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Research Article

Antioxidant Activity and Histochemical Analysis of *Acalypha indica* L. and *Acalypha wilkesiana* Muell. Arg. Vegetative and Generative Organs

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ABSTRACT

Acalypha indica L. and *Acalypha wilkesiana* Muell.Arg. which are member of Euphorbiaceae have ethnopharmacological history. However, the use of *Acalypha indica* L. and *Acalypha wilkesiana* Muell.Arg. have not been optimalized. The objective of this study was to know the antioxidant activity, secondary metabolites content, the location and distribution of secondary metabolites, and secretory structure of *Acalypha indica* L. and *Acalypha wilkesiana* Muell.Arg. Antioxidant activity were determined by DPPH methode using chloroform and methanol extracts of stems, leaves, and the mixture of flowers-fruits-seeds. Secondary metabolites contents were tested using various reagent and measured using spectrophotometre. Moreover, the distribution of secondary metabolites were analyzed by histochemical test. DPPH test showed that methanolic extract of leaves and the mixture of flowers-fruits-seeds of *Acalypha wilkesiana* performed the best antioxidant activity compared to other samples. The IC_{50} value of leaves and flowers-fruits-seeds mixture methanolic extracts were 3,42±0,78 µg/ml and 4,71±0,71 µg/ml respectively. The phenol content of leaves and the mixture of flowers-fruits-seeds methanolic extract of *Acalypha wilkesiana* were 238,89±19,48% GAE and 301,22±32,16% GAE respectively; flavonoids content were 36,93±2,01% QE and 58,67±1,51% QE respectively; and tannin content were 84,18±5,68% GAE and 60,96±0,67% GAE respectively. Antioxidant activity could be due to the phenol, flavonoids, and tannin contents. The histochemical test showed that phenol, flavonoids, tannin, and alkaloids could be found in the stems, leaves, and the mixture of flowers-fruits-seeds of *Acalypha indica* and *Acalypha wilkesiana*. The distribution of various mentioned group of compound were almost in the whole of various tissues. The secretory stuctures were consisted of internal structure and external structure which in the forrm of capitate glandular trichome.

Keywords: *Acalypha indica* L., *Acalypha wilkesiana* Muell.Arg, antioxidant, secondary metabolites, histochemical.

INTRODUCTION

Free radicals are reactive molecules that can cause oxidative stress. They cause many diseases, such as cardiovascular disease, renal disorders, cancer, pulmonary disorders, gastrointestinal diseases, and diabetes[1,2]. Free radicals can be neutralized by antioxidant. Bioactive compounds which have pharmacology and toxicology effects have antioxidant activity. Those are phenol, flavonoids, tannin, alkaloids, and other metabolites[2,3]. Food and herb are natural sources of antioxidant. The use of antioxidant activity from natural product is better than synthetic one. The synthetic antioxidant, such as *Butylated hydroxytoluene* (BHT) and *Butylated hydroxyanisole* (BHA) can change enzyme activity, lipid concentration, and have carsinogenic effects. It can cause chronic health problems in a long term use[2]. *Acalypha indica* L. and *Acalypha wilkesiana* Muell. Arg. belong to Euphorbiaceae had high flavonoids, phenol, and alkaloids contents[4]. *Acalypha indica* L. leaves and roots methanolic extracts have been reported to have antimicrobial and antioxidant activity[4,5]. *Acalypha wilkesiana* Muell.Arg may has the same potential as *Acalypha indica* L due to the same genus of both plants. It was mentioned that the close relation plants have similar bioactive compounds[6]. Indonesian use *Acalypha indica* leaves as traditional medicine especially for purgative and eye disorders. Meanwhile, *Acalypha wilkesiana* is used as antiseptic for skin injury[7].

MATERIALS AND METHODES

This research were determining antioxidant activity, tannin, phenol, and flavonoids contents, also histochemical test for the distribution of various secondary metabolites. Result of antioxidant activity indicated as IC_{50} value was obtained from DPPH test method and was analyzed using regression. Statistical analysis was done using ANOVA Factorial test to know the effects of species, solvent, and plant's organ toward IC_{50} value. Result of phenol, flavonoids, and tannin content were analyzed using correlation and regression of Microsoft Excel 2007. Result of histochemical test was obtained from photograph of fresh transversal section which was stained with spesific reagent, then analyzed descriptively.

