct quantitative measure for antioxidant activity (AOA); highly effective antioxidant is characterized by low EC_{50} value, and vice versa [23]. The EC_{50} values of the different samples taken into account were obtained from the plot of the absorbance against the concentrations and described in fig. 4.

Data showed that EC_{50} = 49.14±4.81 mg/g for GE, 43.45±3.69 mg/g for EAF, 80.38±8.28 mg/g for RE, 130.97±10.48 mg/g for BHT, 93.88±8.45 mg/g for ATS, 85.91±8.59 mg/g for AF, 117.65±11.53 mg/g for BF and 90.23±7.13 mg/g for PE. Based on EC_{50} at fig. 4, showed the orders of ability to scavenging DPPH radicals of samples tested similar to data based on IC_{50}.
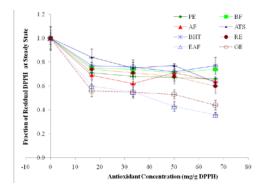

Fig. 4: Fraction of the residual DPPH· left at the steady state plotted versus antioxidant concentration

It means EAF was a potential antiradical. Antioxidant activity based on EC_{50} doesn't involve reaction time. Dawidowicz *et al.* (2012) said that parameter is considered to determine the antiradical activity of compounds including the EC_{50} and the time needed to reach the steady state (TEC_{50}). EC_{50} and TEC_{50} are calculated graphically affected the antiradical capacity. AE is calculated by equation 1. The antioxidant had the lower the EC_{50} and the shorter the reaction time as the higher the antiradical efficiency. AE is a more adequate parameter for selecting antioxidants than the widely used EC_{50}. Table 1 showed that EAF had the higher AE than PE and the other fractions, but its AE lower than it of BHT, GE, and ATS. Based on AE, BHT has been proven as a superior synthetic antioxidant. The classification order of AE for the tested antioxidants was BHT>GE ~ ATS>EAF>RE ~ BF~ PE~ AF.

Table 1: The effective concentration of antioxidant compounds to inhibit 50% DPPH and their antiradical efficiency

Antioxidant	EC_{50} (mg/g DPPH)	Range of time the steady state for antioxidant concentrations (s)	TEC_{50} (S)	AE
GE	49.14±4.81[a]	0.08-0.22	0.1224	0.1663±0.0166[c]
EAF	43.45±3.69[a]	0.19-0.28	0.2224	0.1035±0.0088[b]
RE	80.38±8.28[b]	0.28-0.42	0.2369	0.0525±0.0054[a]
BHT	130.97±10.48[c]	0.09-0.19	0.0154	0.4943±0.0395[d]
ATS	93.88±8.45[b]	0.12-0.20	0.0692	0.1540±0.0139[c]
AF	85.91±8.59[b]	0.30-0.39	0.2618	0.0445±0.0044[a]
BF	117.65±11.53[c]	0.31-0.45	0.1656	0.05133±0.0050[a]
PE	90.23±7.13[b]	0.29-0.26	0.2459	0.0451±0.0036[a]

Note: EC_{50} = effective concentration to inhibit 50% DPPH, TEC_{50} = the time needed to reach the steady state to EC_{50}

$$AE = 1/(EC50 \, x \, TEC50)$$

The difference of antioxidant activity of PE and its fraction was related to structure-activity of some polyphenols. This is supported by [24]. DPPH free radical scavenging is determined by phenolic compounds capacity to donate hydrogen atom or electron and kinetic reaction between phenolic compounds and DPPH free radical. Polyphenolic compounds with aglycon structure could donate hydrogen atom or electron higher than those with glycoside structure. Sanchez-Moreno *et al.* (1998) also reported that monophenols need less process to reach time at steady state. The higher antiradical capacity of EAF than PE and the other fractions was predicted that phytochemical contained was aglycon structure and medium polarity. Deng *et al.* (2011) informed that the antioxidant power is influenced by the capacity to prevent the autoxidation of free radical-mediated oxidation of the substrate in low concentration and the resulting radical after scavenging must be stable.

CONCLUSION

Pluchea extract (PE) and its fraction showed free radical scavenging activity compared with control antioxidant. Based on EC_{50} and AE, EAF was more potentially as DPPH free radical-scavenging activity than PE and its other fractions. AE was exactly parameter to determine antioxidant activity of PE and the other fractions.

ABBREVIATION

Pluchea extract: PE, Rosemary extract: RE, n-Butanol fraction: BF, Alpha-tocopherol succinate: ATS, Aqueous fraction: AF, Green tea extract: GE, Efficient concentration at 50% inhibition: $EC_{50,}$ Ethyl

acetate fraction: AF, Antioxidant activity: AOA, Antiradical efficiency: AE, The time needed to reach the steady state to EC_{50}: TEC_{50}, 2,2-diphenyl-1-picrylhydryzyl: DPPH

CONFLICTS OF INTERESTS

Author declares no conflicts of interest

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