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### Antifungal Activity of Crude Extracts of *Ageratum conyzoides*, *Cyperus rotundus*, and *Amaranthus spinosus* Against Rust Disease

Eriyanto Yusnawan\*) and Alfi Inayati

Indonesian Legumes and Tuber Crops Research Institute, Indonesian Agency for Agricultural Research and Development, Indonesia

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\*) Corresponding author:

E-mail: eyusnawan@litbang.pertanian.go.id

#### ABSTRACT

*Puccinia arachidis* is an obligate pathogen which infects peanut leaves and causes rust disease. Alternative controls of this disease, particularly to limit the frequent use of synthetic fungicide, have been conducted. One of which is by applying botanical fungicides. Crude extracts of *Ageratum conyzoides*, *Amaranthus spinosus*, and *Cyperus rotundus* were used to suppress the rust disease intensity on Bima peanut cultivar. *A. conyzoides* extracts at 2.5% and 5.0% concentrations were the most effective biofungicide to reduce the disease. The disease intensity (29.8 % and 30.2 %) recorded at 10 weeks after planting (WAP) was significantly lower than the untreated crops (41.4 %). Both weed extract applications reduced about 50 % of pustule number compared to untreated crops at 10 WAP. Applications of 2.5 % and 5.0 % of *ageratum* extracts saved yield loss of 67.5 % and 63.5 %. Caryophyllene was observed in the roots, stems, leaves, and flowers of *ageratum* extracts in considerable amounts based on GC-MS analysis and may attribute to its significant antifungal activity. Higher total phenolic and flavonoid contents were observed in *ageratum* extracts than in *amaranthus* and *cyperus* extracts. *Ageratum* extracts at concentrations of 2.5 % to 5 % could be used to control peanut rust disease.

#### INTRODUCTION

*Puccinia arachidis* Speg., an obligate peanut pathogen which causes rust disease, is considered as an economical peanut disease since this pathogen causes yield loss up to 57 % under favorable environment (Subrahmanyam & McDonald, 1987). The pathogen is distributed worldwide wherever peanut is cultivated. However, the variation of incidence and severity occur between locations and seasons (Mondal & Badigannavar, 2015). Initial symptom appears in the field particularly under warm and humid conditions and spread rapidly using uredospores. The orange color of pustules appears on the lower surface of peanut leaves. Then, reddish-brown uredospores are released from the mature pustules and are ready to spread across peanut cultivation area (Mondal & Badigannavar, 2015; Subrahmanyam & McDonald, 1987).

Without chemical controls, severe peanut

rust disease devastates the crops. Considering the increase of food security issue and environmental-unfriendly control, the frequent applications of chemical fungicides need to be taken into account. An alternative strategy to reduce frequent use of synthetic fungicides has been carried out, one of the strategies is utilizing natural plant extracts as botanical fungicides. Plants are rich in secondary metabolites and biologically active compounds. The secondary metabolites are not as crucial as primary metabolites for sustaining their life, yet, it plays an important role for plant survival in their environment such as abiotic and biotic stresses as well as to defend injured plants (Ghasemzadeh & Ghasemzadeh, 2011; Hadacek, 2002; Hussain et al., 2012). Plant secondary metabolites, including phenolic compounds, flavonoids, alkaloids, and terpenoids also have properties to repel or attract insects or microorganisms, and to maintain plant

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structural integrity (Paiva *et al.*, 2010).

Biologically active compounds in plant extracts have been used to inhibit the growth of microorganisms, one of which is to inhibit phytopathogenic microorganisms caused by fungi. Several soil borne and phyllosphere pathogenic fungi can be inhibited or killed by plant extracts. For instance, mycelial growth of *Macrophomina phaseolina*, *Fusarium equiseti*, *Botryodiplodia theobromae*, *Colletotrichum corchori*, *Alternaria alternate*, and *Curvularia lunata* can be 100 % inhibited by ethanolic rhizome extract of *Acorus calamus* at concentration of 1 mg mL<sup>-1</sup> (Begum, Yusuf, Chowdhury, Khan, & Anwar, 2007). Chemical compounds extracted from *Ageratum conyzoides* L., *Amaranthus spinosus* L., and *Cyperus rotundus* L. have been utilized to suppress the growth of some pathogens both bacteria and fungi (Bisht, Bisht, Singh, Gupta, & Singh, 2011; Dayie, Newman, Ayitey-Smith, & Tayman, 2007; Kamboj & Saluja, 2008; Kong *et al.*, 2004; Maiyo, Ngure, Matasyoh, & Chepkorir, 2010; Mushatq *et al.*, 2012; Singh, Maurya, Singh, & Singh, 2011). In nature, these three plant species grow rapidly and are known as weeds due to their invasive growth.

The previous study showed that those three weeds inhibited spore germination and the growth of peanut rust disease both *in vitro* and under greenhouse condition. The spore germination of the rust pathogen was inhibited by applications of 5 % *ageratum* and *cyperus* crude extracts by 78 % to 80 %, and 76 % to 80 % respectively. Phytochemical screening and thin layer chromatography of these weeds have also been conducted to investigate groups of active compounds. However, only a peanut cultivar (Kancil) was used to investigate the effectiveness of the crude extracts (Yusnawan & Inayati, 2016). The use of other cultivars may confirm the effectiveness of the extracts since genotypes also play an important role to inhibit the growth of this obligate pathogen besides human interference to control the pathogen, pathogenicity of the rust and environmental condition.