Materials

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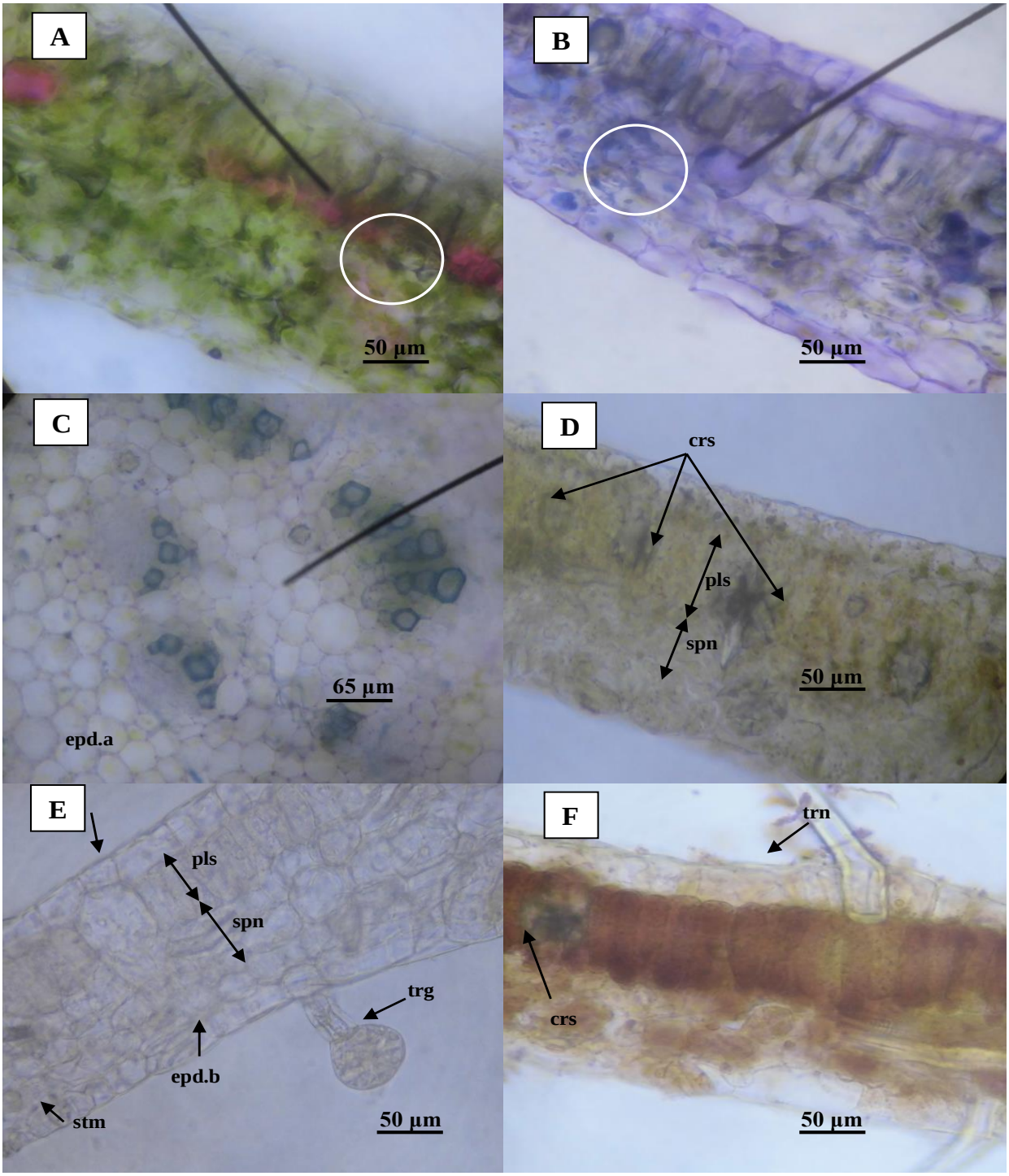

Figure 1. Cross section of *Acalypha wilkesiana* Leaves. A) Before staining, B&C) After staining with Toluidine blue, phenol in mesophyll and xylem showed blue color, white circle was anthocyanins, D) After staining with $AlCl_3$, flavonoids in mesophyll showed yellow color, E) After staining with $FeCl_3$, tannin in glandular trichome and mesophyll showed green darkness color, F) After staining with Dragendorf, alkaloids in mesophyll showed brown color, epd.a=upper epidermis, epd.b=lower epidermis, crs= crystal druse, pls=palisade mesophyll, spn=spongy mesophyll, stm=stoma, trg=glandular trichome, trn=non glandular trichome.

