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Insecticidal Effects of the Flavonoid-rich Fraction of Leaves Extract of Gamal (*Gliricidia sepium*) on the Coffee Mealybugs (*Planococcus citri* Risso.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author NN designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ER and Apriliyani managed the analyses of the study. Author MK managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To find out whether flavonoid-rich fractions of aqueous and methanolic extracts of gamal plant leaves, *Gliricidia sepium* (Jacq.) Kunth ex Walp., can be used for eradicating coffee mealybugs, *Planococcus citri* Risso.
Study Design: Completely randomized design using five concentration levels repeated three times.
Place and Duration of Study: Department of Biology, Faculty of Mathematics and Sciences, University of Lampung, Bandar Lampung, Indonesia between December 2015 and May 2016.
Methodology: Mealybugs that found to infest coffee berries (*Coffea robusta* L.) were reared and grouped into two. Both groups were fed consecutively with flavonoid fractions of aqueous and methanolic extracts of gamal plant leaves with the concentration of 0% (as control), 0.01%, 0.02%, 0.03%, and 0.04%. The percent mortality of insects was examined at the 12th, 24th, 48th and 72th hour.

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Results: Thin layer chromatography analysis of aqueous and methanolic extracts yielded consecutively one and seven fractions of flavonoid. Both type of extract affected mortality rate of the insects in a concentration-related manner, however based on LC_{50} and LT_{50} values the water fractions showed a higher effectiveness than that of methanol.
Conclusion: It is suggested that flavonoid-rich fraction of gamal leaf extracts is potent to be used as bioinsecticide for coffee mealybugs, *Planococcus citri* Risso.

Keywords: Gamal; Gliricidia sepium; Planococcus citri; Coffea robusta; mealybug; bioinsecticide.

1. INTRODUCTION

Robusta coffee is one of the mainstay commodities of Lampung Province, Indonesia, which is famous for its nickname Kopi Lampung (Lampung coffee). The production of this type coffee is all dependent on smallholder plantations with the productivity per year in average is around 700 kg/Ha [1]. There are many challenges facing coffee farmers, especially in the effort to maintain the quality of coffee beans, among others are pests attack. One of the pests commonly attack robusta coffee in this province is mealybug [2].

The most effective way to eradicate mealybugs, needless to say, is using chemical pesticides such as spirotetramat, chlorpyriphos, profenofos, methidathion, methomyl [3,4,5]. However, the use of chemical insecticides may have unexpected effects such as causing pest resistance [6], leaving harmful chemical residues in the coffee beans [7]. That's why safe pest control, such as the use of natural-based pesticides, the biopesticides, still needs to be pursued and developed [8].

There are countless plant based chemicals that are known to affect plant-environment interactions, including flavonoids. These phytochemicals defend plants against various abiotic and biotic stresses including UV radiations, pathogens and insect pests. Flavonoids protect the plant against insect pests by influencing the behavior, growth and development of insects [9,10].

Among plants that are known to have biopesticide properties allegedly due to it flavonoid content is gamal, an Indonesian name for *Gliricidia sepium* (Jacq.) Kunth ex Walp. (Fabaceae Lindl.). The crude leaves extract of this plant has reported to show nematicidal effects, antimicrobial properties, and larvicidal activity on mosquitoes [11,12,13].

Although studies on the pesticide effects of gamal plant extracts have been widely done but research on the insecticide effects of this plant against mealybug, especially *Planococcus citri* Risso., are still lack considerably. In an attempt to find out whether flavonoids in gamal plants can be used and effective for killing mealybu the flavonoids contained in leaf extracts of *Gliricidia sepium* (Jacq.) Kunth ex Walp. have isolated and tested on mealybugs that infect coffee berries of *Coffea robusta*.

The potential advantage of using gamal leaf extracts is that there has been no report on the insecticidal effects of these plant extracts against natural enemies of mealybugs. As has been indicated, there are two types of natural enemy of mealybugs, parasitoid and predator. Among the parasitoids there are *Anagyrus pseudococci*, *Leptomastix dactylopii* (Howard), *Leptomastidea abnormis* (Girault), *Coccidoxenoides peregrinus* (Timberlake), *Rhyzobius lophanthae* and *Scymnus sp.* [14] (Mahfoudhi and Dhouibi, 2009). Whereas common predators of the mealybugs are 31 insect species such as *Chrysoperla carnea* Steph., *Hyperaspis* sp. and *Nephus bipunctatus* (Kugelann) and nine spiders including *Plexippus paykulli* Audouin and *Thyene imperialis* (Rossi) [15].

