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Citronella Extracts: Chemical Composition, *In Vivo* and *In Silico* Insecticidal Activity against Fall Armyworm (*Spodoptera frugiperda* J.E Smith)

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Abstract

Citronella (*Cymbopogon nardus* L.) is a widely used plant in medicinal and agrochemical fields due to its unique secondary metabolite, citronellal. In this study, the chemical composition of citronella stem extract and its biopesticide activity against fall armyworm (*Spodoptera frugiperda*) was studied. Extraction of citronella with 96% ethanol was carried out using three methods, namely maceration, soxhletation, and microwave-assisted extraction (MAE). The chemical content in the extracts was analyzed using a GC-MS instrument. The potential of the extract as a biopesticide was carried out using the Completely Randomized Design (CRD) method by treating instar III larvae to citronella stem extract with a concentration of 0.5%; 1.0%; 1.5%; 2.5%; and 3.5% in triplicate. Data were analyzed using SPSS probit analysis. *In silico* computational study was done through molecular docking analysis. The results showed that the soxhletation method successfully obtained the highest citronellal percentage, 0.28%, compared to maceration (0.03%) and MAE (0.02%). Three citronellal compounds were detected in the extracts, namely citronellol, geranyl acetate, and hydroxy citronellol. The ethanol extract obtained by the soxhletation method was powerfully effective as a biopesticide with an LC_{50} of 0.456% in 12 hours of observation. Molecular docking analysis showed that all citronellal compounds contained in the extract had a binding energy lower than -5 kcal/mol indicating a potential bioactivity. Hydroxy citronellol had the lowest binding energy about -5.81 kcal/mol and two hydrogen bond interactions in Arg314 and Phe313 residues. It can be concluded that ethanolic extract of citronella stem can be used as a biopesticide, particularly against fall armyworm (*Spodoptera frugiperda*).

Keywords: Biopesticide; *Cymbopogon nardus* L.; molecular docking; natural insecticide; soxhletation

1. Introduction

Spodoptera frugiperda was firstly reported in Indonesia, in 2019. It was found in Lampung, West Sumatera, Banten, West Java, and Bali, and was predicted to spread to other provinces due to its great dispersal capacity [1–4]. This pest, a native pest from tropical America, invades Indonesia plantations by transportation from Africa [4]. It is an important pest in maize crops by 20-50% of loss and causes significant damage to other crops, such as rice, sweet potato, banana, strawberry, and many more [5]. The characteristics of *S. frugiperda* attack on corn plants are the presence of holes and fractures in the leaves at the age of 2-8 weeks after planting [3]. The chemical spray as an insecticide has been widely applied to control this pest [6]. However, due to its numerous drawbacks to non-target organisms and the environment [7–11], and the resistance of the pest to chemical insecticide [12], the utilization of biopesticide is highly recommended.

Citronella (*Cymbopogon nardus*) is known as a multifunctional plant, one of which is in the agricultural sector. Including in genus *Cymbopogon*, citronella was firstly found in Sri Lanka, Southeast Asia. It can grow well in Indonesia and can be found in several regions, such as in Aceh [13] and East Nusa Tenggara [14]. Citronella is one of the main components in citronella oil, with high antifungal properties [15]. Citronella is included in the terpenoid group belonging to monoterpenes, which can

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suppress the growth of pathogenic fungi [16]. Other major constituents, such as geraniol and citronellol, also exhibited potential bioactivity in pest management [17].

Several studies revealed that citronella extract was effective in being used as *Aedes Aegypti* repellent [18]. Not only as a pest repellent, citronella can also be used as a fumigant and oviposition inhibitor in stored products [19]. It performed potential used as a biopesticide against several pests, such as *Ulomoides dermestoides* [19], *Tribolium castaneum, Sitophilus oryzae*, and *Drosophila melanogaster*[20] in stored grain, as well as *Cryptolestes sp*, *Rhyzopertha Dominica*, *Palorus subdepressus*, and *Sitophilus zeamais* in stored coffee and cocoa [21, 22]. In addition, citronella oil can also be applied to control the population of field insects, gram pod borer (*Helicoverpa armigera*) [23].

This study aims to study the effectiveness of citronella stem extract as a biopesticide against the armyworm *S. frugiperda*. In addition, we also compare three extraction methods, namely maceration, soxhletation, and microwave-assisted extraction, based on the yields and chemical compositions of the extracts. The results of this study are expected to be the beginning of the development of renewable biopesticides from ethanol extract of citronella stems that can overcome the pathogenesis of the new pest *S. frugiperda* in the corn plantations in Indonesia.