Furthermore, weekly monitoring of disease intensity to investigate the infection rate of the rust after crude extract application has not been reported. In addition, identification of individual compound in the methanolic extracts of these weeds originated from Indonesia using gas chromatography-mass spectrometry (GC-MS) has not been conducted yet. Also, quantification of the secondary metabolites

specifically total flavonoid and phenolic contents in these weeds have not been performed yet. This further study, therefore, aimed to investigate the ability of the crude extracts to reduce disease intensity on Bima cultivar by weekly monitoring, and to investigate individual compound in the methanolic extracts using GC-MS. The quantification of the secondary metabolites particularly total flavonoid and phenolic contents in each part of the weed extracts was also conducted.

## MATERIALS AND METHODS

### Sample Preparation

Preparation and extraction of *ageratum*, *cyperus* and *amaranthus* were conducted in laboratories as described by Yusnawan & Inayati (2016). The laboratory studies were conducted in mycology and central laboratories of Indonesian Legumes and Tuber Crops Research Institute. For a green house experiment, the whole parts of the three weeds were ground separately. Antifungal activity was carried out in the greenhouse of this institute in 2016.

### Sample Extraction

The fine weeds were extracted with methanol (1:10 w v<sup>-1</sup>) for 18 hours after 4 hours of shaking the extract. Centrifugation was conducted to separate the supernatant from the pellet. Excess amount of the solvent was evaporated using a vacuum rotary evaporator. A constant temperature at 50 °C was set during this process. The concentrated extract was transferred in amber bottles and stored at 4 °C prior to be further used (Joko *et al.*, 2018; Yusnawan & Inayati, 2016).

### Greenhouse Study

The extracts of three weeds were used to spray infected peanut of Bima cultivar as conducted by Yusnawan & Inayati (2016). A randomized completely block design with two factors was performed during this experiment. Each treatment was repeated three times. Extracts of *ageratum*, *cyperus*, and *amaranthus* were used as the first factor whereas concentrations of 0.1, 1.0, 2.5, and 5.0 % were as the second factor. Spraying with water only and difenoconazole fungicide according to the recommended dosage as negative and positive controls were conducted. The parameters were disease intensity, number of pustule per cm<sup>2</sup>, and pustule accumulation per cm<sup>2</sup>. These parameters were recorded at seven to nine WAP. Disease

intensity was recorded based on the scoring according to Subrahmanyam *et al.* (1995). Pod yield specifically intact, empty, and immature pods were observed at harvesting.

#### Compounds Identification Using GC-MS Analysis

Methanolic crude extract of flowers, leaves, and roots were qualitatively identified using GC-MS according to Karimi & Jaafar (2011) with modifications as follows: as much as 1  $\mu$ L of samples without derivatization was injected into the GC-MS system (Thermo Scientific ISQ LT equipped with TriPlus RSH autosampler) with split less mode. A TG-5MS capillary column (30 m x 0.25 mm x 0.25  $\mu$ m, Thermo Scientific) was installed to the GC and column temperature was set at 50  $^{\circ}$ C for 2 minutes, increased at 5  $^{\circ}$ C per minute and reaching up to 150  $^{\circ}$ C and hold for 10 minutes, then another increased at 10  $^{\circ}$ C per minute and reaching up to 200  $^{\circ}$ C and hold for 5 minutes, finally increased at 15  $^{\circ}$ C per minute to 320  $^{\circ}$ C and hold for 5 minutes. Carrier gas of hydrogen generated by a hydrogen generator (Thermo Scientific) at a flow rate of 1 mL per minute was set during the compound separation. The injector and transferline were set at 200  $^{\circ}$ C and 320  $^{\circ}$ C respectively. Mass spectra obtained from the respective extracts were matched with mass spectra library of National Institute of Standard and Technology (NIST version 2.2) and Wiley 10<sup>th</sup> edition for compound identification. The identified compounds listed were only the compounds which exceeded 5 % level.

#### Total Flavonoid Content Determination

Flour of three weeds was extracted in 80 % methanol (1:10 w v<sup>-1</sup>) overnight. The supernatant was transferred into a tube after centrifugation. Total flavonoid in the supernatant was measured and expressed as catechin equivalents per gram of sample (mg CE per g sample) (Heimler, Vignolini, Dini, & Romani, 2005; Xu & Chang, 2007).

#### Total Phenolic Content Determination

The supernatant obtained from the extraction of three weeds in flavonoid content preparation was used to determine the total phenolic content. The supernatant was reacted with Folin Ciocalteu's reagent (Singleton, Orthofer, & Lamuela-Raventós, 1999; Xu & Chang, 2007). Gallic acid equivalent was used to express the total phenolic content in the weed extract.

## RESULTS AND DISCUSSION

### Disease Intensity

Application of the three crude extracts at a concentration of 5 % reduced disease intensity compared to the control (Fig. 1), even though less effective than those of fungicide treatment. Weekly monitoring of the rust disease showed that after 7 WAP, the disease intensity on Bima cultivar rose at 10 WAP, even though the increase was not at the same rate as in each weed extract concentration. The rate of disease intensity on crops treated with fungicide decreased as expected. Disease intensity on crops treated with 5 % of *ageratum* extract increased slowly starting from 8 to 10 WAP. The disease intensity rose from 26.6 % to 30.2 %, whereas those disease intensity of control increased from 30.2 % to 41.4 %.