2. MATERIALS AND METHODS

2.1 Plant Samples and Extraction

Leaves of gamal plant were collected from Pekurun Village, sub-district of Abung Pekurun, the district of Lampung Utara, Lampung Province, Indonesia. Taxonomic identification of the plant was done by botanist at the Botany Laboratory, Faculty of Mathematics and Sciences, University of Lampung, Indonesia. The leaves were air-dried at room temperature for 10 days and ground into fine powder using disk-mill machine. These simplicia then prepared and subjected to flavonoid extraction and isolation.

Considering flavonoid and derivatives are best soluble in polar solvents [16,17], separation of the non-polar and or semi-polar biochemicals from the simplicia is crucial to be done. For that reason gamal leaves powder (500 g) were macerated using 1500 ml n-hexane (J.T Baker) for 24 hours with three replications until no more extraction observed. Afterward, the solid residues macerated further using 1000 ml dichloromethane (J.T Baker) with the same duration and replication as n-hexane. After the extracts separated by filtration, the solid residues of gamal leaves powder then macerated further to obtain polar extracts. To obtain methanolic polar extract from gamal plant leaves, the solid residues were soaked in methanol solvent (1200 ml) for 24 hours and repeated for eight times. After evaporated in rotary evaporator, the methanolic extract then concentrated by recristalized technique using freeze dryer for 72 hours until a paste form of crude polar extract was formed.

After methanolic polar extracts were filtered, solid residues of the gamal leaves powder were subjected final maceration using water. The resdidues were soaked in water solvent (1200 ml) for 24 hours and repeated for six times. Afterward the water extract evaporated and purified by recrystallization technique using freeze dryer for 72 hours until a paste form of crude polar extract was formed. Such crude water extracts then hydrolized using hydrochloric acid (HCl) and methanol at the temperature of 60°C for 60 minutes. The hydrolyzed extract was filtered and transferred into separating funnel and NaCl and ethyl acetate (both from JT Baker) were added. After being shaken, the mixture allowed to separate into two phases, water phase and ethyl acetate phase. Water phase was indicated by yellowish color and the presence of precipitated crystals. Both filtrate and precipitated crystals of water phase are purified by crystallization and freeze dry.

2.2 Flavonoids Isolation and Identification

To isolate flavonoids from the polar extracts, both methanolic and aqueous extracts were fractionized using Amberlite XAD-4 column (Merck, Darmstadt, Germany) in which dichloromethane and methanol with the proportion of 4:1 were used as eluent. To identify flavonoids then each fraction obtained was subjected to thin layer chromatography analysis using aluminum oxide (alox) plates with cellulose adsorbent (Merck KGaA 64271 Darmstadt Germany). Pure flavonoid on TLC plates were identified using $AlCl_3$, $CeSO_4$, NaOH 2M and saturated H_3BO_3 reagents (J.T. Baker) by the presence of yellowish, brownish, orange or redish spots [18]. The flavonoid-rich fraction of both methanol and water extracts of gamal leaves then tested for their lethal effects on mealy bug.

2.3 The Mealybugs

The insects tested in this study were mealybug (*Planococcus citri* Risso) that found to infest coffee berries (*Coffea robusta* L.) in the traditional-smallholder coffee plantations of the Pekurun Village, sub-district of Abung Pekurun, the district of Lampung Utara, Lampung Province, Indonesia. The insects along with coffe berries infested (Fig. 1) were transfered to the laboratory and reared until reaching adult stages. The adult females, the developmental stage of mealybug feeding on coffee berries [19], were chosen as the test animal in this study.

Fig. 1. Coffee berries infested by mealybug (Planococcus citri, Risso) from which test insects were collected

2.4 Crude Extract Bioassays

To determine the effective concentration range of extract on the test animal, bioassay of the water and methanol crude extracts on the test insect was done for 72 hours. The LD_{50} values were assigned as the range of concentration in bioassay of flavonid-rich fraction of both extract.