Plant materials used were *Acalypha indica* L. and *Acalypha wilkesiana* Muell.Arg collected from Bulaksumur, Yogyakarta. Plant identification was done by Indonesian Institute of Science (LIPI) Bogor, Indonesia. The herbarium was stored in the same place of identification. The plant's organs used were stems, leaves, and the mixture of flowers-fruits-seeds. The samples were dried and grinded into powders. Chemicals for extraction, DPPH scavenging activity, metabolites contents, and histochemical test were: $methanol_{(aq)}$ p.a.MERCK,

chloroform$_{(aq)}$ p.a.MERCK, DPPH 0,05 mM$_{(aq)}$, ascorbat acid$_{(aq)}$ 1 mg/ml, gallate acid$_{(aq)}$ 1 mg/ml, aquadest$_{(aq)}$, Folin Ciocalteau$_{(aq)}$ 50%, $Na_2CO_{3(aq)}$ (2% and 35%), $NaNO_{2(aq)}$ 5%, $AlCl_{3(aq)}$ 10%, Quercetin$_{(aq)}$ 1 mg/ml, toluidine blue, $FeCl_3$, dragendorf.

Plant compounds extraction

Extraction was done using maceration method. Ten grams of each kind of powdered material was macerated using 150 ml chloroform for 3 days followed by remacerration using 50 ml chloroform. Both extracts were combined, filtered and dried. The debris was then macerated again with methanol using the same as previous method.

Antioxidant Activity Test

Antioxidant activity test was done using DPPH method. Ten milligram of extract was dissolved in 10 ml of methanol. The solution was made to be 5 series, for example 200, 400, 600, 800, and 1000 µg/ml up till to be found the mean of inhibition value as much as 50%. One milliliter of extract solution was mixed with 2 ml of DPPH 0,05 mM, and incubated 30 minutes in dark. Ascorbat acid was used as possitive control and made into 5 series solution (20, 40, 60, 80, dan 100 µg/ml). The absorbance of sample solution was measured using *visible spectrophotometre* with 517 nm wavelenght. The decreasing DPPH absorbance showed the increasing antioxidant activity to scavenge DPPH. Antioxidant activity (AOA) can be formulated as follows:

$$\text{AOA (\%)} = \frac{\text{Ao} - \text{As}}{\text{Ao}} \text{ X } 100$$

Ao = control

As = mixed solution (DPPH and sample extract).

Total phenol content

The total phenol contents were measured by Folin Ciocalteau method[5] with minor modification. Sample extract was dissolved in methanol (1 mg/ml). A hundred microliter of the solution was mixed with 100 µl Folin Ciocalteau 50%. Mixed solution was incubated in room temperature for 3 minutes then mixed with 2 ml Na_2CO_3 2%. The mixture was added with aquadest up to 3 ml than boiled in the water bath with the temperature 100°C for 1 minutes, and cooled down in dark. The sample's absorbance was measured using *visible spectrophotometre* λ=760 nm. Total phenol content was measured using standard curve of gallate acid 1 mg/ml.

Total flavonoids content

Total flavonoids content was measured using spectrophotometre[8]. Five hundred microliter of sample extract (1 mg/ml) was mixed with 2 ml of aquabidest and 150 µl of $NaNO_2$ 5%, then incubated in room temperature for 6 minutes, following by mixing with 150 µl $AlCl_3$ 10% and incubated in room temperature for another 6 minutes. Sample was then mixed with 1 ml NaOH 1M and aquabidest up to 5 ml. Absorbance was measured at 510 nm wave length using same tool with previous measurement. Total flavonoids content was measured using standard curve of Quercetin 1 mg/ml.

Tannin content

Tannin content was measured using Folin Ciocalteu[9] with minor modification. Five hundred microliter of sample extract 1 mg/ml was mixed with 500 µl Folin Ciocalteu and 1 ml Na_2CO_3 35 %, following by mixing with aquadest up to 10 ml volume. The mixed solution was shaken and kept in room temperature for 30 minutes. Gallate acid was used as a standard and made into 5 series (20, 40, 60, 80 and 100 µg/ml). Sample's absorbance was measured using *visible spectrophotometre* with 725 nm lenght.

Histochemical test

Histochemical test was done by preparing fresh section of sample then stained with spesific reagent[10,11]. Toluidine blue was used for phenol detection which gives blue possitive color[11]. $AlCl_3$ was used to detect flavonoids which gives yellow possitive color[12], while $FeCl_3$ was used to detect tannin which gives green darkness or blue dark possitive color[13-15]. Dragendorf was used for alkaloids detection[14]. Sample was analyzed using monoculer light microscop NIKON and photographed using digital camera CASIO Exilim EX-ZS6.