The fungicide treatment, however, reduced the disease intensity from 16.4 % to 14.0 % at 8 to 10 WAP. Unlike application of the *ageratum* extract, the disease intensity of crops sprayed with *cyperus* and *amaranthus* extracts increased from 7 to 9 WAP and reduced at 10 WAP. Surprisingly, application of 2.5 % *ageratum* extract was effective in suppressing the disease intensity as that of 5 % concentration at 10 WAP. At the end of observation (10 WAP), the disease intensity of 2.5 % application was 29.8 %, while 5 % concentration was 30.2 %. This result suggested that 2.5 % *ageratum* extract could be used to reduce the growth of rust disease as effective as the application of 5 % concentration.

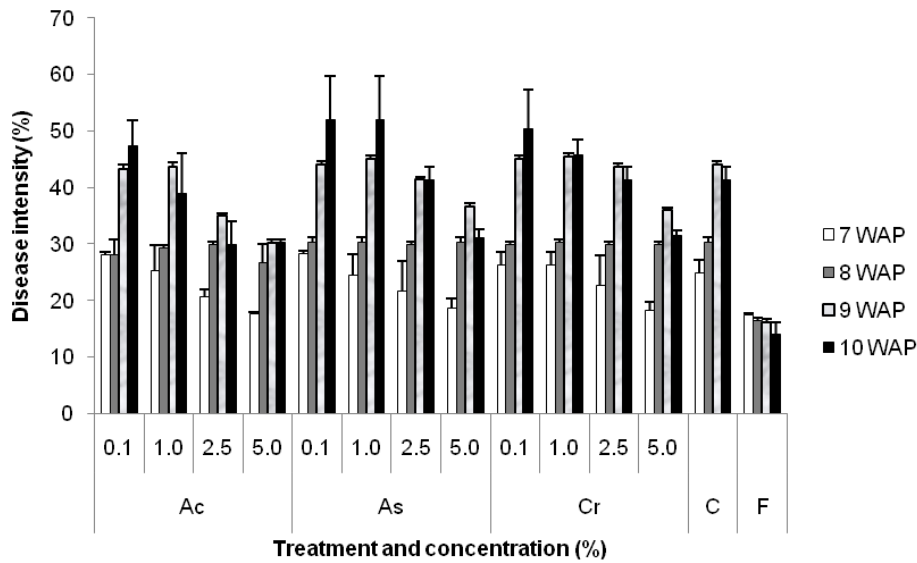
Disease intensity on Kancil cultivar was also increased at 8 and 9 WAP, however, the disease rate was unable to be monitored since the observations were only recorded at 8 and 9 WAP or at the third and fourth weeks after weed extract applications (Yusnawan & Inayati, 2016). After application of 5 % *ageratum* extract, both Kancil and Bima cultivars had similar disease intensity. On Kancil cultivar, the disease intensity was recorded at 25 % to 30 % at 8 and 9 WAP (Yusnawan & Inayati, 2016). While on Bima cultivar, the disease intensity was around 27 % to 30 % at the same time interval of observation (Fig. 1). This finding showed that cultivars did not affect the rust disease intensity after the crude extract applications.

### Pustule Number and Pustule Accumulation

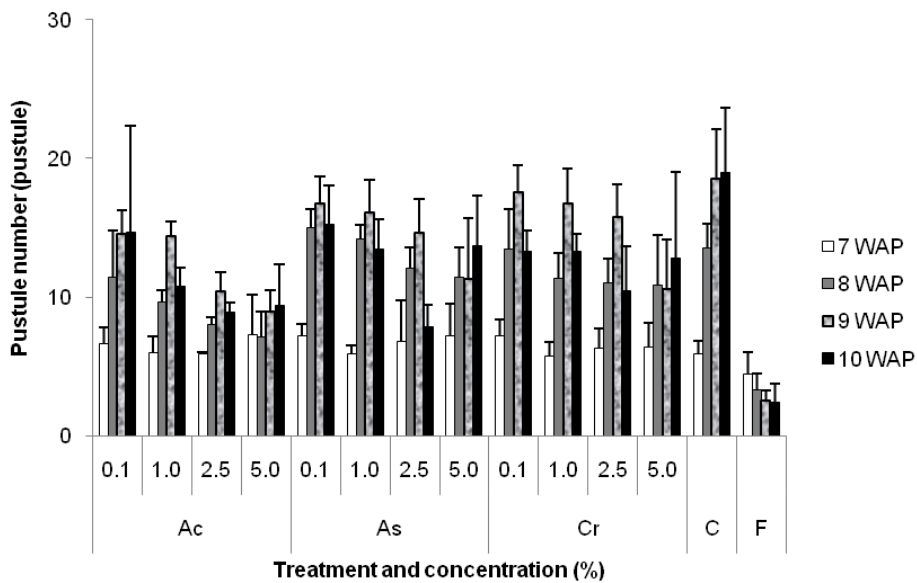
A number of rust pustule observed in all application treatments showed variations (Fig. 2). In general, application of the weed extracts in lower

concentration was unable to reduce the pustule number. In line with the disease intensity reduction, the applications of 2.5 % and 5.0 % of *ageratum* extracts reduced the number of pustules. Both weed extract applications reduced about 50 % of pustule number compared to untreated crops at 10 WAP. However, the pustule number was about 4 folds higher than that of the fungicide application.

