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1 Repellent Activity of *Piper* spp. Leaves Extracts on Rice Ear Bugs (*Leptocorisa oratorius* Fabricius) and the Characters of Its Volatile Compounds

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Authors' contributions

This work was carried out in collaboration among all authors. Author LHN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RP and RCHS managed the analyses of the study. Author RCHS managed the literature searches. Authors ERS and SW developed modified olfactometer. Author Jekli identified *Piper* spp. Authors YSH and IQL applied and analysed GC-MS. All authors read and approved the final manuscript.

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ABSTRACT

1 Rice ear bug (*Leptocorisa oratorius* Fabricius) is still classified as the top of five pests that cause the most losses in rice farming productivity. The attack causes the grain to become empty resulted in reducing the yield of grain up to 40% even to 100%. Betel plants (Genus *Piper*) are easy to grow and grown by almost all households in Indonesia. Ethnobotany showed that betel is used as one of the insect controllers. This research aimed to study the repellent activities of five species of *Piper*

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1 spp. and their phytochemical composition based on CG-MS chromatograms. Moreover, the active compounds were analyzed in potent plant. The repellent activities of chloroform and methanol extract of *P. betle*, *P. aduncum*, *P. nigrum*, *P. longum*, and *P. crocatum* were applied using modified olfactometer, while the phytochemical composition were analyzed using GC-MS. The compounds with insecticidal activity in the potent species were analyzed by the references. The results showed that chloroform extract of *P. crocatum* was the most potent extract. It contains 28 specific compounds which were not found in other species. Literature studies showed that compounds that had repellent activities are monoterpene and sesquiterpene groups.

Keywords: Rice ear bug; repellent; *Piper* spp.; volatile compounds; biopesticide.

1. INTRODUCTION

Rice ear bug (*Leptocorisa oratorius* Fabricius) of the family, Alydidae order Hemiptera, is a pest, either as adult insect or nymph, which can attack the grain of the flowering phase until the ripe milk. It is very dominant in lowland rice fields [1]. This pest caused 5.59%-14.23% damage to rice in the reproductive phase (ripe milk) in the paddy fields of the Dusun Besar Village of Bengkulu, Indonesia [2]. In Maluku Province of Indonesia, the damage occurred by 22.95% due to rice ear bug and 3.30% caused by white rice borer [3].

Various methods of pest control, such as biological, mechanical and chemical have can be used in controlling the pest. Pest control using chemical synthetic pesticides is still the main choice even though it has been known to have a serious impact on ecosystems and may be toxic to animals, humans and non-target organisms. Excessive use of synthetic pesticides also causes pest resistance to various pesticides [4]. As an alternative, currently there many integrated pest control efforts with Integrated Pest Management (IPM) which include the development of pest-resistant varieties, and the development of biopesticides or vegetable pesticides. Biopesticides have been developed as insecticides which are directed to the discovery of compounds that are not only effective in pest control but also have selective activities against certain insect pests. Rational control agents can change insect growth regulators and change the behavior of insects, such as pheromones and allelochemistry [5].

Vegetable pesticides come from plant parts such as roots, tubers, leaves, skin, stems, fruit and seeds. The ingredients are processed into various forms, including flour, extract or resin which is the secondary result of metabolite fluids from plant parts as insecticides [6]. Environmental pest controls have been carried out by several researchers using *Piper* plant extracts. In a research conducted by Yunianti [7],

the application of a green betel (*Piper betle*) leaves water extract with a concentration of 75% can kill pests of stinky rice pest as much as 60%. Jayalakshmi et al. [8] reported that the methanol leaves extract of *P. betle* contain of flavonoids, tannins, steroids, glycosides, proteins and carbohydrates, while chloroform leaves extracts of *P. betle* contained alkaloids, glycosides and proteins.

Leaves extracts compounds of other members of the genus *Piper* are also identified. The methanol leaves extract of red betel (*Piper crocatum*) was conducted by Lister et al. [9], the results showed that the leaf extract of the plant contain of alkaloids, flavonoids (flavonols, flavanols, isoflavones, aurons, catechins, antocyanidines, chalcones), saponins, triterpenoids, and tannins. In current research, therefore, the repellent activity of *Piper* spp. leaves extracts and its secondary metabolite compositions were analyzed and detected.

