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THE EFFECTIVENESS OF POLAR AND NON POLAR FRACTIONS OF *AGERATUM CONYZOIDES* L. TO CONTROL PEANUT RUST DISEASE AND PHYTOCHEMICAL SCREENINGS OF SECONDARY METABOLITES

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ABSTRACT

***The effectiveness of polar and non-polar fractions of* Ageratum conyzoides *L. to control peanut rust disease and phytochemical screenings of secondary metabolites*.** Peanut rust disease caused by *Puccinia arachidis* is one of the important diseases which causes yield loss more than 50%. An alternative control which is more environmentally friendly can be conducted by applying botanical fungicides extracted from weeds. This study aimed to obtain effective concentrations of polar and non-polar fractions of roots, leaves, and flowers of *Ageratum conyzoides* as well as to investigate secondary metabolites in each extract. A spore germination test was conducted to determine the effectiveness of each extract. An application of 5% polar fraction of the leaves resulted 98±2% ungerminate spores which was not different with the application of 5% polar fraction of the roots which resulted 95±2% ungerminate spores. The use of a polar solvent extracted more diverse secondary metabolites compared with that of a non-polar solvent. Phytochemical screening tests on the three extracts showed that the leaf polar fraction contained more diverse secondary metabolites as compared with the others. The polar fraction of the leaves contained alkaloids, flavonoids, tannins, saponins, and terpenoids. A further test to confirm the existence as well as to separate these compounds with thin layer chromatography showed that alkaloids, flavonoids, saponins, and terpenoids separated into 7, 9, 6, and 8 spots, respectively. These secondary metabolites may be responsible to inhibit the spore germination of *P. arachidis*.

Key words: *Ageratum conyzoides*, botanical fungicide, peanut rust disease, secondary metabolite

ABSTRAK

Efektivitas fraksi polar dan non polar* Ageratum conyzoides *L. untuk mengendalikan penyakit karat kacang tanah dan skrining fitokimia metabolit sekunder. Penyakit karat kacang tanah yang disebabkan oleh *Puccinia arachidis* merupakan salah satu penyakit penting yang mampu menurunkan hasil polong hingga lebih dari 50%. Pengendalian ramah lingkungan dilakukan salah satunya dengan aplikasi fungisida nabati dari ekstrak gulma. Penelitian bertujuan untuk mendapatkan konsentrasi efektif fraksi polar dan non polar dari akar, daun, dan bunga *Ageratum conyzoides* dan mengetahui golongan senyawa metabolit sekunder yang terkandung dalam ekstrak gulma yang digunakan. Uji perkecambahan spora secara *in vitro* dilakukan untuk mengetahui keefektifan masing-masing ekstrak. Aplikasi fraksi polar daun dengan konsentrasi 5% menghasilkan spora yang tidak berkecambah sebanyak 98±2% yang hasilnya tidak berbeda dengan aplikasi 5% fraksi polar akar, yaitu 95±2% spora tidak berkecambah. Pelarut polar mampu mengekstrak lebih beragam metabolit sekunder dibandingkan dengan pelarut non polar. Skrining fitokimia awal yang dilakukan menunjukkan bahwa fraksi polar daun mengandung golongan metabolit sekunder yang lebih beragam dibandingkan bagian akar dan bunga. Fraksi polar daun mengandung alkaloid, flavonoid, tannin, saponin, dan terpenoid. Uji konfirmasi keberadaan metabolit sekunder dengan kromatografi lapis tipis untuk memisahkan golongan senyawa tersebut menunjukkan bahwa alkaloid, flavonoid, saponin, dan terpenoid masing-masing menghasilkan 7, 9, 6, dan 8 noda. Kandungan metabolit sekunder tersebut diduga berperan dalam menggagalkan perkecambahan spora *P. arachidis*.