The other two weed extracts were less effective than *ageratum* extracts at 2.5 % and 5.0 % concentration. At the highest concentration, *amaranthus* and *cyperus* extracts were only reduced 28 % and 33 % of pustule number compared to the control at 10 WAP. The fungicide application was the most effective treatment in reducing the pustule number. This application reduced up to 87 % of pustule compared to the untreated crops.



**Fig. 1.** Disease intensity on Bima cultivar treated with crude extracts of *A. conyzoides* (Ac), *C. rotundus* (Cr), *A. spinosus* (As), difenoconazole fungicide (F), and water (C) at seven to ten weeks after planting (WAP). Bars represent standard errors



**Fig. 2.** A number of rust pustules per cm² on Bima cultivar after weed extract applications (*A. conyzoides* (Ac), *C. rotundus* (Cr), *A. spinosus* (As)) compared to fungicide (F) and untreated crops (C). Bars represent standard errors

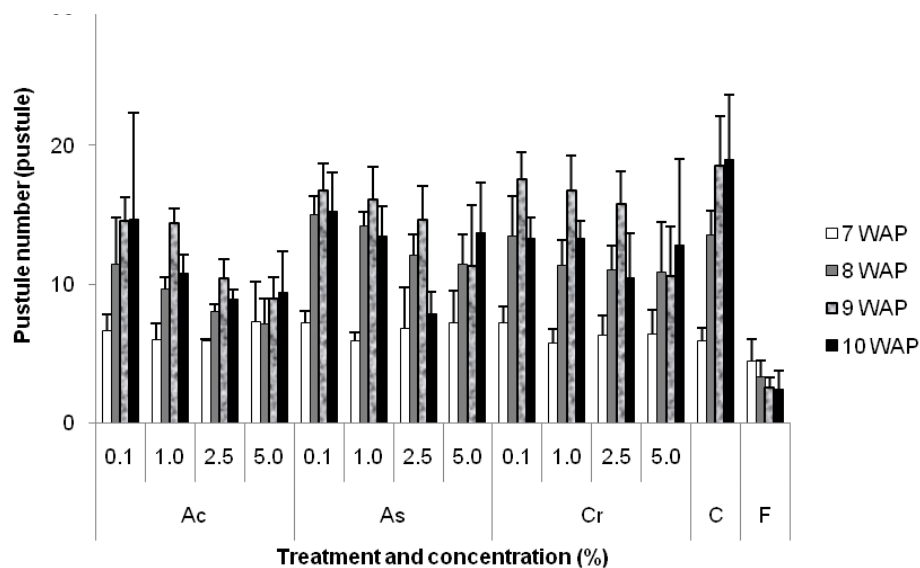
Accumulation of pustules observed from all weed treatments, fungicide application, and untreated crops increased from 7 to 10 WAP (Fig. 3). Among the three weed extracts, applications of *ageratum* extracts showed the least number of pustule accumulation at 10 WAP. Again, applications of 2.5 % and 5 % of *ageratum* extracts were the most effective treatments in reducing the rust pustules. Pustule accumulation reduction of 43 % was recorded in those weed extract treatments. Other weed extract applications at the highest concentration only reduced 25 % and 30 % for *amaranthus* and *cyperus* extracts, respectively. The highest reduction up to 76 % was observed only in the fungicide application as expected.

Pustules containing uredospores are potential inoculums in the field. These uredospores are airborne (Mondal & Badigannavar, 2015; Subrahmanyam & McDonald, 1987). Premature defoliation as observed during this study occurs on severe infected crops. The infected crop debris contaminated with uredospores is potential inoculums in the peanut cultivation area (Subrahmanyam & McDonald, 1987). Continuous warm temperature (> 22 °C) with high humidity (> 78 %) during wet weather is favorable for rust epidemic (Mondal & Badigannavar, 2015). The reduction of pustule number after weed extract applications also reduced the potential inoculums to infect the healthy crops (Yusnawan & Inayati, 2016).

### Pod Yield

The significant difference of pod distribution from the crops treated with four concentration levels of the three weed extracts was observed (Table 1). Among the three weed extracts, total wet pods obtained from the application of 2.5 % and 5.0 % of *ageratum* extracts were 28.9 and 27.2 g per plant. The similar result was found in the intact pods which were also not different. At the same concentration levels, the application of *amaranthus* and *cyperus* extracts only yielded total pods of 17.0 and 13.3 g per plant as well as 19.4 and 20.5 g per plant for 2.5 and 5.0 % respectively. Higher disease intensity and a number of pustules on the peanut crops treated with these two crude extracts caused early defoliation, then disturbing pod filling and reducing pod weight. This result was in line with the study conducted by Yusnawan & Inayati (2016).

Surprisingly, as many as 20.7 g per plant of total pods was harvested from the untreated crops. Total wet pods from the fungicide application were the highest. The application of fungicide was able to suppress both disease intensity and a number of pustules; therefore peanut growth was not much affected. As a result, the crop produced the highest yield. Similar to the wet weight of pods, dry weight of pods harvested from the weed extract applications varied significantly (Table 2), and the trend was quite similar. Applications of 2.5 % and 5.0 % of *ageratum* extract saved yield loss of 67.5 and 63.5 % respectively.