RESULTS AND DISCUSSION

Antioxidant Activity

Antioxidant activity of *Acalypha indica* and *Acalypha wilkesiana* methanolic extracts were higher than chloroform extract which were indicated by IC_{50} values. IC_{50} is antioxidant that is needed to scavenge 50% concentration of DPPH[16,17]. The lower IC_{50} means the higher antioxidant activity[18]. The lowest IC_{50} was found in methanolic extract of *Acalypha wilkesiana* leaves (3,42±0,78 µg/ml), and methanolic extract of the mixture of *Acalypha wilkesiana* flowers-fruits-seeds (4,71±0,71 µg/ml). Those IC_{50} were close to ascorbat acid's IC_{50}, which was 1,78±0,02 µg/ml. IC_{50} value from methanolic and chloroform extract of *Acalypha indica* and *Acalypha wilkesiana* was shown on Table 1. The IC_{50} mean value of *Acalypha indica*t was higher than *Acalypha wilkesiana*, so that antioxidant activity of *Acalypha indica* was lower than those of *Acalypha wilkesiana*. Although *Acalypha indica* and *Acalypha wilkesiana* are same in the case of genus and may have similar metabolite compounds[6], however the

Table 1: IC_{50} value (µg/ml) from chloroform and methanolic extract of *Acalypha indica* and *Acalypha wilkesiana*.

Species	Plant's organ	Extracts	
		Chloroform	Methanol
Acalypha indica	Stem	242,85 ± 8,26	139,04 ± 11,28
	Leaves	213,20 ± 40,95	239,61 ± 16,32
	Flower-Fruit-Seed	371,34 ± 10,13	79,14 ± 7,04
Acalypha wilkesiana	Stem	203,53 ± 44,31	161,39 ± 28,52
	Leaves	186,45 ± 29,60	3,42 ± 0,78
	Flower-Fruit-Seed	357,40 ± 39,95	4,71 ± 0,71

Table 2: Phenol, flavonoids, and tannin content from chloroform and methanolic extract of *Acalypha indica* and *Acalypha wilkesiana*.

Species	Plant's organ	Extracts	Phenol content (%) GAE	Flavonoid content (%) QE	Tannin content (%) GAE
Acalypha indica	Stem	Chloroform	10,11±1,35	5,47±0,23	2,62±0,04
		Methanol	21,00±2,33	10,13±2,20	3,71±0,04
	Leaves	Chloroform	4,11±1,68	3,60±0,40	2,07±0,35
		Methanol	43,33±3,53	13,60±0,69	6,73±0,20
	FFS	Chloroform	0,33±0,88	2,93±3,63	1,87±0,13
		Methanol	49,33±2,08	25,33±4,77	19,84±2,41
Acalypha wilkesiana	Stem	Chloroform	13,67±2,03	5,60±0,80	6,58±0,81
		Methanol	24,33±4,00	6,80±3,42	12,27±0,31
	Leaves	Chloroform	9,44±4,76	2,00±2,43	1,42±0,08
		Methanol	238,89±19,48	36,93±2,01	84,18±5,68
	FFS	Chloroform	2,78±0,77	3,20±1,06	1,62±0,04
		Methanol	301,22±32,16	58,67±1,51	60,96±0,67

Table 3: Regression and correlation analysis of phenolic compounds and its IC_{50} values.

Compound group	Equation	Correlation coefficient	Determination coefficient
Phenol	Y=237,4-0,900X	0,76958	52,9%
Flavonoid	Y=261,4-5,366X	0,7984	63,7%
Tannin	Y=241,2-3,398X	0,7818	61,1%.

Table 4: Distribution of secondary metabolites.

Secondary metabolites	*Acalypha indica*			*Acalypha wilkesiana*		
	Stems	Leaves	Flowers-Fruits-Seeds	Stems	Leaves	Flowers-Fruits-Seeds
Phenol	Glandular trichome, xylem	Xylem	Glandular trichome, mesocarp, endocarp,outer integument, epidermis endosperm, placenta, xylem	Xylem	Xylem, mesophyll	Exocarp, mesocarp, endocarp, outer & inner integument, embryo
Flavonoids	Glandular trichome, parenchyma cortex, parenchyma stele	Mesophyll	Glandular trichome, exocarp, placenta	Parenchyma cortex, xylem, parenchyma stele	Mesophyll	Exocarp, mesocarp, endocarp, outer & inner integument, placenta
Tannin	Glandular trichome, epidermis, parenchyma cortex, cambium, xylem, parenchyma stele	Mesophyll, phloem, xylem	Glandular trichome, exocarp, endocarp, outer & inner integument, endosperm, parenchyma embryo, placenta	Epidermis, parenchyma cortex, xylem, phloem, parenchyma stele	Glandular trichome, mesophyll, xylem	Exocarp, mesocarp, endocarp, epidermis endosperm
Alkaloids	Glandular trichome, parenchyma cortex, phloem, xylem, parenchyma stele	Mesophyll, phloem, xylem	Exocarp, mesocarp, outer integument, epidermis endosperm, embryo	Epidermis, parenchyma cortex, xylem, phloem, parenchyma stele	Mesophyll	Exocarp, mesocarp, endocarp, inner integument, endosperm