Kata kunci: *Ageratum conyzoides*, fungisida nabati, karat kacang tanah, metabolit sekunder

INTRODUCTION

Peanut rust disease caused by *Puccinia arachidis* Speg. is one of the important diseases which causes pod yield loss from 6% to 57% depending on the susceptibility of crops (Subrahmanyam & McDonald, 1984). Favorable environment, especially temperature at around 25 ^{o}C and relative humidity more than 87% supports the infection and pustule development (Arsule & Pande, 2011; Sunkad & Kulkarni, 2007). Urediospores

germinate to form germ tubes and penetrate host cells as initial infection (Saleh, 2010). The spread of this disease was supported by the wind and water splashing. In Indonesia, peanut rust disease spreads at the central productions of peanuts such as East Java, Bali, and West Nusa Tenggara (Semangun, 1991).

The effective treatment to control this disease is by carrying out integrated pest management (IPM). This approach involves the combination of one of the following components: the use of resistant varieties, alternative host eradication or sanitation, crop rotation, biological controls, and fungicide applications. The application of chemical fungicides as a component in the IPM has been proven effective to reduce infection rate, however, less environmental friendly. Natural fungicides extracted from plants could be considered to minimize intensive applications of the chemical fungicides. The extracts have been used as antimicrobial agents and anti insects because of the secondary metabolite contents, including flavonoids, alkaloids, terpenoids, saponins, and tannins (Harborne, 1998; Kamboj & Saluuja, 2008; Patil *et al*., 2009).

A. conyzoides has been known as an invasive weed because of its fast growth and disturbing the growth of crops. The weed released allelochemical compounds which suppressed the crop growth (Kong, 2006). Since ancient era, the weed has been used to cure several human diseases such as skin diseases, skin wound, diseases caused by bacterial infections, and headache (Kamboj & Saluuja, 2008). Extract of this weed also suppressed the growth of *Candida albicans*, *Cryptococcus neoformans*, *Sclerotium rolfsii* and *Trichophyton mentagrophytes* (Okunande, 2002).

Applications of this weed extract to control plant pathogens were still limited. Kong (2006) reported that essential oil of Ageratum which contained ageratochromones, monoterpepenoids, and sesquiterpenoids inhibited *Rhizoctonia solani*, *Botryris cinerea*, and *S. rolfsii*. However, studies on the application of Ageratum extract to control peanut rust disease have not been reported yet. Therefore, this study aimed to determine effective concentrations of the root, leaf, and flower extracts of Ageratum to suppress the growth of rust spores *in vitro* and to screen the secondary metabolites.

MATERIALS AND METHODS

Sample preparation and extraction. Ageratum weeds were collected from around Kendalpayak Research Station, Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Malang East Java. Roots, leaves, and flowers were separated, air dried, and ground to obtain fine particles. Maceration in methanol and *n*-hexane (1:10 w/v) was conducted separately for 18 h after shaking the suspension using an orbital shaker for 4 h at 100 rpm. Supernatant was collected after filtration and extracts containing the solvents were evaporated using a vacuum rotary evaporator. The crude extracts were stored at 4°C in the dark before used (Agbafor and Nwachukwu, 2011; Yusnawan, 2013).

Spore germination test. Treatments were arranged in a completely randomized design with three factors and three replicates. The first factor was polarity of solvents used to extract samples (methanol as a polar solvent and *n*-hexane as a non polar solvent). The second factor was three parts of Ageratum (roots, leaves, and flowers), and the third factor was four concentration levels (0.1%, 1%, 2.5%, and 5%). Spores of *P. arachidis* were collected from 10-week infected crops cultivated in an ILETRI greenhouse. The infected leaves were harvested and incubated in petridishes for two days. The humidity was maintained at around 95% by placing wet cotton layers inside the petridishes. Mature spores were harvested and suspended in sterile water. Spore germination was tested in the polar and non polar crude extracts with four concentration levels. Phosphate buffer was used as the control. The numbers of germinate and ungerminate spores were recorded after 24 h of incubation (Yusnawan, 2013).