Table 1.

The yield of extracts by using three different extraction methods

Sample	Sample Weight (g)	Dry Extract Weight (g)	Yield (%)
CE-1	20.0029	0.2901	1.45
CE-2	20.0022	2.5092	12.55
CE-3	20.0025	1.5081	7.54

2. Experimental

2.1. Plant Collection and Preparation

Citronella (C. nardus L) plants were collected from Palakka Sub-District, Bone District, South Sulawesi, Indonesia. The citronella stems were separated from the leaves, cut into small pieces and air-dried until constant weight. The dried samples were ground and sifted using a sieve of 20 mesh.

2.2. Sample Extractions

Three extraction methods were used in this stage, namely maceration, soxhletation, and MAE (Microwave-assisted extraction). All of them were carried out in the ethanol 96% (Merck) as a solvent with a 1:10 sample/solvent ratio. The three extraction processes were done using the following conditions: For maceration, the extraction was performed in 3x24 hours, while for soxhletation, it was performed for 90 minutes at 50 ^{o}C, and for MAE, it was extracted at 100 watts for 3x8 minutes using domestic microwave (Sharp Microwave Oven R21A1 22 L) with slight modification. All mixtures were filtered out, evaporated, and dried until dry extracts resulted. The chemical constituents of these three extracts were analyzed using the phytochemical screening method [24] and GC-MS (Gas Chromatography-Mass Spectrometry) QP2010 Ultra Shimadzu.

2.3. GC-MS Analysis

Each sample of 1 µL was injected into GC-MS, which was operated using a glass column with 25 m in lenght, 0.25 mm in diameter and 0.25 m in thickness, with a stationary phase of CP-Sil 5CB with an oven temperature programmed between 70-270 °C with a temperature rise rate of 10 °C/min. Helium carrier gas pressure was 12 kPa, the total rate was 30 mL/min and the split ratio was 1:50. The results in chromatogram graphs were obtained from GC analysis and molecular weight graphs from MS.

2.4. Rearing of Larva

The collection of *S. frugiperda* larva was done randomly from corn plantations in Takalar and Gowa Districts, South Sulawesi, Indonesia. The samples were reared until they got enough larvae in the third instar phase, which is then used to study biopesticide activity.

2.5. Biopesticide Activity of Extract

The Preliminary test was carried out to determine the concentration range of the extract to be applied in the toxicity study, which used 0%, 5%, 7.5%, and

10% of extract in ethanol 70% (Merck) as solvent. The 10 test larvae were dripped into 0.5 ml of each solution on the dorsal thorax and the ventral part of the insect body, namely the spiracle, as a natural hole or breathing hole. The test larvae that had been treated were placed in a petri dish and fed baby corn. The mortality of larvae was observed for 5 hours. The test was performed in 3 repetitions. The concentration ranges from the preliminary test, which gave significant larva mortality, were chosen to make the series of the extract solution to be used in the toxicity study. The procedure of the toxicity study was similar to the preliminary study, except for the observation duration, which was conducted for 12 hours. The study was performed in three repetitions.

2.6. Data Analysis

Analysis of data was carried out using normality, homogeneity, and ANOVA tests. The parameter used in this test is the mortality of the armyworm *S. frugiperda* to determine whether the method used is effective and efficient for the parameters to be achieved. The normality test used the Shapiro-Wilk test, and the homogeneity test used the Levene test. The toxicity data were analyzed using probit analysis with the SPSS Statistics Version 23 to determine the LC_{50} value.

2.7. Protein Structure Modelling and Molecular Docking Analysis

In this study, molecular docking of chemical contents in Citronella extracts was done against electrophorus electricus acetylcholinesterase enzyme (eeAChE). Some researchers reported that eeAchE is the target enzyme for inhibition of some difference insecticide. Due to the absence of this enzyme in protein data bank, the structure modelling was done through I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) by using UniProt code 042275. The best predicted model resulted in I-TASSER was determined based on C-score, the higher C-score value indicated the higher confidence of the model.

Molecular docking analysis of chemical contents was done using AutoDock4 with the help of AutoDockTools. The docking preparation including adding hydrogen, adding charge, etc was done in Chimera software. Docking protocol of each ligand was set to produce 10 conformations using The Lamarckian Algorithm and box grid size 40 x 40 x 40 with spacing 0.375 Å. Visualisation of interaction was displayed using Discovery Studio Visualizer program.

