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Antimicrobial Activity of *Tithonia diversifolia*, *Elephantopus scaber*, and *Kigelia africana* Against Plant Pathogens

Desi Rejeki[1], Suharto[1], Hardian Susilo Addy[1, 2, *]

[1]Agrotechnology Study Program, Faculty of Agriculture, University of Jember, Jember, Indonesia

[2]Center for Development of Advanced Sciences and Technology (CDAST), University of Jember, Jember, Indonesia

Email address:
hsaddy.faperta@unej.ac.id (H. S. Addy)

[*]Corresponding author

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Abstract: Synthetic pesticides, one among agricultural inputs, have been used and applied to crop production, particularly during plant pathogen attacks. Although promisingly, possible effect that the application of pesticides on agro-ecosystem may have to be concerned to support health of food, consumers and to the environment. Alternatively, exploration of the potential plants that probably have natural antimicrobial compounds is important step to discover natural pesticide as component of plant disease management. Some of plants with low economical value such as *Tithonia diversifolia, Elephantopus scaber,* and *Kigelia africana* have been known to have antimicrobial substances and successfully demonstrated against food and human pathogens. These, bring us to study their potency in controlling several plant pathogens of important crops, either fungal such as *Phytophthora nicotianae* and *Rhizoctonia solani*, or bacterial pathogens such as *Ralstonia solanacearum* and *Xanthomonas oryzae*. Leave extracts of both, *T. diversifolia* and *E. scaber*, and fruit extract of *K. africana* were obtained and concentrated using methanol. Our results showed that all extracts contained flavonoid, tannin, and alkaloid but the amount of the content of each extract was different. Among extracts used in this study, fruit extract of *K. africana* was known to contain the highest flavonoid and tannin content of 21, 54 µg QE/ml and 28.95 µg GAE/ml, respectively, with low content on alkaloid (3.32 µg AE/ml) compared to other plant extracts. To test it potency as biopesticides, antimicrobial activity against fungal pathogens were evaluated using poisoned food technique method while antimicrobial activity against pathogenic bacteria were evaluated using spot test method. The result showed that extract from *K. africana* fruit was able to inhibit fungal pathogen *R. solani*, while extracts of *E. scaber* and *T. diversifolia* were have inhibition ability against *P. nicotianae*. In addition, the *E. scaber* extract was also able to inhibit bacteria *R. solanacearum* and *X. oryzae*. In average, 5 mg/ml of extracts were demonstrated to give the best performance in inhibit plant pathogens.

Keywords: Biopesticide, Plant Pathogen, Plant Disease, Fungal Disease, Bacterial Disease

1. Introduction

Synthetic pesticide in agriculture is one of the agricultural inputs that supports crop production and alternatively belongs to integrated pest management. Intensive use and inappropriate dose of these pesticides for the control of plant pathogens may raise harmful impact on the environment and adverse effects on human health [1]. Therefore, it is a challenge in agricultural practices, particularly in disease management that ideally support and contribute to the sustainability of disease management through protecting crop yields, maintaining and improving profitability for crop producers, and reducing the negative environmental impacts of diseases [2]. Alternatively, several techniques to control plant disease have been developing with low negative impact on environment with high efficacy such as the use of biological control agent or natural products that are grouped to biopesticides [3]. However, the annual growth rate of biopesticides is relatively low than the rate of synthetic pesticide in past five year [4].

In general, natural products (microorganism-derived, higher plant-derived, and animal-derived products) and

microorganisms (viruses, bacteria, fungi, nematodes, and protozoa) have been known to have promising result in control disease caused pathogens [5]. Particularly, natural product from higher plant-derived is known to be successfully tested against several human [6], and animal pathogens [7]. Against plant pathogens, it has been shown that several plants contained antimicrobial substances that able and have potential effect to combat pathogens with limited test in challenge to fungal or bacterial pathogens [8]. In addition, the mechanisms of inhibition activities by using plant-derived product against pathogens are literally explained by Upadhyay *et al*. [7] due to the properties of plant substances like flavonoids, quinones, alkaloids, tannins, coumarins, terpenoids, lectines, and peptides.

Indonesia, with larger plant diversity, has natural resources that may explore and discover plant-derived biopesticide with antimicrobial activity against plant pathogen. Some of them are plants with low economical value, such as *Tithonia diversifolia*, *Elephantopus scaber,* and *Kigelia africana*, which are easy to find elsewhere and abundant in nature as wild plants. It has been shown that all plants are able to inhibit the growth of pathogens. *E. scaber* has been reported to be able to inhibit the growth of *Staphylococcus aureus* [9], *T. diversifolia* against human pathogens, *S. aureus* and *Escherichia coli* [10], and *K. africana* against human pathogens, *S. aureus*, *Bacillus subtilis*, *Corynebacterium diphtheriae*, *Aspergilus niger*, *A. flavus*, *Candida albicans* and *Aureobasidium* sp. [11] indicating that all plants are able to inhibit pathogen from either fungi or bacteria.

