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Radical Scavenging Activity Combination of Sambiloto (*Andrographis paniculata* Nees.) and Patikan Kebo (*Euphorbia hirta* L.) Ethanolic Extracts on 2,2-Diphenyl-1-Picrylhidrazyl (DPPH)

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ABSTRACT

Research of radical scavenging activity of sambiloto (*Andrographis paniculata* Nees) and patikan kebo (Euphorbia hirta L) ethanolic extracts and their combination toward 2,2-Diphenyl-1-Picrylhidrazyl (DPPH) has conducted. Subjects of this experiment comprise sambiloto ethanolic extract, patikan kebo ethanolic extract, dechlorophyllated extract of sambiloto and combinations among those three. Dechlorophyllation by electrocoagulation method is done to reduce chlorophyll content thereby increasing the level of active compound in the sambiloto extract. The results of dechlorophyllation showed that only total flavonoid content in the extract of sambiloto increased, meanwhile the radical scavenging activity results showed that patikan kebo ethanolic extract has the lowest IC_{50} value (22,36 µg/mL) compared to sambiloto ethanolic extract (IC_{50} value 499,03 µg/mL). Extract combination may increase DPPH radical scavenging activity of the sambiloto extract (IC_{50} value 76,06 µg/mL) but not higher than that of patikan kebo single extract.

Key words: extracts combination; radical scavenging; patikan kebo; sambiloto

INTRODUCTION

Free radicals have been known to possess an important role in causing several chronic and degenerative diseases. Human body naturally undergoes the process of forming free radicals, as a result of metabolic processes or immune system response. Excessive free radicals could react with lipids, proteins and DNA. The intake of exogenous antioxidant compounds could help the body to overcome these radicals. Antioxidants are molecules that can neutralize free radicals by donating or receiving electrons. Today, natural antioxidants are preferred over synthetic antioxidants because they have lower side effects (Basma *et al.,* 2011).

Some plants have been proven to possess antioxidant activities; one of them is *Sambiloto* (Andrographis paniculata Nees.). This plant contains andrographolide bioactive compounds. Other compounds reported found in this plant are flavone class of flavonoid compounds (Jarukamjorn and Nemoto, 2008; Okhuarobo *et al.,* 2014).Methanol and water extract of this plant are reported to have antioxidant activity determined based on DPPH radical capture method, Cupric ion method reducing antioxidant activity (CUPRAC), Ferric ion reducing antioxidant parameters (FRAP) and inhibition of lipid peroxidation (Akowuah *et al.,* 2006; Hossain *et al.,* 2014; Kurzawa *et al.,* 2015).

Euphorbia hirta L. (euphorbiaceae family) or *patikan kebo*, is an herb that has been used in various regions of Asia. The leaf extract of this plant is reported to have immunomodulatory activities (Ramesh and Padmavathi, 2010). The compounds contained in *patikan kebo* flowers indicate potential as scavengers of free radical, superoxide and nitric oxide anion radicals (Kumar *et al.,* 2010). Ethanolic extract of *patikan kebo* with active compounds β-amyrin can inhibit the function of iNOS protein and the induction of NO. Thus, it has the potential as an anti-inflammatory agent (Shih *et al.,* 2010). Basma *et al.,* (2011) reported that the methanol extract of E. hirta leaves had DPPH radical capture activities (IC_{50} 803 µg/mL). The main ingredient of the *patikan kebo* herb is flavonoid compounds (Patil *et al.,* 2009).

Effective, efficient method and processes of extraction are needed to obtain extracts with maximum levels of active substances and minimizing concomitant substances. To get a cleaner extract from concomitant substances, in which the activity of active compounds is expected to increase, it is necessary to make semi-purified extracts or multilevel extractions to obtain fractions (List and Schmidt, 1989). Electrocoagulation is an environmentally friendly method that can be used to precipitate chlorophyll

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in an extract (Jumpatong *et al.,* 2006). Efforts in reducing chlorophyll levels in extracts or dechlorophilization are expected to increase the level of active ingredient. In the case of trichlorophilization of the *sambiloto* extract, the active compounds to be increased are andrographolide and flavonoids.

In this study, the extraction of *sambiloto* herbs followed by dechlorophilization of the extract was carried out by using electrocoagulation method. Further observation in the effect of the combination of *sambiloto*'s ethanolic extract and dechlorophilized extract with *patikan kebo* herb's ethanolic extract on free radical scavenging is carried out.