**Fig. 3.** Accumulation of pustule number per cm<sup>2</sup> on Bima cultivar after weed extract applications (*A. conyzoides* (Ac), *C. rotundus* (Cr), *A. spinosus* (As)) compared to fungicide (F) and untreated crops (C). Bars represent standard errors



**Table 1.** The wet weight of intact, empty, immature and total pods of Bima peanut cultivar treated with three botanical extracts

Concentration (%)	Extract	Distribution of wet weight of pod (g) per plant			
		Intact	Empty	Immature	Total
0.1	Ac	17.58±3.39 <sup>d-f</sup>	0.21±0.27 <sup>c</sup>	0.73±0.11 <sup>cd</sup>	18.53±3.17 <sup>d-g</sup>
1.0	Ac	26.03±7.96 <sup>bc</sup>	0.64±0.72 <sup>bc</sup>	0.85±0.37 <sup>cd</sup>	27.52±7.91 <sup>bc</sup>
2.5	Ac	27.33±7.00 <sup>b</sup>	0.59±0.73 <sup>bc</sup>	0.98±0.14 <sup>b-d</sup>	28.90±7.19 <sup>b</sup>
5.0	Ac	24.18±3.14 <sup>b-d</sup>	0.26±0.25 <sup>c</sup>	2.72±2.14 <sup>a</sup>	27.16±4.26 <sup>bc</sup>
0.1	As	15.87±2.15 <sup>d-f</sup>	0.40±0.70 <sup>c</sup>	1.26±0.41 <sup>b-d</sup>	17.53±2.36 <sup>d-g</sup>
1.0	As	14.81±5.57 <sup>ef</sup>	0.89±0.22 <sup>bc</sup>	1.69±0.57 <sup>bc</sup>	17.39±5.96 <sup>d-g</sup>
2.5	As	15.18±3.60 <sup>ef</sup>	0.61±0.77 <sup>bc</sup>	1.24±0.76 <sup>b-d</sup>	17.03±3.41 <sup>e-g</sup>
5.0	As	12.04±6.23 <sup>f</sup>	0.70±0.31 <sup>bc</sup>	0.58±0.45 <sup>d</sup>	13.32±6.81 <sup>fg</sup>
0.1	Cr	15.11±0.55 <sup>ef</sup>	1.93±2.15 <sup>a</sup>	1.18±0.37 <sup>b-d</sup>	18.22±1.92 <sup>d-g</sup>
1.0	Cr	10.93±7.31 <sup>f</sup>	0.56±0.55 <sup>c</sup>	0.86±0.74 <sup>cd</sup>	12.35±7.93 <sup>g</sup>
2.5	Cr	17.22±2.72 <sup>d-f</sup>	0.51±0.50 <sup>c</sup>	1.69±0.50 <sup>bc</sup>	19.42±2.65 <sup>c-g</sup>
5.0	Cr	18.33±8.61 <sup>c-f</sup>	0.23±0.40 <sup>c</sup>	1.95±0.53 <sup>ab</sup>	20.51±8.83 <sup>c-g</sup>
	Wt	18.89±1.71 <sup>c-f</sup>	0.53±0.59 <sup>c</sup>	1.31±0.09 <sup>b-d</sup>	20.73±2.10 <sup>b-f</sup>
	Fs	41.10±2.02 <sup>a</sup>	0.97±0.42 <sup>a-c</sup>	0.71±0.38 <sup>cd</sup>	42.78±1.76 <sup>a</sup>

Remarks: Ac = *A. conyzoides*, As = *A. spinosus*, Cr = *C. rotundus*, Wt = water, Fs = fungicide. Numbers followed by the same letter in the same columns were not significantly different based on the LSD ( $\alpha = 5\%$ )

**Table 2.** The dry weight of intact, empty, immature and total pods of Bima peanut cultivar treated with three botanical extracts

Concentration (%)	Extract	Distribution of dry weight of pod (g) per plant			
		Intact	Empty	Immature	Total
0.1	Ac	11.27±1.72 <sup>c-f</sup>	0.04±0.05 <sup>de</sup>	0.08±0.04 <sup>f</sup>	11.39±1.68 <sup>d-h</sup>
1.0	Ac	15.69±5.50 <sup>bc</sup>	0.08±0.05 <sup>de</sup>	0.14±0.12 <sup>ef</sup>	15.91±5.56 <sup>b-e</sup>
2.5	Ac	18.41±4.29 <sup>b</sup>	0.32±0.49 <sup>c-e</sup>	0.38±0.29 <sup>c-f</sup>	19.12±4.88 <sup>b</sup>
5.0	Ac	15.68±3.78 <sup>bc</sup>	0.41±0.71 <sup>b-d</sup>	0.67±0.70 <sup>a-f</sup>	16.63±3.63 <sup>b-d</sup>
0.1	As	8.67±2.05 <sup>e-g</sup>	0.05±0.09 <sup>de</sup>	0.36±0.49 <sup>c-f</sup>	9.08±2.43 <sup>gh</sup>
1.0	As	8.52±3.31 <sup>e-g</sup>	0.31±0.26 <sup>c-e</sup>	0.53±0.60 <sup>b-f</sup>	9.36±3.99 <sup>fh</sup>
2.5	As	8.93±1.36 <sup>e-g</sup>	0.27±0.25 <sup>c-e</sup>	0.46±0.41 <sup>b-f</sup>	9.66±1.67 <sup>fh</sup>
5.0	As	7.84±1.62 <sup>fg</sup>	0.29±0.31 <sup>c-e</sup>	0.12±0.10 <sup>ef</sup>	8.25±1.32 <sup>gh</sup>
0.1	Cr	8.10±0.99 <sup>e-g</sup>	0.12±0.18 <sup>de</sup>	0.28±0.21 <sup>d-f</sup>	8.50±0.96 <sup>gh</sup>
1.0	Cr	5.54±2.46 <sup>g</sup>	0.22±0.19 <sup>c-e</sup>	0.10±0.10 <sup>f</sup>	5.86±2.56 <sup>h</sup>
2.5	Cr	10.30±2.53 <sup>d-g</sup>	0.10±0.09 <sup>de</sup>	0.15±0.09 <sup>d-f</sup>	10.55±2.49 <sup>e-h</sup>
5.0	Cr	10.13±3.48 <sup>d-g</sup>	0.04±0.07 <sup>de</sup>	1.04±1.08 <sup>ab</sup>	11.21±4.33 <sup>d-h</sup>
	Wt	11.80±1.84 <sup>c-f</sup>	0.33±0.38 <sup>c-e</sup>	1.14±0.43 <sup>a</sup>	13.28±2.06 <sup>c-g</sup>
	Fs	27.94±1.67 <sup>a</sup>	0.72±0.31 <sup>ab</sup>	0.63±0.31 <sup>a-f</sup>	29.29±1.84 <sup>a</sup>