3. Results and Discussion

3.1. Citronella Extraction

The yield of the extracts, namely CE-1 (Maceration), CE-2 (Soxhletation), and CE-3 (MAE), are shown in Table 1. Several studies revealed that the extraction method can affect the yield and chemical content of the extract, besides affecting the effectivity of extraction process [25, 26, 27]. Compared to maceration and MAE methods, extraction using the soxhletation method in ethanol produced the highest yield. Moreover, the extract yield by soxhletation method was higher than extracted citronella oil using the distillation method [28].

Table 2.

Phytochemical Screening of Citronella Extracts

Phytochemical Screening	CE-1	CE-2	CE-3
Alkaloids	+	+	+
Flavonoids	+	+	+
Terpenoids	-	-	-
Saponins	+	+	+
Tannins	+	+	+
Steroids	-	-	-
Polyphenol	+	+	+

Soxhletation can obtain a large amount of extract and is relatively efficient in terms of duration and chemical used [29]. The temperature used, which was 50˚C, could not damage the chemical compounds in the extract. Extraction with the soxhletation method provides an advantage compared to other processes. In the soxhletation process, the solvent will continuously recirculate the sample to allow effective extraction [30].

Table 2 shows the phytochemical content of all extracts, which each contain alkaloids, flavonoids, saponins, tannins, and polyphenols. These results are also in accordance with the related studies conducted by Balakrishnan [31] regarding phytochemical

Table 3

Citronellal compounds in citronella extracts

Retention Time (min)	Chemical Compound	Structure	CE-1 (%)	CE-2 (%)	CE-3 (%)
9.56	Citronellol		-	1.26	0.37
17.86	Geranyl Acetate		2.26	2.72	0.33
23.21	Hydroxy Citronellol		2.13	0.51	0.98
		Total Citronellal	4.39	4.49	1.68

screening for citronella oil.

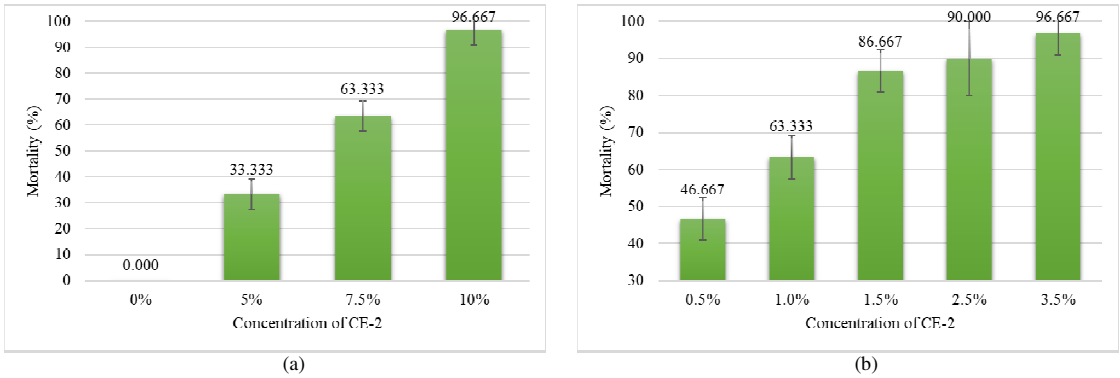
The chemical compounds of the citronellal group that were successfully analyzed from all extracts were citronellol, geranyl acetate and hydroxy citronellol, as shown in Table 3, except for CE-1, which contains no citronellol. This data is in accordance with previous report about chemical constituent of citronella stem[32].These compounds can act as natural insecticides such as repellants, antifeedants, and plant diseases control [17]. Moreover, they also have shown bioactivity as oviposition inhibitors as well as contact poisons which cause dehydration and result in death [16, 33].

This result showed that CE-2 extract contained higher total citronellal than CE-1 and CE-3. Therefore, CE-2 was selected to be used in the next stage, which is the bioactivity study of the extract as a biopesticide against fall armyworm,*S. frugiperda.*

3.2. Biopesticide Activity of Citronella Extract

Based on Figure 1(a), it can be concluded that the mortality of *S. frugiperda* is dose-dependent on citronella extract concentration. The morphological condition of the dead test larvae showed that the application of 10% extract greatly affected the mortality of the test larvae, so preliminary tests were carried out in the concentration range of 0 to 10%.

The use of extract with a concentration of 5% has shown significant mortality in test larvae at 5 hours of observation. Therefore, the selected concentration for the toxicity test is below 5% so that the mortality can be monitored better. Therefore, the concentration of 3.5%; 2.5%; 1.5%; 1.0%; and 0.5% were used in



(a) (b)

Fig. 1. Mortality of *S. frugiperda* by the treatment of CE-2 in preliminary test (5 h of observation) (a), and in toxicity test (12 h of observation) (b)

the toxicity test to determine the LC_{50} value for 12 hours of observation.