2. MATERIALS AND METHODS

2.1 Sample Preparation

The five species of *Piper*: *Piper betle*, *P. aduncum*, *P. nigrum*, *P. longum*, and *P. crocatum*, were identified in the laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The specimens and leaves samples were stored at the Laboratory of Plant Structure and Development, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Preparation of five *Piper* spp. leaves extract using the maceration method with chloroform and methanol solvent. The leaves were cleaned of dirt, dried under the sunlight while covered by black cloth, then dried in an oven at 40 °C and mashed into powder. Five grams of the powder was put into an Erlenmeyer flask, soaked using 150 ml of chloroform and covered with aluminum foil for 24 hours while occasionally shaken. After

24 hours the pulp was separated from the filtrate by filter paper. The chloroform filtrate was placed on a porcelain cup and concentrated with a fan. The pulp which has been separated from the filtrate was dried in an oven at 40°C and after drying the pulp was macerated for 24 hours using a second solvent, 150 ml of methanol. After 24 hours, methanol filtrate was separated from the pulp by filter paper, then concentrated with a fan. Next, the chloroform and methanol extract from *Piper spp.* were stored in a cold room for further test.

2.2 Repellent Activity Test

Piper spp. leaves extracts were tested for their repellent activity to rice ear bug using modified olfactometer from Netto [10] with three different concentrations. Each kind of *Piper spp.* leaves extracts were diluted using water added with DMSO to 25%, 37.5% and 50% of extracts concentrations [11,7].

Repellent test was done by placing one pot of rice mature milk in each arm of the olfactometer (35cm x 35cm x 75cm). The extract solution was sprayed on the treatment of rice plants for bioassays. Each test unit was used as many as 10 rice ear bugs that have been acclimatized for 24 hours, then were released into the olfactometer body. Observations were carried out for 24 to 36 hours. The behavior of the rice ear bug observed was the preference of the bug to choose either rice plants that treated with *Piper spp.* leaves extract treatment or control. The treatments were carried out with 3 replications.

2.3 Identification of Chemical Compounds

The chemical compounds of *Piper spp.* chloroform leaves extracts were analyzed by GC-MS (Agilent GC 6890N 5975B MSD). The capillary column was Agilent 19091S-433 model, HP-5MS 5% Phenyl Methyl Siloxane. The oven temperature was programmed as follows: initial temperature at 100°C, initial time for 1.00 minute, final temperature at 300°C for 10.0 minute. Identification of components in sample used Wiley7Nist05.L data based.

2.4 Data Analysis

Determination the repellency of chloroform leaves extract of *Piper spp.* which has the highest repellent by calculating the value of Inhibition Concentration (IC) using probit analysis [12]. To calculate the IC value required percent inhibition data from the tests conducted. Percent

inhibition can be calculated using the formula Gour and Tembhe [13] as follows:

$$\% \text{ inhibition} = \frac{\text{Control inhibition} - \text{treatments}}{\text{Control inhibition}} \times 100\%$$

Chromatograms of *Piper spp.* leaves extract were compared based on the kind of components and the concentration of each component. Data were correlated with the results of repellent activity test. The characteristic compounds (resulted from GC-MS chromatograms) in the potent sample were predicted as the potent compounds.

3. RESULTS AND DISCUSSION

3.1 Modified Olfactometer

In this study, the olfactometer was used based on Netto's research [10] as shown in Fig. 1. The use of Netto's olfactometer design failed due to no movement of rice ear bugs into a box filled with ripe rice milk when optimizing olfactometer was done. Therefore, the olfactometer design was modified by removing the rice bug storage box, the connecting hose between the rice bug storage box and the milk-cooked rice box, and the glass cover. In new modified olfactometer design, the glass cover was replaced with a cardboard with a hole in the bottom so that the movement space was more widespread, the side of the cardboard was affixed with transparent plastic, so that light can enter the cardboard, as shown in Fig. 2 and Fig. 3. The olfactometer design as the results of the modification were further used in the repellent test because at the time of the optimization, it was shown that rice ear bugs were able to move freely and alighted on the mature rice milk plant.

3.2 Repellent Activity

Determination of the most potential extracts to drive out rice ear bug using probit analysis was seen from the IC₅₀ value. Based on the probit analysis, it was found that the IC₅₀ values of the *P. crocatum* chloroform extract and methanol extract were 3.71% and 19.61%, while those of the chloroform and methanol extract of *P. betle* was 24.99 (Fig. 4). It was known that the *Piper* extract which has the most potential to drive out rice ear bugs was the chloroform and methanol extract of *P. crocatum*. Next, the chloroform extract of *P. crocatum* was used in analyzes of phytochemical composition based on GC-MS chromatograms.