Remarks: Ac = *A. conyzoides*, As = *A. spinosus*, Cr = *C. rotundus*, Wt = water, Fs = fungicide. Numbers followed by the same letter in the same columns were not significantly different based on the LSD ( $\alpha = 5\%$ )

The high yield of pods both wet and dry weight harvested from untreated crops suggested that Bima cultivar was categorized into tolerant to the peanut rust disease. Considering these untreated crops showed high disease intensity and abundance of pustule compared to the treated crops with 5.0 % of three weed extracts. Tolerance

was defined as ability of the crops to maintain its performance or to limit yield loss under diseases stress (Ney *et al.*, 2013; Savary, Teng, Willocquet, & Nutter, 2006). Compared with Kancil cultivar in the study conducted by Yusnawan & Inayati (2016), Kancil cultivar was considered as susceptible to the rust disease which was reflected in low yield of

total peanut pods on untreated crops. This present study suggested that genotypes also influence the tolerance of peanuts to the rust disease.

### GC-MS Analysis

Compound identifications in the roots, stems, leaves, and flowers of the crude extracts of *ageratum*, *amaranthus* and *cyperus* using GC-MS were shown in Table 3. Compounds which had more than 5 %

composition in the crude extracts are listed. Gudžić, Djokovic, Vajs, Palić, & Stojanovic (2002) highlighted that only five compounds exceeded 5 % composition in the essential oil of *Hypericum maculatum* Crantz, although this essential oil consisted a large numbers of components (seventy one were detected) in low percentages. A large numbers of compounds which had low percentages were also detected in the crude extracts in this present study.

**Table 3.** The compound composition of methanolic extract of *A. conyzoides*, *A. spinosus*, and *C. rotundus* which exceeded 5 % composition

Extract	Part	Identified compound	
<i>Ageratum</i>	Root	Caryophyllene (RT = 16.72, 8.78%)	
		(E)- $\alpha$ -Famesene (RT = 17.21, 12.42%)	
		7-Methoxy-2,2-dimethyl-2H-chromene (RT = 17.37, 6.55%)	
		5-(5'-Hydroxymethyl-2'-furoyl)-2(1H)-pyrimidinone (RT = 22.47, 17.02%)	
	Stem	Hexadecanoic acid, methyl ester (RT = 31.06, 13.40%)	
		Caryophyllene (RT = 16.73, 18.35%)	
		(E)- $\alpha$ -Famesene (RT = 17.21, 8.29%)	
		7-Methoxy-2,2-dimethyl-2H-chromene (RT = 17.37, 13.80%)	
	Leaf	Acremine E (RT = 22.48, 28.08%)	
		Caryophyllene (RT = 16.73, 13.99%)	
		2H-1-Benzopyran-2-one (RT = 16.97, 6.17%)	
		5-(5'-Hydroxymethyl-2'-furoyl)-2(1H)-pyrimidinone (RT = 22.53, 35.71%)	
Flower	$\zeta,\zeta$ -Dimethylallyl - $\alpha$ -Phenylethynyl Sulfoxide (RT = 25.26, 6.11%)		
	Neophytadiene (RT = 34.97, 7.77%)		
	Caryophyllene (RT = 16.73, 14.95%)		
	methyl 4,4,7-trimethyl-4,7-dihydroindan-6-carboxylate (RT = 22.56, 32.95%)		
<i>Amaranthus</i>	Leaf	$\alpha$ -nitrobenzo (3,4)tricyclo [3.2.1.0(2,7)]octene (RT = 25.27, 6.58%)	
		Licarin A (RT = 34.1, 7.71 %)	
		Butyl 9,12-octadecadienoate (RT = 34.42, 9.92%)	
		9-Octadecenoic acid (Z)-, methyl ester (RT = 34.61, 22.02%)	
	Stem	Phytol (RT = 35.00, 14.15%)	
		Methyl stearate (RT = 35.34, 5.82%)	
		3-(5-bromo-3-nitro-1H-1,2,4-triazol-1-yl)tricyclo[3.3.1.1~3,7~]decane-1-carboxylic acid (RT = 39.22, 5.32%)	
		Boeavinone F (RT = 39.59, 8.07%)	
	Root	Phytol isomer (RT = 34.98, 6.56%)	
		Hexadecanoic acid, methyl ester (RT = 31.08, 25.43%)	
		8,11-Octadecadienoic acid, methyl ester (RT = 34.51, 30.10%)	
		9-Octadecenoic acid (Z)-, methyl ester (RT = 34.67, 15.26 %)	
Flower	Hexadecanoic acid, methyl ester (RT = 31.07, 18.75%)		
	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (RT = 34.50, 9.74%)		
	(9E,12E)-9,12-Octadecadienoyl Chloride (RT = 34.66, 9.21%)		
	Phytol (RT = 34.97, 24.05%)		
<i>Cyperus</i>	Root	Bicyclo[8.1.0]undeca-2,6-diene, 3,7,11,11-tetramethyl-, (RT = 16.43, 11.77%)	
		$\alpha$ -Bulnesene (RT = 22.37, 5.27%)	
	Leaf	Hexadecanoic acid, methyl ester (RT = 31.06, 8.94%)	
		Hexadecanoic acid, methyl ester (RT = 31.06, 10.38%)	
		9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (RT = 34.65, 17.20%)	
	Flower	Phytol, acetate (RT = 34.96, 4.54%)	
		Hexadecanoic acid, methyl ester (RT = 31.07, 22.91%)	
		9,12-Octadecadienoic acid (Z,Z)-, methyl ester (RT = 34.50, 14.73%)	
		9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (RT = 34.66, 20.81%)	
			1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (RT = 40.97, 0.59%)