Phytochemical screening. The polar and non polar crude extracts were screened to determine the presence of alkaloids, flavonoids, tannins, saponins, and terpenoids according to a method developed by Trease & Evans (1983). Briefly, alkaloids were detected using Mayer dan Wagner reagents. Sedimentation or precipitation on the bottom of the extracts showed positive results. Flavonoids were determined using Mg and HCl. Red colour indicated the presence of flavonoids. Tannins were detected using $FeCl_3$ and gelatin. Change in colour from light green to dark green or dark blue indicated the presence of tannins after being reacted with $FeCl_3$ or white sedimentation after being added gelatin. Saponins were detected by the ability of the extracts to form foam for at least 30 s after shaking. Terpenoids were detected using Liebermann-Burchard reagent and H_2SO_4, and the presence of terpenoids was shown by reddish brown rings after being reacted with concentrated H_2SO_4 (Harborne, 1998; Trease and Evans, 1983).

Separation of active compounds using thin layer chromatography (TLC). Separations of active compounds were performed on F_{254} silica gel plates. Crude extracts (5 µl) were spotted on the plates and separated using suitable mobile phases. Methanol:chloroform (0.5:9.5 v/v) was used to separate alkaloids (Wagner & Bladt, 1996), chloroform:methanol (9:1 v/v) to separate flavonoids (Harborne, 1998), *n*-hexane:acetone (4:1 v/v) to separate saponins (Marliana *et al*., 2005), and *n*-hexane:ethyl acetate (2:8 v/v) to separate terpenoids (Wagner & Bladt, 1996).

RESULTS AND DISCUSSION

Spore germination test. The polar and non polar extracts of the roots, leaves, and flowers with four concentration levels were tested to determine the effectiveness of the extracts against spore germination of peanut rust. At the same concentration of the polar and non polar extracts, especially 1.0% and above, the polar extracts inhibited more germinate spores compared to those of the non polar extracts, for example 1.0% of the polar extract of the roots inhibited 77% of germinate spores, whereas the counterpart only inhibited 64% of germinate spores (Figure 1). The effective concentrations to inhibit spore germination were 5% of the polar fraction of the roots (95±2%) and 5% of the polar fraction of the leaves (98±2%). The numbers of germinate spores of these two extracts were only 5±2% and 2±2% for the root and leaf extracts. Interestingly, only the non polar fraction of the flowers resulted more ungerminate spores (64±4%) than the polar fraction