METHODOLOGY

Materials and Equipments

The ingredients used were *sambiloto* and *patikan kebo* herbs taken from Banyuanyar, Surakarta and Tangen, Sragen, Central Java. The selected plants were those which have begun to flower, and cut on the stems, 10 cm from the ground. 96% Ethanol technical (Brataco), 2.2-Diphenyl Picrilhydazyl (DPPH) reagent (Sigma), Silica Gel 60 F_{254} HPTLC plate (Merck), Distilled water (Brataco), 98% Andrographolide (Sigma), 90% rutin trihydrate (Fluka), aluminum plates obtained from the local hardware store, Sodium chloride, Sodium acetate, Aluminum chloride, Hexane, Methanol, Ethylacetate, Absolute ethanol each with p.a from Merck.

The tools used were chromatography developing chambers (Camag), Linomat 5 TLC sample applicator (Camag), TLCScanner3 (Camag), Uv-Vis Optima SP 300 spectrophotometer, DC 30 Volt Power supply (Thunder). analytic balance (AD-6 Autobalance controller, Perkin Elmer) for comparative andrographolide weighing, analytic balance (AL 204, Mettler Toledo) for rutin and extracts' comparative weighing.

The course of Research

Material identification and preparation

To ascertain the correctness of the material used, plant determination was carried out in the Pharmacognosy Laboratory of the Faculty of Pharmacy, Gadjah Mada University. Herbs were made into simplicia by washing it under running water, draining and drying it in a drying cupboard at a temperature of 50°C for 12 hours, until easily destroyed simplicia when kneaded was obtained. The dried simplicia obtained were then powdered with a milling machine and sieved with a 30 mesh sieve size, to obtain powder with particle diameter of <600 μm.

The making of ethanolic extract

In a closed Erlenmeyer flask, 50 g simplicia powder of *sambiloto* were added with 80% ethanol in the amount of 350 mL and placed on an orbital shaker with rotational speed of 250 RPM for 8 hours. A total of 300 mL of extract obtained were used for the dechlorophilization stage, while the rest was evaporated until thick extracts were obtained with drying shrinkage value of less than 10%. *Patikan kebo* extraction was carried out in the same way as *sambiloto* extraction, but using 50% ethanol. The use of ethanol solvents with different levels was based on the results of previous studies, in which solvent *sambiloto* herbs with high ethanol content would extract andrographolide better, whereas in *patikan kebo* herbs, ethanol 50% was a solvent which would give the highest total of flavonoid content (Puspitasari, 2013).

Dechlorophilization of *sambiloto* ethanolic extract

The electrocoagulation method for extract dechlorophilization refers to the method used by Jumpatong *et al.,* (2006). Dechlorophilization was carried out on 300 mL of liquid *sambiloto* extract obtained from the extraction stage using the electrocoagulation method. Two Aluminum electrode plates (size 15 X 4 cm) were placed at a distance of 3 cm and submerged in an extract solution as deep as 5 cm. 0.2% sodium chloride was added as an electrolyte. Direct current (0.2 A,24 V) from the DC power supplier was applied into the sample solution. Electrocoagulation was carried out for 90 minutes. Chlorophyll compounds would be precipitated. The precipitate was separated by centrifuging at the speed of 10000 RPM for 10 minutes. The supernatant was taken and evaporated on a water bath until a thick extract was obtained with a shrinkage value of less than 10%.

Determination of andrographolide levels

The andrographolide levels in the ethanolic extract of *sambiloto* (EES) and the dechlorophilized ethanolic extract of *sambiloto* (EEDS) were determined by the High-performance Thin-layer Chromatography (HPTLC) method as used by Akowuah *et al.,* (2006) and Saxena *et al.,* (2000). The system used was: Silent phase of silica 60 60 F_{254} HPTLC plate, thickness of 0.15 mm with mobile phase of hexane-ethyl acetate 1: 4 (v/v) 2 times development. Detection was carried out under 254 nm UV light. Comparatives of andrographolide (1.0 mg/mL in methanol) and sample (10.0 mg/mL in ethanol) was sprayed with Camag Linomat 5 sample applicator. On plates with

a size of 14 x 10 cm, a comparative andrographolide serial was sprayed consisting of 5 bands (1.0-10 µg/band) and 6 sample bands (concentration of 10 µg/band). The operational spraying parameters were: nitrogen gas flow rate 100 nL/sec, 4 mm bandwidth, starting point 15 mm from the bottom and 12 mm from the right-left side while the inter-band distance was 11 mm.

After the plate was developed with a development distance of 8 cm, the plate was taken and left to dry at room temperature, then the plate was developed again as far as 8 cm in the same developing chamber without changing the mobile phase.

Quantitative analysis of andrographolide was carried out by measuring the peak area of the chromatogram using TLC scanner 3 which was equipped with WinCATS (Camag) software. The operational parameters specified in the reading were 232 nm wavelength, slit dimensions 8 x 0.2 mm, scanning speed 80 mm/s and data resolution of 100 µm/step. A calibration curve was made between the andrographolide levels versus the chromatogram peak area. This curve was used in determining the andrographolide content in the sample.