In the other hands, some plant pathogens are known difficult to control using chemical pesticides due their characteristic and nature. *Rhizoctonia solani* and *Phytophthora nicotianae* are adverse fungal plant pathogen that rapidly growth and infect valuable crops in Indonesia [12, 13] In addition, two of important bacterial disease caused by *Ralstonia solanacearum* and *Xanthomonas oryzae* are dangerous plant pathogens on several common crops cultivated in Indonesia [14, 15]. Several control techniques have been tested and enforced to obtain the best composition of disease management. Alternatively, natural products (biopesticides) as well as plant-derived substances are promising to list on disease management strategy.

In this paper, we presented the analysis the phytochemical content such as alkaloid, tannin, and flavonoid as well as its ability to inhibit pathogens.

2. Materials and Methods

2.1. Plants and Microorganisms

Plants used in this research were collected from field around the University of Jember in January 2017. The leaf of *Tithonia diversifolia*, *Elephantopus scaber*, and the fruit of *Kigelia africana* (Figure 1) were collected as fresh material prior to sample extraction. Microorganisms used in bioassay were obtained from the collection of Department of Plant Protection, Faculty of Agriculture, the University of Jember such as two species of fungi, *Phythophthora nicotianae* (causative agent of black shank on tobacco), and *Rhizoctonia solani* (causative agent of sheath blight on rice), and two species of phytopathogenic bacteria, *Ralstonia solanacearum* (causative agent of bacterial wilt), and *Xanthomonas oryzae* pv. *oryzae* (causative agent of bacterial leaf blight on rice). Routinely, fungi species were grown on potato dextrose agar (PDA) medium, while bacterial species were grown on nutrient Agar (NA) for *X. oryzae* and cassamino acid peptone glucose (CPG) for *R. solanacearum* at 28°C.

Figure 1. *Morphology of Tithonia diversifolia (a), Elephantopus scaber (b), and Kigelia africana (c).*

2.2. Plant Samples Preparation and Extraction

All small cut of leaf and or fruit of plant samples were air-dried at 32°C for two weeks prior to extraction. The dried bulk samples were grounded using a blender, and sieved through a 2 mm^2 wire mesh to obtain simplitia powder.

Each simplisia powder (50 g) was macerated by adding methanol at ratio of (1:3), and was incubated for 48 hours with shaking. The aqueous extract was filtered through a plug of absorbent cotton wool in a Buchner funnel before concentrated using a rotary evaporator at 45°C. All concentrated samples were then dissolved in steril water to obtain final concentration of 0 mg/ml, 1 mg/ml, 5 mg/ml, and 10 mg/ml following by passing through 0.22 µm membrane filter.

2.3. Phytochemical Analysis of Crude Extracts

All crude extracts were screened for phytochemical contents of alkaloid, tannin, and flavonoid through qualitative and quantitative analysis.

2.3.1. Qualitative Analysis

For alkaloid test, a 3 mL of extract (in water) was mixed with 1 ml of HCl following by heating for 20 minutes. Sample was then cooled and filtered prior to adding with Wagner reagent [16].

Tannin was tested by mixing 1 mL extract in methanol and added by several drops of 1% of $FeCl_3$ for color changes from dark green to dark blue [17].

Flavonoid was tested from 1 ml extract (in methanol) using several drops of 1% of NaOH and was allowed to stand for color changes [18].

2.3.2. Quantitative Analysis

The plant extract (1 mg) was added by 1ml of 1,5 N HCl and filtered. A hundred microliters solution was mixed with 1 ml of chloroform followed by transferring 100 µl to a glass

tube. A 500 µl of bromocresol green solution and 500 µl of phosphate buffer were added and the mixture was shaken vigorously. Chloroform fraction that formed after stand was adjusted using chloroform to have a final volume of 1 ml. A set of standard solutions of atropine (20, 40, 60, 80 and 100 µg/ml) was prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract [19].