Remarks: Numbers in the brackets are retention time (RT) and relative area (%) which represents peak area relative to the total peak area

The crude extract of *ageratum* which had a notable activity against peanut rust disease contained caryophyllene in all parts of the botanical extracts. This compound made up considerable amounts of 8-18 % composition of the extracts (Table 3). Caryophyllene is a sesquiterpene compound which has anti fungal properties (Cheng, Wu, Chang, Kao, & Chang, 2004; Mondal, Mahapatra, Mirdha, & Naik, 2007; Yang, Michel, Chaumont, & Millet-Clerc, 2000). Two forms of caryophyllene isolated from *Calocedrus formosana* leaves at 100  $\mu\text{g mL}^{-1}$ , i.e. caryophyllene oxide and  $\beta$ -caryophyllene exhibited 32.3 % and 24.8 % of antifungal activity against *Laetiporus sulphureus* (Cheng, Wu, Chang, Kao, & Chang, 2004). Mode of action as anti fungi, however, was not influenced by a certain compound only. Actions as antimicrobial compounds depends on the interactions of each compound which affects bioactive mode of action, the concentration in which the compounds are present, and the chemical structures (da Cruz Cabral, Fernández Pinto, & Patriarca, 2013)

#### Total Flavonoid Content

Total flavonoids of different parts of the three weeds were quantified to estimate its contents. In general, *ageratum* extracts contained more flavonoid contents than the other two weed extracts which may contribute to its effectiveness to reduce the disease intensity of peanut rust disease. Flavonoids in leaf ( $4.55 \pm 0.04 \text{ mg CE.g}^{-1}$ ) and flower ( $3.82 \pm 0.01 \text{ mg CE.g}^{-1}$ ) extracts of *ageratum* were more than four times higher than those in stem and root (Fig. 4). In total, flavonoid contents of root, stem, leaf and flower of *ageratum* extracts were  $9.75 \pm 0.02 \text{ mg CE.g}^{-1}$ . While, in *amarantus* and *cyperus* extracts, total flavonoid was lesser at  $6.33 \pm 0.02 \text{ mg CE.g}^{-1}$  and  $6.40 \pm 0.02 \text{ mg CE.g}^{-1}$ . Higher flavonoid contents in leaf and flower than in stem and root was also observed in *amaranthus* and *cyperus* extracts. In the case of *cyperus*, both root and stem were combined since these two parts were difficult to separate during the sample preparation.

The action mode of plant secondary metabolites as antimicrobial properties is not comprehensively understood, however several studies have been carried out to investigate the active compound action (Paiva *et al.*, 2010). The role of flavonoids as anti plant pathogenic microbes is related to their antioxidative properties (Mierziak, Kostyn, & Kulma, 2014). The flavonoids are able to

reduce or quench reactive oxygen species (ROS). This ROS is produced by pathogens as well as by plants as a reaction to the infection (Blount, Dixon, & Paiva, 1992; Dai *et al.*, 1996).

Several mechanisms are involved in the reduction or quenching ROS, some of which is inhibition of enzymes produced by pathogens by chelating metals for enzyme activation, particularly enzymes which digest the plant cell wall, free-radical reaction quenching in lipid peroxidation, and the use of a byproduct of other antioxidants (Arora, Byrem, Nair, & Strasburg, 2000; Cotellet *et al.*, 1996; Harborne & Williams, 2000; Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994; Mierziak, Kostyn, & Kulma, 2014; Treutter, 2005). Other studies showed that the activities of flavonoids are to inhibit nucleic acid synthesis or DNA gyrase, to inhibit the function of cytoplasmic membrane and to inhibit energy metabolism (Cushnie & Lamb, 2005; Ikigai, Nakae, Hara, & Shimamura, 1993; Ohemeng, Schwender, Fu, & Barrett, 1993).