Fig. 1. First modified olfactometer



Fig. 2. Second modified olfactometer

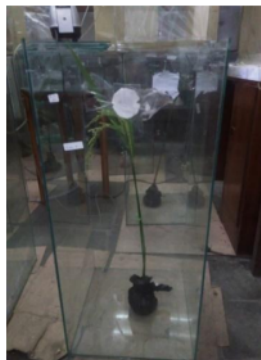


Fig. 3. Detail of the second modified Olfactometer. From left to right – Cage with rice plant, the hole bottom of cardboard as glass cover replacement, connection of cage, covers and aerators

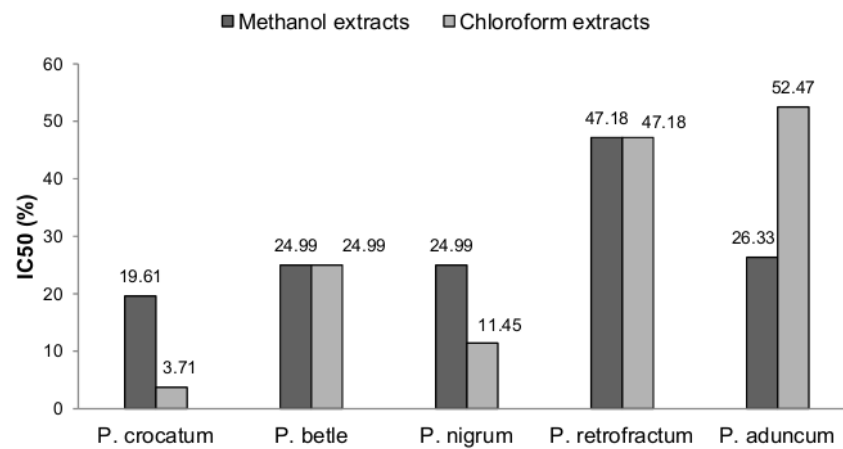


Fig. 4. Repellent activities of chloroform and methanol extract from five species *Piper* on rice ear bug (*Leptocorisa oratorius* Fabricius)

From the Fig. 4, it can be seen that chloroform extract of *Piper crocatum* was the most potent extract since it had the lowest IC₅₀. The lower repellent concentration IC₅₀ means the highest repellence activity. The activities of plant extract were depending on the phytochemicals composition of each solution which had active compound chase rice ear bug from the mature milk rice. The most active compounds were normally secondary metabolites. It is known that secondary metabolites are characteristic for each species although known that the production are affected by their environment.

Repellence of rice ear bugs caused by *Piper spp.* leaves extract was suspected through several ways, namely fumigant and nervous system. Moreover, it can occur as fumigant by the statement of Matsumura [14], respiratory poison is an effect when a compound enters the body of an insect and can interfere with the performance of the respiratory tract. Insecticidal compounds can enter the body of insects in the form of gas through stigma or spiracles that are in the respiratory tract and into the trachea and finally can enter the tissue.

Piper spp. compounds, especially from *P. crocatum* extract, is quickly evaporates and captured by the olfactory receptor neurons in the sense of smell and antenna of rice ear bugs. This will affect nerves function by inhibiting the performance of the enzyme acetylcholinesterase. When the performance of the acetylcholinesterase enzyme is inhibited, the hydrolysis of acetylcholine becomes disrupted, so that the accumulation of acetylcholine increases and causes the muscle performance to continue to be active, quickly tense, and it can cause paralysis and even death effects on insects [15,16]. Non-polar compounds are also found in *Piper spp.* extracts, such as alkaloids and terpenoids. In addition, the compounds in *Piper spp.* leaves extract can be antifeedant, when the extract in high concentration, insects will not eat the grains of rice affected by the extract. This can cause a lack of food intake and energy production in the stink bugs, so the stink bugs will move, and even lead to death [17].

3.3 Volatile Compounds of *Piper spp.*

Based on mass spectra of gas chromatograms followed by identification of the spectra based on the similarity with Wiley7Nist05.L data based, it were known that there were high variation of secondary metabolites. Total number of

compounds found in five leaves extracts of *Piper spp.* were 88 compounds. *P. crocatum* and *P. longum* leaves extracts contained the highest number of compounds (71 kind of compounds) followed by *P. nigrum*, *P. betle*, and *P. aduncum* leaves extracts with the number of compounds were 45, 42, 37 compounds respectively (Table 1).

3.4 Specific Compounds of *P. crocatum* Chloroform Leaves Extract

Concerning the repellent activities results, it found that highest repellent activities was chloroform extract of *P. crocatum*. The number of specific compounds in *P. crocatum* played an important role in the repellent activities considering that chloroform extract of *P. longum* had the same number of compounds. Therefore, the analysis was continued to identify a specific compound in the chloroform extract of *P. crocatum* which were not found in other extracts using GC-MS. The compounds were showed in Table 2. The example of GC-MS chromatogram from the chloroform leaves extract of *P. crocatum* could be seen in Fig. 5.