2.5 Experimental Design and Treatments

Five concentration levels of flavonoid-rich methanolic and water extract of the gamal plant leaves were prepared for treatment based on the LD_{50} of crude extracts. Coffe berries obtained from the same coffee plantation from which the

test insects are obtained were soaked in treatment solution for 10 minutes. After being air-dried, the fruits were put into glass jars and then infested with 10 imago mealybugs. Each treatment repeated three times. The percent die of insects were examined and recorded at the 12th, 24th, 48th and 72th hour.

2.6 Study Parameters and Data Analysis

Mortality rate of test insect was expressed as percentage, whereas effectiveness of extract in killing the insect was expressed as LD_{50} (lethal dose 50%) and LT_{50} (lethal time 50%). Both LD_{50} and LT_{50} were determined using Probit EXE Analysis Program. Mean difference between parameters were analyzed using two-way ANOVA and post hoc test from Tukey at α=5%.

3. RESULTS AND DISCUSSION

3.1 Crude Extracts and Fractions

The multistage maceration of the 1500 g dried powder of gamal plant leaves yielded 25 grams of methanol extract and 4 grams of water extract. From methanol extracts obtained 42 fractions that based on the TLC analysis known that seven of them are flavonoids. All seven fractions were mixed and labeled as flavonoid-rich methanol extract. The TLC analysis of the hydrolysis end product of water extract only produces one single flavonoid rich fraction. The TLC chromatogram of and Rf values of flavonoid-rich fractions of the water and methanol extracts of gamal plant leaves using four different reagents are shown in Fig. 2.

3.2 Bioassays of Crude Extract

Bioassays of water and methanol crude extracts on adult female mealybugs resulting LD_{50} values of 0.04% dan 0.034% respectively. The highest values (0.04%) of LD_{50} then used as the maximum concentration level in bioassay of the flavonoid-rich fractions of the water and methanol extracts. Five levels of extract concentration set for bioassay of the flavonoid-rich fractions of the water and methanol extracts are 0% (as control), 0.01%, 0.02%, 0.03%, and 0.04%.

3.3 Bioassays of Flavonoid-rich Extracts

Mortality rate of mealybugs by flavonoid-rich fractions of water and methanol extracts of gamal plant leaves are presented in Table 1. The two-way ANOVA applied to the bioassay data resulted in the statistical parameters presented in Table 2.

Based on the statistical data in Table 2 it is clear that all independent variables, extract types, concentration levels, and hours of exposure are significantly contributing in insect mortality rate ($P < 0.05$). However, only concentration levels and hours of exposure that show interaction effects ($P < 0.01$)

Tukey's post hoc test of the mean mortality rate by concentration levels and hours of exposure of the flavonoid-rich fraction of water and methanol extracts of gamal plant leaves concecutivlely presented in Table 3 and Table 4. By the end of hour 72, it is evident that flavonoid-rich fraction of both water and methanol extracts of gamal plant leaves showed lethal effects that increase with the increase of concentration ($\alpha < 0.05$). Correspondingly, by the highest level of concentration (0.04%) of flavonoid-rich fraction of both water and methanol extracts of gamal plant leaves, hours of exposure showed an increased lethal effects by the duration of exposure ($\alpha < 0.05$).

Table 1. Mean of mortality rate of mealybugs by flavonoid-rich extracts of gamal plant leaves

Extract	Concentration (%)	Percent die of insects by hour of exposure			
		12h	24h	48h	72h
Water	0	0	0	0	0
	0.01	0	6.67	16.67	26.67
	0.02	0	10	23.3	33.33
	0.03	3.33	16.67	26.67	43.33
	0.04	6.67	26.67	36.67	56.67
Methanol	0	0	0	0	0
	0.01	0	3.33	13.33	23.33
	0.02	3.33	6.67	20	30
	0.03	0	10	26.67	36.67
	0.04	3.33	16.67	36.67	46.67

Table 2. Results of two-way ANOVA of the mortality rate of mealybugs by water and methanol extracts of gamal plant leaves