Figure 1(b) shows the average mortality which above 50% except for concentration of 0.5%. Furthermore, the mean mortality at a concentration of 3.5% did not statistically different to the concentrations of 1.5% and 2.5%. During the toxicity test, the larvae were observed in convulsion orirregular movement activities, and not long died. In the thoracic part of the insect body, there is a thoracic ganglion or ganglia or central nerve in the thorax which is connected to all nerve cells throughout the body. The ganglion in the thorax is connected to the Deuterocerebrum in the brain, which coordinates the movements of the thoracic segments. If there is a disturbance in the thoracic ganglion, then the coordination of movement to the central nervous ganglia in the Deutoceremrum (brain segment) will be hampered, resulting in irregular (uncoordinated) movements which are commonly called convulsive movements and will then die [33, 34].

Table 5.

Duncan test

	Subset Score*		
Treatment	1=a	2=b	3=c
0.5%	4.67		
1.0%		6.33	
1.5%			8.67
2.5%			9.00
3.5%			9.67
Sig	1.00	1.00	0.117

* same notation shows no significant difference. Different notation shows significant difference.

The citronellal content in the citronella stem extract plays an important role as a biopesticide, which has toxic properties of dehydration. Citronellal is a contact poison to pest that can lead to death due to continuous fluid loss. Insects exposed to this poison can die due to the lack of body fluids [33, 35, 36].

Based on Table 4, the normality test of the data obtained through the Shapiro-Wilk test showed that the data are normally distributed. Moreover, the homogeneity test of the data through the Leven Test shows that the homogeneity requirements in the regression model has been met, which means that the data is homogeneous and comes from the same variance. Meanwhile, the ANOVA test result showed a significance value of 0.000 <0.05, where there is a significant difference in the data. Thus, a further test was carried out, namely Duncan Test. Based on the

Table4.

Data analysis

Test	Significance
Normality	0.166
Homogeneity	0.866
Anova	0.000

results of Duncan's test in Table 5, the results obtained at concentrations of 0.5% and 1.0% were significantly different, while at a concentration of 1.5%; 2.5% and 3.5% were not significantly different.

Based on statistical analysis, LC_{50} (LCL-UCL) value of citronella stem extract against *S. frugiperda* for 12 hours of observation after treatment is 0.456% (0.357 - 0.872%). Compared to another biopesticide study of plant extracts against *S. frugiperda*, such as tobacco and papaya leaf extracts(50% of effective concentration for both)[37, 38], it can be stated that citronella extract showed better biopesticide activity against *S. frugiperda*. Thus, it has potential application as a natural insecticide. However, before it can be applied in the field, further studies must be conducted, such as the biopesticide activity against other pests as well as the toxicity to non-target organisms.

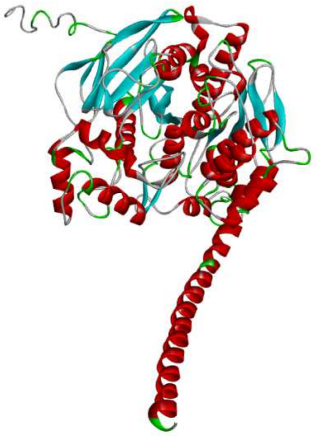


Fig. 2.Tertiary structure of *ee*AChE model resulted by I-TASSER server

Table 6. Docking result of three chemical contents in citronella extract

Chemical Contents	Binding Energy (kcal/mol)	Inhibition Constant (μM)	Hydrogen bond interaction
Citronellol	-5.33	124.91	Arg314; Phe313
Geranyl Acetate	-5.18	159.12	Phe313
Hydroxy Citronellol	-5.81	54.84	Arg314; Phe313