Tannin was determined as previously described by Singh et al. [20]. Briefly, 10 µl of methanol extract was added with 50 µl of Folin-Ciocalteu reagent and 100 µl of 35% of Na_2CO_3 %. Volume of H2O was added to adjust mixture to obtain final volume of 1 ml followed by shaking for 30 minutes at room temperature. A set of reference standard solutions of gallic acid (20, 40, 60, 80, and 100 µg/ml) was prepared in the same manner. The absorbance for test and standard solutions were determined against the reagent blank at 725 nm with an UV/Visible spectrophotometer. The total tannin content was expressed as mg of GAE/g of extract.

Total flavonoid content was measured by the aluminium chloride colorimetric assay. The reaction mixture consists of 100 µl of extract and 400 µl of distilled water was taken in a glass tube. To the tube, 30 µl of 5 % sodium nitrite was treated and after 5 minutes, 30 µl of 10 % aluminium chloride was mixed. After 5 minutes, 200 µl of 1M Sodium hydroxide was treated and diluted to 1 ml with distilled water. A set of reference standard solutions of quercetin (150, 300, 450, 600, and 750 µg/ml) was prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract.

2.4. Antimicrobial Assay

The plant extracts were tested for antifungal activity using poisoned food technique [21]. A one cm in diameter of the test organisms (*R. solani* and *P. nicotianae*) were transferred on tested PDA medium containing final concentrations of plant extracts of 0 mg/ml, 1 mg/ml, 5 mg/ml, and 10 mg/ml. The plates were incubated at 28 C for 4 days. Antifungal activities were evaluated by calculating the percentage of inhibition of diameter of fungal colony and diameter of hyphae using light microscope DM2500 (Leica, Germany). Percentage of inhibition (I) was calculated using following equation:

$$I = \frac{r1-r2}{r1} \; x \; 100 \qquad (1)$$

Where, r1 = the diameter of fungal colony on control agar plate, while r2 = the diameter of fungal colony on agar plate containing plant extracts.

The plant extracts were also tested for antibacterial activity using diffusion method. The test organisms (100 µl of 10^7 CFU/ml) were lawn on agar medium (NA for *X. oryzae* and CPG for *R. solanacearum*) followed by spotting 3 µl of 10% plant extracts. The plates were incubated at 28 C for 48 hours. As a positive control, ampicillin was spotted in the same manner on bacterial lawn. Antibacterial activities were evaluated by measuring the diameters of zones of inhibition in mm against the test bacteria.

All data were subjected to analysis of variance (ANOVA) and the means were compared using Duncan's multiple range test (DMRT) at P=0.05 level of significance.

3. Results

3.1. Phytochemicals Analysis of Plant Extracts

Phytochemicals analyses of plant extracts were done to know the content of alkaloid, tannin, and flavonoid in samples. Qualitative analysis showed that all samples contained all phytochemical substances in different amount. The more dark color the more total content of substance (Figure 2). In addition, our quantitative analysis proved that, extracts from *K. africana* contained higher flavonoid and tannin compared to other extracts about 21,54 µg QE/ml and 28,95 µg GAE/m, respectively. Although lower in flavonoid and tannin contents, extracts of *T. diversifolia* and *E. scaber* contained higher amount of alkaloid (Figure 3).

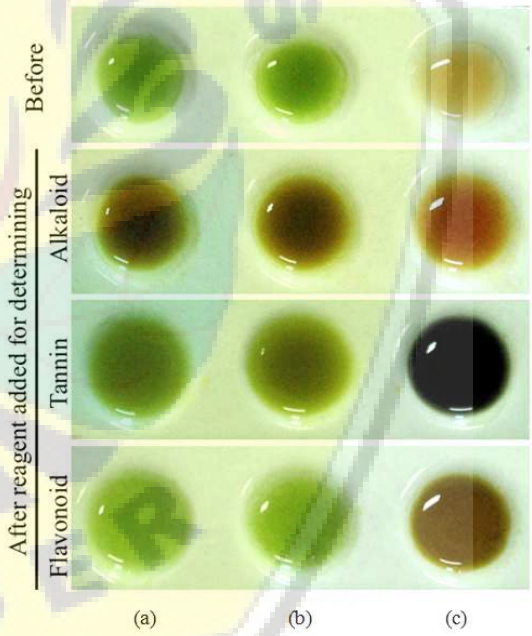

Figure 2. *Qualitative determination of phytochemical from plant extracts of T. diversifolia (a), E. scaber (b), and K. africana (c).*

3.2. Antimicrobial Activity of Plant Extracts

Antimicrobial assays were done using plant extracts against plant pathogens. Our results showed that all extracts were able to inhibit the growth of phytopathogenic fungi *P. nicotianae* especially *K. africana* as shown as the smallest of colony of fungi on medium compared to others and control (Figure 4). However, the extract abolished to inhibit the growth of *R. solani*. This result somehow indicates that extract of *K. africana* support the growth of *R. solani* resulting higher diameter of colony compared to control (Figure 4).