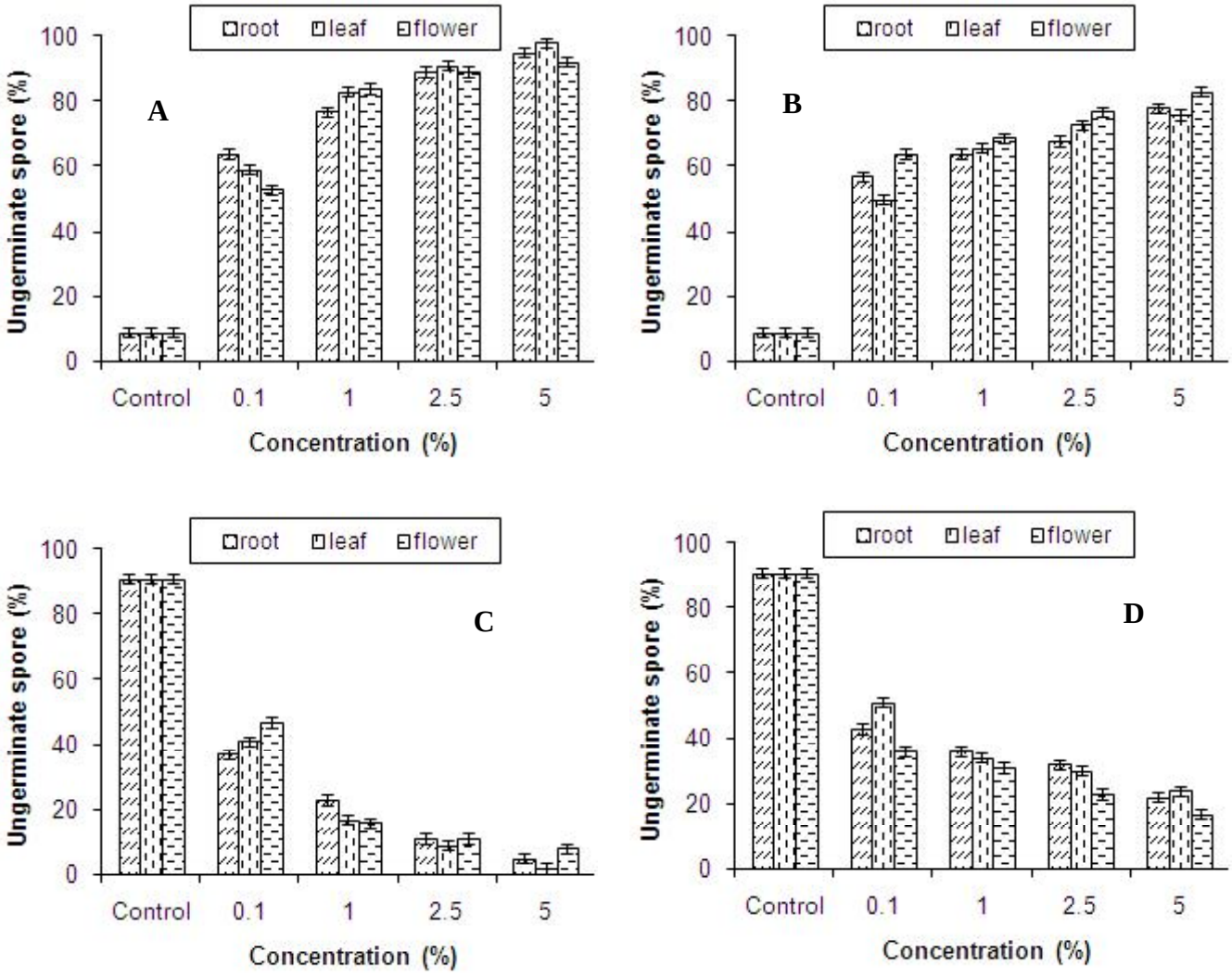

Figure 1. Percentages of ungerminate spores of *P. arachidis* treated with the polar fractions (A), ungerminate spores treated with the non polar fractions (B), and germinate spores treated with the polar fractions (C), germinate spores treated with the non polar fractions of *A.conyzoides* (D). Bars show deviation standards.

(53±2%), which was not observed in the non polar fractions of the roots and leaves at all concentration levels. Microscopic examination of ungerminate spores showed that the spores were either shrinking as indicated by the reduction of the spore size or lyses as indicated by the breakdown of the spore wall.

Crude extracts from plants extracted with polar solvents including methanol and other alcohol solvents showed more effective to inhibit fungal growth (Cimanga *et al*., 2004; Sharma *et al*., 2007). According to Sharma *et al*. (2007), the effectiveness of the methanol fraction extracted from *Millingtonia hortensis* as antifungal compounds showed stronger activities than those of less polar solvents, chloroform and ethyl acetate. In another study, the use of 80% methanol to extract *Mitracarpus scaber* leaves showed stronger inhibition to fungal growth than the use of *n*-hexane (Cimanga *et al*., 2004). In the case of the non polar fraction of Ageratum flowers inhibiting more germinate spore than the polar fraction, it may be related to the presence of essential oil in the flowers. According to Patil *et al*. (2009), essential oil of Ageratum which had reddish-orange in colour and a powerful odor constituted up to 0.18% (v/w) of the macerated tissue. In addition, three main compounds of this essential oil, i.e. caryophyllene, precocene I (dimethoxi ageratocromene) and precocene II were effective to inhibit the growth of toxigenic strains of *Aspergillus flavus* and *A. parasiticus* (Nogueira *et al*., 2010; Patil *et al*., 2009).