Source	df	SS	MS	F	*P*
Treatment	39	291.925	7.485	37.426	0.000
Extract	1	2.408	2.408	12.042	0.010
Concentration	4	109.883	27.471	137.354	0.000
Hour	3	133.825	44.608	223.042	0.000
Extract × Concentration	4	1.217	0.304	1.521	0.204
Extract × Hour	3	1.025	0.342	1.708	0.172
Concentration × Hour	12	42.383	3.532	17.66	0.000
Extract × Conc. × Hour	12	1.183	0.099	0.493	0.913
Residual	80	16.000	0.200		
Total	119	307.925			

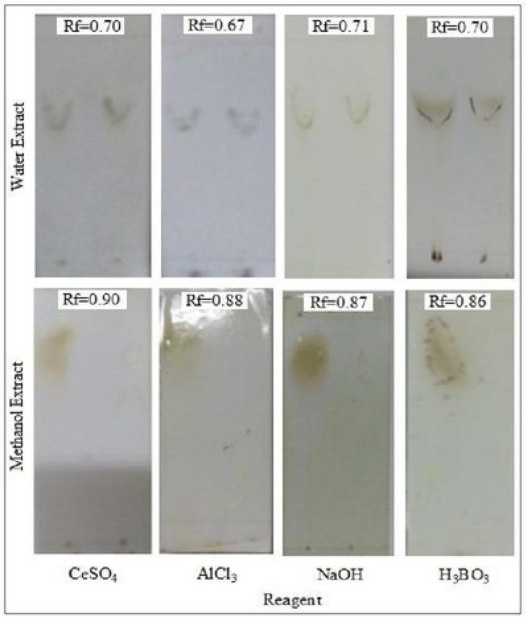

Fig. 2. TLC chromatogram and Rf values of flavonoid-rich fractions of the water and methanol extracts of gamal plant leaves by four different reagents

Table 3. Mortality of insects on hours 72th gamal leaf extracts at different concentration

Concentration	Water extract (Mean±SD)	Methanol extract (Mean±SD)
0.00	0.00±0.00a	0.00±0.00a
0.01	2.67±0.58b	2.33±0.58b
0.02	3.33±0.58c	3.00±1.00b
0.03	4.33±1.00c	3.67±0.58c
0.04	5.67±0.58d	4.67±1.00d

Mean±SD values in the same column followed by different superscript are differ significantly at α < 0.05 based on Tukey's test

Table 4. Mortality of insects by gamal leaf extracts (0.04%) on different hour of treatment

Hour	Water extract (Mean±SD)	Methanol extract (Mean±SD)
12	0.67±0.58a	0.33±0.58a
24	2.33±0.58b	1.67±0.58b
48	3.67±0.58c	3.67±0.58c
72	5.67±0.58d	4.67±0.58d

Mean±SD values in the same column followed by different superscript are differ significantly at α < 0.05 based on Tukey's test

3.4 Probit Analysis

The application of Probit EXE Analysis Program on insect mortality data yields LD_{50} and LT_{50} values presented in Tables 5 and 6, respectively. The data trend in Table 5 shows that LC_{50} decreases with increasing hours of exposure. While the data trend in Table 6 shows the decrease of LT_{50} with increasing concentration of flavonoid-rich fraction of both water and methanol extract of gamal plant leaves. To compare the effectiveness of the two types of fractions, a box plot analysis was used and the results are presented in Fig. 2. Based on the box plot depicted in Fig. 2 it is assumed that flavonoid-rich fraction of water extract of Gliricidia sepium plant leaves more effective than that of ethanol extract.

Table 5. Mean of LC_{50} of gamal leaf extracts

Hour	Mean LC_{50} (%)	
	Water extract	Methanol extract
12	0.063	0.123
24	0.059	0.067
48	0.048	0.051
72	0.033	0.039

Table 6. Mean of LT_{50} of gamal leaf extracts

Concentration	Mean LT_{50} (Hours)	
	Water extract	Methanol extract
0.00	**	**
0.01	94.849	96.979
0.02	85.298	93.970
0.03	71.460	80.309
0.04	63.113	70.143