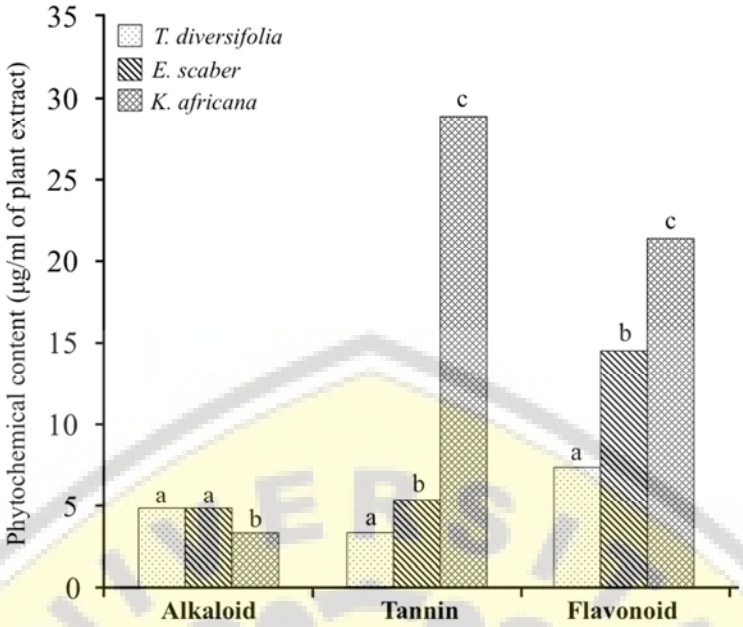

Figure 3. *Estimation of phytochemical from plant extracts of T. diversifolia (a), E. scaber (b), and K. africana (c).*

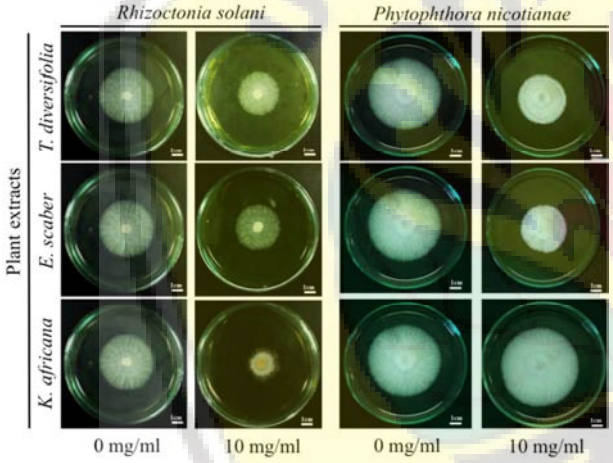

Figure 4. *Antifungal assay of plant extracts against phytopathogenic fungi. Pictures were taken at 2 days post incubation (dpi) for P. nicotianae and 4 dpi for R. solani.*

Interestingly, statistical analysis of microscopic observation supported the data that the all plant extracts inhibited the growth of hyphae resulting inhibition of fungal colony but not hyphae diameter. In addition, inhibition of the growth of hyphae was also related to the observation of the length between septa around mycelia tips (case of *R. solani*) that inhibited-hyphae had shorter length of cell (between septa) (Table 1). This result suggested that inhibition of development of fungi colonies because of the inhibition of the growth of hyphae. In general, *K. africana* had higher ability to inhibit the growth of *R. solani* about 50% compared to control. Moreover, our result suggested that plant extracts of *T. diversifolia* and *E. scaber* had broad antifungal activity compared to *K. africana* since both plant extracted were able to inhibit either *P. nicotianae* of *R. solani*.

Table 1. *Characteristics of fungi grown on antifungal assay medium containing plant extracts.*

No	Plant extracts	Conc. (mg/ml)	Growth characteristics of …				
			P. nicotianace		*R. solani*		
			Colony Diameter (cm)	Hyphae Diameter (µm)	Colony Diameter (cm)	Hyphae Diameter (µm)	Length between septa (µm)
1.	*T. diversifolia*	1	4,11bc	1,33a	3,85c	2,15b	66,71b
		5	3,33d	1,27a	3,15e	1,98b	42,17c
		10	3,13d	1,03a	2,58h	1,73b	32,82cd
2.	*E. scaber*	1	4,03c	1,89a	4,15b	2,13b	69,28b
		5	3,41d	1,74a	3,4d	1,7b	35,12cd
		10	3,01d	1,53a	3,03f	1,58b	31,44cde
3.	*K. africana*	1	4,56abc	1,03a	2,85g	2,01b	23,13def
		5	4,9 ab	2,12a	2,23i	1,8b	15,61ef
		10	4,93a	2,14a	2,22i	1,3b	10,97f
4.	Control	0	4,78ab	2,07a	4,55a	6,35a	113,03a

Number followed by similar alphabet indicating that both results were not in significant difference using DMRT (P=0.05).