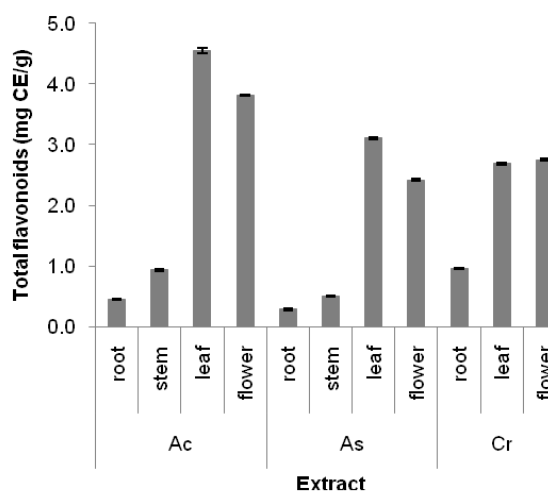


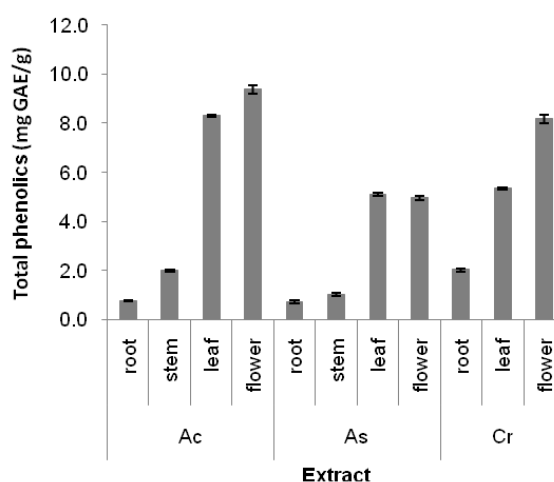
Fig. 4. Total flavonoid contents of different botanical parts of Ac = *A. conyzoides*, As = *A. spinosus*, and Cr = *C. rotundus*. Bars represent standard errors

#### Total Phenolic Content

The different proportion of total phenolic contents in roots, stems, leaves, and flowers was observed in the three botanical extracts. In general, total phenolic contents of whole *ageratum* extracts (root, stem, leaf, and flower were combined) were the highest ( $20.49 \pm 0.07 \text{ mg GAE.g}^{-1}$ ) compared to the other weed extracts ( $11.83 \pm 0.06 \text{ mg GAE.g}^{-1}$ )



and  $15.57 \pm 0.09$  mg GAE.g<sup>-1</sup> for *amaranthus* and *cyperus*) (Fig. 5). Both flowers of *ageratum* and *cyperus* contained more phenolic contents than root, stem, and leaf. Interestingly, both phenolic and flavonoid contents of roots were the least amount.



**Fig. 5.** Total phenolic contents of different botanical parts of Ac = *A. conyzoides*, As = *A. spinosus*, and Cr = *C. rotundus*. Bars represent standard errors

Phenolic compounds consist of many compounds including phenols and phenolic acids, some of which pose antimicrobial activity and function as a defense against phytopathogenic microorganisms (da Cruz Cabral, Fernández Pinto, & Patriarca, 2013; Gurjar, Ali, Akhtar, & Singh, 2012). Phenolic compounds disturb cell permeability which allow macromolecules transfer from the inside cells to the outside. These compounds also cause deformation and dysfunction of the membrane protein structures (da Cruz Cabral, Fernández Pinto, & Patriarca, 2013; Fung, Taylor, & Kahan, 1977). These secondary metabolites disrupt adenosine triphosphate (ATP) as generating energy system in the cell, inhibit enzyme activity, prevent utilization of substrate in cell for generating energy (de Oliveira et al., 2011; El-Mogy & Alsanius, 2012). According to da Cruz Cabral, Fernández Pinto, & Patriarca (2013), these all mode of actions resulted in inhibiting spore germination, suppressing mycelial growth, and germ tube elongation, therefore, inhibiting disease intensity and spore production.

Based on the phytochemical screening, the study conducted by Yusnawan & Inayati (2016) showed that those three crude extracts

also contained alkaloids, tannins, terpenoids, and saponins. In addition, analysis using thin layer chromatography separated its secondary metabolites into several spots, which may represent different compounds (Yusnawan & Inayati, 2016). The presence of sesquiterpene compound particularly caryophyllene, flavonoids, phenolic compounds, alkaloids, tannins, and saponins in the *ageratum* extracts may enhance the antifungal activity against *P. arachidis*. Crude extracts are widely used instead of specific compounds or fractions since the crude extracts gave advantages such as more effective to combat the pathogens because of synergetic effects of each compound, broader spectrum in inhibiting the pathogens and reducing the resistance of pathogens to the compound mixtures (Yazdani, Tan, Zainal Abidin, & Jaganath, 2011).

## CONCLUSION

*Ageratum* extracts at concentration of 2.5 % and 5.0 % exhibited significant inhibition to the rust disease intensity. The other two crude extracts, i.e. *amaranthus* and *cyperus* extracts were less effective in suppressing peanut rust disease. At the end of observation, the crops treated by those two concentrations of *ageratum* extracts exhibited 29.8 % and 30.2 % of the rust disease intensities which were much lower than those of untreated crops 41.4 %. A significant reduction of pustule number by 50 % showed the considerable potential inoculum suppression in the field. Yield losses reduction by 67.5 % and 63.5 % suggested significantly effective treatments of those two extract concentrations. *Ageratum* extracts contain considerable biologically active compounds particularly caryophyllene. At these two concentration levels, *ageratum* extracts are potential for biofungicide to control peanut rust disease.

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