***Cannot be determined*

Column chromatography of the crude methanol extract of gamal plant leaves yielding 42 fractions which seven of them were identified as flavonoids. In contrast, from crude water extract of the solid residues there was found only one fraction identified as flavonoid. These findings seem to confirm several previous studies that methanol solvent tend to dissolve flavonoid better than that of water. Methanol is the most effective of solvent to yield total flavonoid contents of extracts from *Zingiber officinale* Roscoe [20], *Marrubium peregrinum* L [21], leaves and flowers of *Elaeagnus angustifolia* L. [22], and leaves of *Amomum chinense* C. [17]. However, given the maceration technique employed in this study is multi-stage, so these findings cannot be concluded that flavonoids are less soluble in water, but because the aqueous solvent only dissolves the remaining residues. As has been indicated, flavonoids are phenolic and are mainly water soluble compounds [23], mainly in certain plants such as *Medicago sativa* [24] and *Helicteres hirsuta* Lour [25].

Whatever the effectiveness of methanol and water in dissolving flavonoids, both the crude methanolic and water extracts of the gamal leaves are all le[illegible] to the coffee mealybug *Planococcus citri* with LD50 values of 0.034% and 0.04% respectively. These findings are seemingly the answer to the study reported by Nazli et al. [26] that the crude leaf extracts of *Gliricidia sepium* has a repellent activity against mosquito insects, *Aedes aegypti*, even up to 78%. The result of bioassay of flavonoid-rich fraction of both extracts, as presented in Tables 1-4, confirmed that the the toxic component of the phytochemicals extracted from gamal plant leaves are flavonoids.

Among flavonoid compounds, isoflavones are well known for their toxic effects. One of the isoflavonoids that has been used as pesticide is rotenone. In California (USA) for instance, rotenone is widely use as a botanical insecticide for organically grown lettuce and tomatoes [27]. In United States, due to its highly selective toxicity to fishes, rotenone has also been used for controlling or eradicating of non-native fish in streams and lakes across the country [28]. Interestingly, isoflavones are much rarer in their occurrence, except in one sub-family of the Leguminosae or Fabaceae [29].

Flavonoid compouds that are also known to be toxic to insects are biochanin and pinocembrin. Biochanin was suggested as promising phytochemical with ability to reduce fecundity in primary reproductives of the termite [30]. Meanwhile, pinocembrin was known to show anti-feeding and mortality effects in the larvae of butterfly *Spodoptera frugiperda* [31]. Biochanin and pinocembrin, in fact, are also theflavonoids commonly found in leguminous plants such as *Dalbergia odorifera* [32]. Very likely the lethal activity of the flavonoid-rich fractions of the aqueous and methanolic extracts of gamal leaves was related to the high-specific content of such isoflavonoids. Given *Gliricidia sepium* is belonging to pea plants family, the Fabaceae (Leguminosae), it is quite natural that the flavonoid-rich fractions of aqueous and methanolic extracts of the plant effectively kill mealybugs.

Other types of flavonoids that are no less toxic to insects are quercetin, rutin and naringin. These three types of phytochemicals were reported causing nymphs mortality in the woolly apple aphid, *Eriosoma lanigerum* [33]. Rutin, in particular, considered to be interfered with physiological processes in the insects at the time of molting leading to delay in development [34]. Quercetin adversely affected egg hatching and decreased significantly larval periods in the second and third instar of melon fruit fly, *Bactrocera cucurbitae* (Coquillett) [35]. *Gliricidia sepium*, as indicated, also found to contain such flavonoids including quercetin, rutin and naringin [36].

Regarding the mechanism of flavonoids affecting insects, based on the test against mosquito larvae of*Cullex pipiens, Aedes aegypti*, and *Aedes albopictus*, Perumalsamy et al. [37] assummed that acetylcholinesterase (AChE) is the main site of action of the flavonoids. Due to the great role of AChE in controlling impulses transmission between nerve cells, interference with this enzym this enzyme by itself will adversely affect the insects [38]. There are still many shortcomings from this study among others the characterization of flavonoid-rich fractions of the aqueous and methanolic extracts of the plant was not performed.

4. CONCLUSION

Based on the mortality rate, LC50 and LT50 obtained, this study results suggest that both crude aqueous and methanolic extracts of gamal (*Gliricida sepium*) leaves are toxic and lethal to the coffee mealybugs, *Planococcus citri*. Flavonoid-rich fraction of water extract of the gamal leaves extract is more effective in killing mealybug in comparison to the methanolic extract.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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