Determination of total flavonoid levels

The total flavonoids levels in the ethanolic extract of *patikan kebo* (EEP), EES and EEDS were determined by procedure carried out by Chang *et al.,* (2002), which was modified.

The test solution was made by weighing 100.0 mg extract and dissolving it in 10.0 mL 70% ethanol. Rutin was used as a comparative in making a calibration curve. The comparative solution was made by weighing 10.0 mg and dissolved in ethanol to 25.0 mL, hereinafter referred to as a stock solution. Dilution was carried out with ethanol to the stock solution to obtain a series of levels of 38-760 µg/mL, at 4 levels of concentration. 100 uL of the test solution or rutin standard solution were taken, then methanol is added to 1.5 mL in the test tube. The next step, added reagents consisting of 0.1 mL $AlCl_3$ 10%; 0.1 mL Na-acetate; and 2.8 mL of distilled water. The solution was mixed with vortex until homogeneous, then left at room temperature for 30 minutes. Test blank solution containing samples and reagents was also made, without the addition of $AlCl_3$. Absorbate of rutin standard solutions, blank and test solutions was read on the spectrophotometer at a wavelength of 415 nm. The total flavonoid level was calculated based on the rutin comparative calibration curve, so that the levels are expressed in % ER (Rutin equivalent).

Radical scavenging activity test

0.1 mL test sample with varied concentration was put in a conical flask tube, then added with 3.9 mL methanol and 1 mL of 0.4 mM DPPH solution. The solution was homogenized with vortex. After being incubated for 30 minutes, absorbate was read at a maximum wavelength of 695 nm along with control in the form of sample solution in methanol. Also measured the blank solution absorbate containing a mixture of 1 mL of 0.4 mM DPPH solution and 4 mL of methanol. Activities were expressed as % of radical scavenging calculated based on the equation:

$$\%\ \mathrm{Pr} = \frac{\text{Abs. blangko} - (\text{abs. sampel} - \text{abs. konrol})}{\text{Absorbansi blangko}} \mathrm{X}\,100\%$$

Pr = Penangkapan radikal

Data Analysis

Data on the total andrographolide and flavonoids levels were analyzed by independent t test, with the help of SPSS for Windows 22.0 software.

RESULTS AND DISCUSSION

The extraction produced green EES liquid, which after being evaporated in the form of dark green extract and tasted very bitter and EEP which was also dark green with a slightly bitter taste. The results of EES dechlorophilization were dark brown thick extracts.

Determination of andrographolide levels

To determine the effectiveness of the dechlorophilization process, the determination of andrographolide was carried out on the extract of ethanolic extract of *sambiloto* and extracts after dechlorophilization (Table I).

In (Table I), it can be observed that the process of dechlorophilization could not increase the andrographolide level in the extract. There is no significant differences in andrographolide levels between EES and EEDS. This is likely due to dechlorophilization done in this study was carried out only for 90 minutes, so the concomitant substances to be removed, in this case the chlorophyll compound, have not precipitated properly. Jumpatong *et al.,* (2006) reported that dechlorophilization of ethanolic extract of *sambiloto* by electrocoagulation method for 150 minutes could increase the andrographolide levels up to 38%, while Chairungsi *et al.,* (2006) reported

Table I. Andrographolide content in extracts

Extracts	Peak chromatogram area	Sample weight (mg)	andrographolide content (µg/band) #	Solvent (µL)	Spraying volume (µL)	Andrographolide content (%)
Sambiloto	12350.53	10.1	1.63	1000	10	16.18
	13784.55	10.4	1.99	1000	10	19.13
	13351.57	10.2	1.88	1000	10	18.45
Dechlorophilization of sambiloto	12710.07	10.2	1.72	1000	10	16.89
	12946.17	10.2	1.78	1000	10	17.47
	11954.48	10.1	1.78	1000	10	17.84
	Test material					**Content % w/w***
	Ethanolic extract of *sambiloto*					17.92 ± 1.55[a]
	Dechlorophilized extract of *sambiloto*					17.34 ± 0.40[a]

Reference:
#Calculated based on the equation of calibration curve y=4029.7x + 5767.4 with R^2 = 0.9981
*The average of the 3 test times, the same letters behind the numbers show no statistically significant difference (p <0.05)

Table II. Calculation of calibration curve and Total flavonoid content in extracts