concentration may be different. Methanol was the best solvent. The higher antioxidant activity from methanolic extract of *Acalypha indica* and *Acalypha wilkesiana* compared to other solvent means that polar compounds played an important role in antioxidant activity compared to those of non-polar compounds. Polar compounds are soluble in the polar solvent, just like methanol[19,20]. The different organs (stems, leaves, and the mixture of flowers-fruits-seeds) also affected the IC_{50}.

Phenol, flavonoids, and tannin content and their correlation with IC_{50}

The antioxidant activity had correlation with phenol, flavonoids, and tannin contents. The results showed that each organ had various phenolic content. The methanolic extract of *Acalypha wilkesiana* leaves and the mixture of flowers-fruits-seeds had the highest content of mentioned compound groups (Table 2). Correlation analysis showed that there was negative correlation between phenol, flavonoids, or tannin contents and the IC_{50} values. Its mean that the higher phenol, flavonoids, or tannin content was the lower IC_{50} values. The lower IC_{50} values was the higher antioxidant activity (Table 3). Phenol, flavonoids, and tannin are secondary metabolite compounds which have antioxidant activity[2,3]. Phenolic compound has antioxidant activity and act as a reductor and hydrogen donor, neutralize singlet oxygen, and bind metal. Their antioxidant activity can neutralize free radical[21]. Flavonoids act as an antioxidant because it is a good reductor so it can prevent oxidative stress[22,23]. Tannin act as an antioxidant by binding free radical, metal, and protein with enzime activity[6,22].

Histochemical test for location of secondary metabolites

Secondary metabolites of *Acalypha indica* and *Acalypha wilkesiana* distributed in some spesific location or almost in the whole tissues of plant (Table 4). Figure 1 which is the cross section of *Acalypha wilkesiana* leaves was one of histochemical test results. Phenol was distributed in the xilem of stem, leaves, as well as flower-fruit-seed of *Acalypha indica* and *Acalypha wilkesiana*. Moreover, phenol was also distributed in mesophyll of *Acalypha wilkesiana* leaves. Anthocyanins shown in the Figure 1 A & B showed blue color when it is stained with toluidine blue. Phenolic compounds will give blue color when stained with toluidine blue[11]. The high antioxidant activity of *Acalypha wilkesiana* leaves may be due to anthocyanins content. Anthocyanins has antioxidant activity, so it is an important group of compound to prevent many diseases which was related to oxidative stress[24]. Histochemical test showed that flavonoids gave yellow color when stained with $AlCl_3$ (Fig.1D)[12,25]. Flavonoids was stored in vacuole[26] while tannin showed green darkness color when stained with $FeCl_3$[13-15]. Tannin in leaves of *Acalypha wilkesiana* could be found in mesophyll, capitate glandular trichome (Fig.1E), and costa xilem. It showed that the compounds was stored in xilem cell wall[26]. Figure 1F showed that alkaloids gave brown color when stained with Dragendorf[14]. The result showed that the compound stored in the mesophyll of *Acalypha wilkesiana* leaves. It was explained that alkaloids is stored in the vacuole, although some of plants have laticifer in storing the alkaloids[26]. Instead of histochemical method, the study of compounds distribution could be done by separating the tissue and identified the compounds from separating tissue. It was found many compounds in different tissues of *Jacobaeae vulgaris* leaves[27].

CONCLUSION

Methanolic extract of *Acalypha indica* and *Acalypha wilkesiana* had higher antioxidant activity compared to chloroform extract. Methanolic extract of *Acalypha wilkesiana* leaves and the mixture of flowers-fruits-seeds had the highest antioxidant activity compared to other extract . The high antioxidant had correlation with phenol and tannin, espescially flavonoids contents. Phenol, flavonoids, tannin, and alkaloids could be found in the stem, leaves and flowers-fruits-seeds of *Acalypha indica* and *Acalypha wilkesiana*, and distributed in almost of the whole tissues. Secretory structure in *Acalypha indica* and *Acalypha wilkesiana* were internal secretory and external secretory which was in the form of capitate glandular trichome.

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