The use of different solvents for extraction influenced compositions and groups of chemical compounds (Dehkharghanian *et al*., 2010). Polar solvents including alcohol groups were commonly used to extract organic compounds from natural products including plant secondary metabolites. The polar solvents were able to increase cell permeability and to penetrate inside the cells, therefore, extracting more endocellular secondary metabolites, both polar and less polar compounds compared to the use of non polar solvents, such as *n*-hexane (Cannell, 1998; Seidel, 2012). Non polar solvents only dissolved non polar compounds. The secondary metabolites of *A. conyzoides* extracted from the polar solvent may increase the effectiveness to inhibit *P. arachidis* spores as observed in this study.

Phytochemical screening. This study aimed to determine active compounds from the polar and non polar extracts of the roots, leaves, and flowers. Changes in colour of the extracts or sedimentation forms after being reacted with the suitable reagents were recorded to determine the presence of the active compound tested. The use of polar solvent for maceration resulted more diverse in the secondary metabolite groups as observed in Table 1. At least, five and two active compound groups were detected in the polar and the non polar extracts, respectively. The polar fraction of the leaves showed the most diverse in secondary metabolites compared to those of the other fractions. This fraction contained alkaloids, flavonoids, tannins, saponins, and terpenoids. The diverse compositions of these secondary metabolites may be responsible to inhibit spore germination of peanut rust. The polar fraction of the roots which had the same effectiveness as the polar fraction of the leaves to inhibit spore germination contained alkaloids, flavonoids, and terpenoids.

According to Seidel (2012), high polarity index of methanol was able to extract the secondary metabolites which had polar properties such as flavonoid glycosides, tannins, and several alkaloids. Also, this solvent was effective to extract phenolic compounds with low molecular weights and medium levels of polarity (Yu Lin *et al*., 2009), aglycons of flavonoids (Dehkharghanian *et al*., 2010), anthocyanins, terpenoids,

Table 1. Phytochemical screenings of the polar and non polar extracts of the roots, leaves, and flowers of *A. conyzoides*

Active compound	Polar fraction			Non polar fraction		
	Root	Leaf	Flower	Root	Leaf	Flower
Alkaloid	++	++	+	-	++	+
Flavonoid	++	++	++	-	-	-
Tannin	-	+	-	-	-	-
Saponin	-	+	-	++	-	-
Terpenoid	++	++	++	+	++	++
Steroid	-	-	-	-	-	-

++ showed more compound/dark colour, + showed less compound/light colour, - showed no compound detected.

saponins, flavons, and polyphenolic compounds (Cowan, 1999). Non polar solvents such as *n*-hexane which had zero of polarity index only dissolved liphophilic compounds, including alcanas, waxes, colour pigments, sterols, several terpenoids and alkaloids, therefore, extracted less secondary metabolites.

Separation of secondary metabolites using TLC. Extracts which had the most effective inhibition to *P. arachidis* spores, i.e. the polar extracts of the leaves and roots were separated using TLC techniques. This study was conducted to confirm the presence of the active compounds as a further step after phytochemical screenings. Also, this step aimed to determine the number of active compounds of the secondary metabolites which were visualized as spots on the TLC plates.

In general, the numbers of spots found in the polar fraction of the leaves (Table 2) were more vary than those spots in the polar fraction of the roots (Table 3). However, more total numbers of the spots did not reflect more effective to inhibit the germinate spores and vice versa, as observed in this study when the effectiveness of the polar fractions of the leaves and the roots were compared. The concentration of each secondary metabolite group may contribute to the effectiveness of the extract rather than the spot numbers.