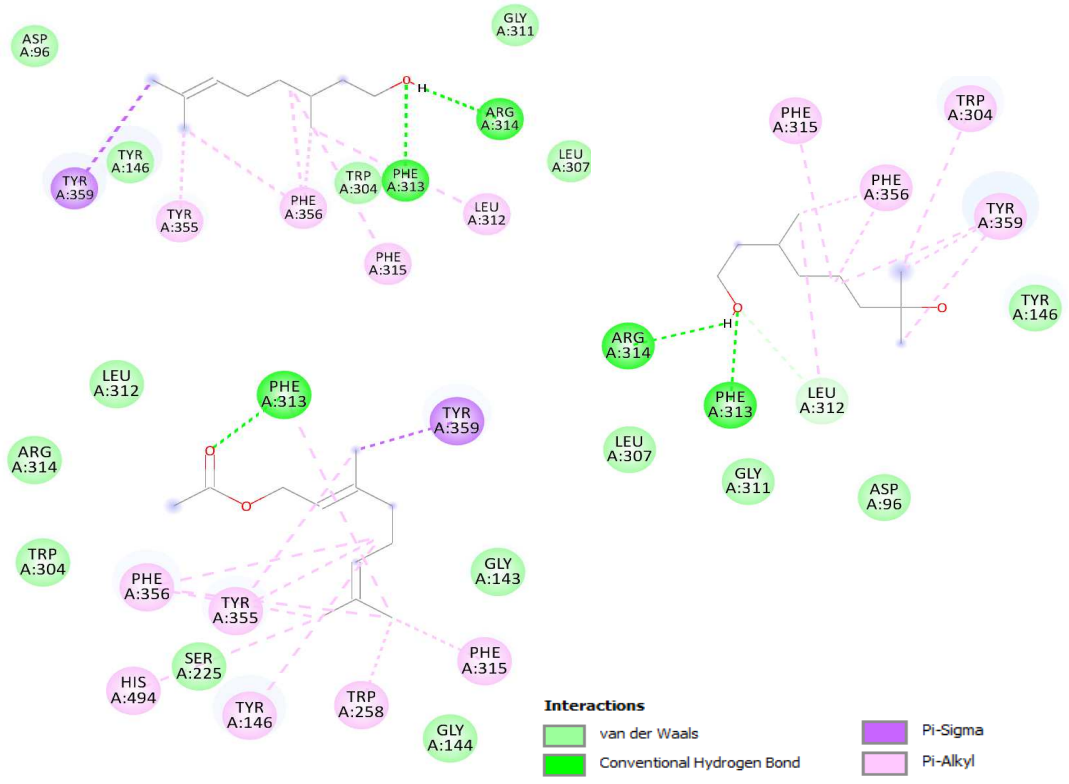


Fig. 3. 2D-interaction between chemical content and *ee*AChE enzyme: (a) citronellol, (b) geranyl acetate, (c) hydroxy citronellol

3.3. Molecular Docking Analysis

To perform molecular docking analysis, we had to predict the tertiary structure of *ee*AChE. This enzyme was used to study insecticide activity controlling agents against some pests, including*Spodoptera frugiperda*[39]. Jintana *et al.* revealed that there was significant correlation between the symptont showed by the pest after insectide exposure and the inhibition of AChE activity [40].Since there was no report of this enzyme in the protein data bank (PDB), we did protein structure modelling using I-TASSER server [41-43]. Result of modelling gave us top 5 protein models which were ranked by C-score of each model. The selected model is the one that has a highest C-score model. C-score is a confidence score for estimating the quality of predicted models by I-TASSER. Figure 2 showed the selected model that has C-score value of -0.28 and estimated TM-score of 0.68 ± 0.12. This model was then used in molecular docking analysis.

There were three chemical contents that docked in the active site of *ee*AChE structure. Table 6 was the summary of docking result from three chemical contents in citronella extracts. A comparison of each content in binding energy showed that hydroxy citronellol has the lowest energy value, which is caused by the interaction resulted from the ligands and enzyme structure. Binding energy was the sum of interaction energy between ligand and protein, the more interactions resulted in the lower binding energy. Figure 3 showed the 2D-interaction of each ligand in the active site of eeAChE enzyme. All chemical contents showed hydrogen interactions in Phe313 residue indicating that all chemical contentswere in the same site of the enzyme. Additional hydrogen bond interaction was found between Arg314 residue and hydroxyl group in citronellol and hydroxy citronellol. Binding energy result of all chemical contents showed that all content could be classified as potential bioactive compounds because they had a binding energy lower than -5 kcal/mol [44].

4. Conclusion

Compared to the maceration and MAE methods, soxhletation extraction gave the highest extract yield and citronellal content, including citronellol, geranyl acetate, and hydroxy citronellal. Ethanol extract of Citronella stem was effective as a biopesticide against fall armyworm *S. frugiperda* with an LC_{50} value of 0.456% in 12 hours of observation. Molecular docking study showed hydroxy citronellol had the lowest binding energy against eeAChE enzyme about -5.81 kcal/mol and two hydrogen bond interactions in Arg314 and Phe313 residue.

5. Conflicts of interest

There are no conflicts to declare.

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