Table 2. *Antibacterial assay of plant extracts against phytopathogenic bacteria.*

No	Plant extracts	Inhibition against bacterial pathogen(s)	
		X. oryzae	***R. solanacearum***
1.	*T. diversifolia*	-	-
2.	*E. scaber*	+	+
3.	*K. africana*	-	-
4.	Positive control (ampicillin)	+	+
5.	Negative control (H_2O)	-	-

Positive (+) indicates clear zone appearance as inhibition activity. Negative (-) indicates no inhibition activity

4. Discussion

Wild plants like *T. diversifolia, E. scaber,* and *K. africana*, which are easy to find elsewhere and abundant in nature in Indonesia, have been known to have ability to inhibit the growth of pathogens [9 -11] due their bioactive compounds including phenolic and alkaloid. In the results, qualitative determination was able to predict the quantity of the tested-compound (Figure 2). For alkaloid, extract from *K. africana* was more bright than other extracts after reaction indicating that *K. africana* contains less alkaloid comparing to others extracts (Figure 3). Moreover, similar patterns were also shown during analysis of tannin and flavonoid that darker in color higher content of compounds.

Alkaloid, tannin, and flavonoid are bioactive compounds that have antimicrobial activity as previously reported [9-11]. The result indicated that these bioactive compounds were somehow correlating with the ability in inhibiting plant pathogens (Table 1 and 2). Alkaloid is known to have a role in inhibition of fungal pathogen by affecting the genetic material of fungi as well as inactivation of genetic material of fungi by inhibiting DNA or RNA replication [22] resulting inhibition and abnormality growth of mycelium. Similar result was also shown flavonoid against fungal pathogen. Interestingly, extract of *K. africana*, however, supported the growth of *P. nicotianae* but drastically reduced the growth of *R. solani*. Our prediction of this phenomenon is due to tannin and flavonoid content in *K. africana* (Figure 3 and 4). As reported by Fan *et al*., [23] that supplying tannin as 100 mg/L in culture media enhanced the growth of *Pleurotus* sp. In addition, flavonoid also induces of specific pathogenicity genes as well as stimulation of development needed for pathogenesis including germination of zygospore of *Gigaspora* sp. and *Glomus* sp. as well as report by Cheo [24] that increasing the concentration of tannin induces the growth and rhizomorf formation of *Armilaria mellea.*

On the other hand, *T. diversifolia* and *E. scaber* contained higher level on alkaloid content than *K. africana* (Figure 3). Accordingly, it showed higher ability on inhibition the growth of *P. nicotianae* (Figure 2, Table 1). The effect of alkaloid on inhibition the growth of fungal pathogen was also observed by Nes *et al*. [25] on *P. cactorum* that increasing alkaloid concentration decreasing spores production. This indicates that different pathogens may have different respond against alkaloid, tannin and flavonoid or vice versa. Besides against fungal pathogens, all extracts were tested against bacterial pathogen. The only extract from *E. scaber* showed antibacterial activity against both bacterial pathogen, *R. solanacearum* and *X. oryzae* (Table 2). Unfortunately, It is unable to find the reason of this phenomenon according to phytochemical content with speculation that *E. scaber* contains another particular substances that may specifically effective against bacterial pathogens. Daisy *et al.,* [9] tested *E. scaber* found that terpenoids, another bioactive compound in *E. scaber*, was able to inhibit *S. aureus* by inhibiting autolysin enzyme that essential to make openings for continuing network of mucopeptides resulting an addition of the new building blocks during growth, essential for the remodeling of the cell wall in making the septum that divides one bacterium into two daughter cells, necessary to break the two newly formed bacteria apart from each other. As consequence, the loss or inhibition of the autolytic enzyme activity will cause inhibition of bacterial growth.

5. Conclusion

In conclusion, extract of *K. africana* is effective to inhibit the growth of *R. solani*, while extracts from *T. diversifolia* and *E. scaber* are able to control *P. nicotianae*. In addition, only extract from *E. scaber* is able to control phytopathogenic bacteria, either *R. solanacearum* or *X. oryzae*.

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