Extracts	Absorbency	Sample weight (mg)	Measured content (µg/mL) #	Solvent volume (µL)	Sample volume (µL)	Total flavonoid content (% ER)
Sambiloto	0.210	50.1	18.37	500	100	0.18
	0.293	50	25.32	500	100	0.25
	0.268	50.1	23.22	500	100	0.23
Dechlorophilization of *sambiloto*	0.675	50.2	57.42	500	200	0.29
	0.689	50.1	58.62	500	200	0.30
	0.669	50	56.97	500	200	0.29
Patikan kebo	0.102	10.1	9.32	500	25	1.85
	0.095	10.2	8.73	500	25	1.71
	0.104	10.1	9.49	500	25	1.88
	Test material					**Total flavonoid content % w/w ER (Equivalent Rutin)***
	Ethanolic extract of *sambiloto*					0.22 ± 0.04[a]
	Dechlorophilized extract of *sambiloto*					0.29 ± 0.00[b]
	Ethanolic extract of *patikan kebo*					1.81 ± 0.09[c]

Reference:
Calculated based on the equation of calibration curve y=0.0119x – 0.0086 with R^2 = 0.9925
*The average of the 3 test times, the same letters behind the numbers show no statistically significant difference (p <0.05)

the precipitation of chlorophyll in extracts with ethanol solvent using electrocoagulation techniques for 120 minutes.

Determination of total flavonoid levels

Level determination was carried out on EES, EEDS and EEP, considering that flavonoid compounds were also one of the ingredients of the *sambiloto* extract, so it is necessary to know the effect of dechlorophilization process on the levels of flavonoids in the extracts. The results of the level determination could (Table II).

Table II shows that flavonoid compounds are the main content of *patikan kebo* extract. According to Nurfitri and Puspitasari (2011), the electrocoagulation process in the ethanolic extract of mango leaves also precipitated phenolic compounds. Thus, dechlorophilization of plant extracts containing phenolic compounds could not be carried out by this method. However, in this study, it was observed that while flavonoids belong to the class of polyphenol compounds, flavonoid compounds contained in EES were not precipitated in the electrocoagulation process. Rao *et al.,* (2004)

Table III. DPPH Radical Scavenging Activities

Test Material	Regression line equation	IC_{50} (µg/mL)
Ethanolic extract of *sambiloto* herb (EES)	y=0.0678x+16.166 (R^2=0.9855)	499.03
Ethanolic extract of *patikan kebo* herb (EEP)	y=1.9758x+4.4514 (R^2=0.9943)	22.36
Dechlorophilized ethanolic extract of *sambiloto* herb (EEDS)	y=0.0559x+6.4534 (R^2=0.9844)	799.01
EES-EEP combination (1:1)	y=0.6613x-0.2974 (R^2=0.9971)	76.06
EES-EEP combination (0.5:0.5)	y=0.3488x-2.6845 (R^2=0.9903)	151.03
EEDS-EEP combination (0.5-0.5)	y=0.2811x-2.0821 (R^2=0.9937)	185.27

and Hossain *et al.,* (2014) stated that the flavonoid compounds in *sambiloto* contained in the form of methoxy flavone and methoxy flavanone. These compounds may not be precipitated during the electrocoagulation process, so there is a higher total flavonoids in EEDS than EES.

Free Radical Scavenging Activities

To determine the effect of the combination of extracts on antioxidant activity, measurements of free radical scavenging activity were carried out on the test samples stated in the Inhibitory concentration $(IC)_{50}$, i.e. the ability of the test material to capture 50% DPPH radicals (Table III).

The results in (Table III) show that EEP has the highest radical scavenging activities. Neither the combination of EEP with EES or EEDS could increase the radical scavenging activity higher than the extract of *patikan kebo*. *Sambiloto* is reported to have antioxidant activity with a mechanism to enhance antioxidant enzymes and inhibit lipid peroxidation (Okhuarobo *et al.,* 2014). The results of this study are in accordance with Akowuah *et al.,* (2006) which states that although the *sambiloto* methanolic extract has the ability to scavenge free radicals, its activity is much lower than quercetin. However, if combined with EEP, EES and EEDS radical scavenging activities would be significantly increased compared to their single extracts. Flavonoid compounds are often associated with radical scavenging activities of plants. According to (Agati *et al*., 2012), only flavonoids with orthodihydroxy groups in the B ring alone which have a significant relationship with these activities. In this case, although the levels of total flavonoids in EEDS are higher than EES, radical scavenging activity is shown to be lower. This is caused by the flavonoids contained in *sambiloto* is a methoxy group of flavones and flavanones which do not have a free hydroxy group on ring B. In this case, the combination with the *patikan kebo* extract could increase the radical scavenging activity of *sambiloto* extract.

CONCLUSION

Dechlorophilization of *sambiloto* extract only increase total flavonoid levels. The ethanolic extract of *patikan kebo* herbs has the highest radical scavenging activity. The combination with *patikan kebo* extract could increase the radical scavenging activity of *sambiloto* herbs' ethanolic extract, but its activity is still lower compared to the *patikan kebo* herbs' ethanolic extract.

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