Table 2. Number of spots and secondary metabolite characteristics of the polar fraction of the leaves after separation using thin layer chromatography

Secondary metabolite	Number of spot	Retardation factor/ Rf value (cm)	Spot colour	
			Visible light	UV light
Alkaloid	7	0.13	Green	Brown
		0.21	Green	Brown
		0.31	No colour	Purple
		0.37	Yellow	Brown
		0.53	Dark yellow	Light purple
		0.68	Yellow	Dark purple
		0.81	Dark yellow	Light brown
Flavonoid	9	0.10	Light red	Brown
		0.18	Light brown	Dark purple
		0.31	Dark green	Brown
		0.33	Light green	Brown
		0.47	Dark green	Dark yellow
		0.60	Yellow	Dark yellow
		0.66	No colour	Dark purple
		0.72	Green	Yellow
		0.83	Light green	Dark blue
Saponin	6	0.06	Green	Purple
		0.12	Yellow	Yellow
		0.21	Light green	Purple
		0.25	Light green	Light yellow
		0.37	No colour	Purple
		0.55	No colour	Light purple
Terpenoid	8	0.23	Dark yellow	Purple
		0.30	Green	Brown
		0.33	Dark green	Brown
		0.46	Green	Brown
		0.61	Yellow	Purple
		0.71	No colour	Dark purple
		0.85	No colour	Purple
		0.88	No colour	Dark blue

Table 3. Number of spots and secondary metabolite characteristics of the polar fraction of the roots after separation using thin layer chromatography

Secondary metabolite	Number of spot	Retardation factor/ Rf value (cm)	Spot colour	
			Visible light	UV light
Alkaloid	7	0.08	Green	Purple
		0.21	No colour	Purple
		0.25	No colour	Brown
		0.36	No colour	Brown
		0.61	Yellow	Brown
		0.70	No colour	Purple
		0.82	No colour	Light red
Flavonoid	3	0.66	No colour	Brown
		0.79	No colour	Purple
		0.83	No colour	Brown
Saponin	-	-	-	-
Terpenoid	4	0.42	No colour	Purple
		0.48	No colour	Purple
		0.77	No colour	Purple
		0.87	Yellow	Dark red

One of the main functions of plant secondary metabolites is for plant defense against pathogen infections (Vickery & Vickery, 1981). With regards to the plant defense, alkaloids which were separated into 7 spots both in leaf and root extracts function to disturb components of peptidoglycans on the cells, therefore, the cell layers did not develop completely, resulting the death of the cells (Robinson, 1995). Alkaloids also inhibited cell respiration; inhibit esterase and DNA as well as RNA polymerases (Aniszewki, 2007). Flavonoids which were separated more spots in the leaf extract (9 spots) than in the root extract (3 spots) acted to form complex compounds on the extracellular protein which disturb cell membrane integrity and as protein coagulators. These active compounds also function as molecule signals and to protect plants against pathogen infections (Boue *et al*., 2009; Samantha *et al*., 2011).

In plants, saponins are carbohydrate deposits or waste products of plant metabolisms. Unfortunately, saponins were not detected in the root extract. Saponins are effective compounds to combat pathogens and pest insects since these chemicals have bitter properties. In addition, these compounds act as haemolysis agents and have ability to form complex compounds with cholesterols and other sisteroids (Robinson, 1995). Terpenoids are one of the phytoalexins, i.e. antimicrobial compounds produced by plants which are synthesized in higher amounts when the plants are infected by pathogens (Harborne, 1998; Cowan, 1999). According to Cowan (1999) and Das *et al*. (2010), tannins may disturb protein metabolism by forming hydrogen bonds, hydrophobic interactions, as well as covalent bonds. As a result, pathogens are unable to continue their growth normally. The compositions of alkaloids, flavonoids, tannins, saponins, and terpenoids in *A. conyzoides* may play an important role in inhibiting spore germination of *P. arachidis*, especially when polar fractions of roots and leaves at concentration of 5% were applied.

CONCLUSIONS

The application of 5% polar fractions of leaves and roots of *A. conyzoides* showed the highest result to inhibit spore germinations (98±2% and 95±2%, respectively), which may be potential to be utilized as botanical fungicides to suppress the infection rate of *P. arachidis* infected crops. Alkaloids, flavonoids, tannins, saponins, and terpenoids in the leaves which may be responsible to inhibit the spore germinations can be considered to be purified to determine the effectiveness of each purified compound.

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