From data in Table 2, it was known that there were 28 compounds in the chloroform extract of *Piper crocatum*, which were not found in other extracts. The compounds consists of terpenes, monoterpenes, sesquiterpense, hydrocarbons, and fatty groups. Myrcene (monoterpene) was found to be the highest content. Based on literature studies it is known that some of them were known had repellent activities. The description is displayed in Table 3. From the literature studies, it is known that some of the specific compounds found in chloroform extract of *P. crocatum* showed repellent and toxic activities. Therefore, it was the reason why chloroform extract of *P. crocatum* played an important role in rice ear bug repellent.

3.5 Terpenes as a Chemical Defense

Terpenes are the largest group of natural products from plants comprising essential oils, flavours, fragrances, and lipid-soluble plant pigments. These hydrophobic compounds are usually stored in plants in resin ducts, oil cells glandular trichomes. They are derived from 5-carbon isoprene units, such as C₅ hemiterpenes, C₁₀ monoterpenes, C₁₅ sesquiterpenes, C₂₀ diterpenes, C₂₅ sesterpenes, C₃₀ triterpenes, C₄₀ tetraterpenes, and C₅₀ band over polyterpenes [33].

The mixtures of terpenes containing compounds with different physical properties may be more toxic with longer persistence of defenses. Terpenes synergize the effects of other toxins by acting as solvents to facilitate their passage through

membranes. An example of such synergism seems to occur in conifer resin, which is a mixture of monoterpene olefins with anti-herbivore and anti-pathogen activity, and diterpenes that are toxic and deterrent to herbivores [16].

Table 1. Volatile compounds of five *Piper spp.* chloroform leaves extract

No.	Compound names	<i>P. aduncum</i>	<i>P. betle</i>	<i>P. crocatum</i>	<i>P. longum</i>	<i>P. nigrum</i>
1	1-Hexadecanol	0	0	1	1	0
2	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-	1	0	0	0	0
3	9,12-Hexadecadienoic acid, methyl ester	0	0	0	0	1
4	9,12-Octadecadienoic acid	1	0	0	0	1
5	9-Eicosyne	1	1	0	0	0
6	Benzenepropanoic acid, methyl ester	0	0	0	1	0
7	Benzylnalonic acid	0	0	0	1	0
8	Cholest-5-en-3-ol (3.β.)-, 22-hexadecanoate	1	1	0	0	0
9	Cyclohexane, 2-chloro-4-methyl-1-(1-methylethyl)-	1	0	0	0	0
10	Cyclohexane, eicosyl-	1	0	1	0	0
11	Ergost-5-en-3-ol, (3.β.)-	1	1	0	0	1
12	Eugenol	1	1	0	0	0
13	Hexadecanoic acid	1	1	0	1	1
14	Hexadecanoic acid, methyl ester	1	1	1	1	0
15	Hinokinin	0	0	0	0	1
16	Neophytadiene	1	0	1	1	0
17	Octadecanoic acid	1	1	1	1	0
18	Octadecanoic acid, 10-oxo-, methyl ester	1	0	0	0	0
19	Octadecanoic acid, methyl ester	1	0	1	0	0
20	Phytol	0	1	1	1	1
21	Piperine	0	0	0	1	1
22	Stigmast-4-en-3-one	1	0	1	0	0
23	Stigmast-5-en-3-ol, (3.β.,24S)-	0	0	0	1	1
24	Stigmasta-5,22-dien-3-ol, acetate, (3.β.,22Z)-	1	1	0	0	0
25	Stigmasterol	0	1	1	1	1
26	Tridecanol	1	0	0	0	0
27	Vitamin E	0	1	1	1	1
28	Eugenyl acetate	0	1	0	0	0
29	Globulol	0	1	0	0	0
30	Octadecanal	0	1	0	0	0
31	9,12-Octadecadienoic acid, methyl ester	0	1	1	0	0
32	Tetrahydroionone	0	1	0	0	0
33	Diethyl phthalate	0	1	1	1	1
34	2-Palmityl-1,3-diacetin	0	1	0	0	0
35	2-Linolenyl-1,3-diacetin	0	1	0	0	0
36	Isomyrcenylacetate	0	1	0	0	0
37	Dicholesteryl succinate	0	1	1	0	1
38	Epiaplysterylacetate	0	1	0	0	0

No.	Compound names	P. aduncum	P. betle	P. crocatum	P. longum	P. nigrum
39	Chavicol	0	1	0	0	0
40	Kauren-18-ol, acetate, (4.beta.)-	0	1	0	0	0
41	5.alpha.-Ergost-8(14)-ene	0	1	0	0	0
42	Myrcene	0	0	1	0	0
43	Limonene	0	0	1	0	0
44	Ocimene	0	0	1	0	0
45	Terpinolene	0	0	1	0	0
46	Myrcenol	0	0	1	0	0
47	Geraniol	0	0	1	0	0
48	4-Tolualdehyde	0	0	1	0	0
49	1,6-Octadiene, 3,5-dimethyl-	0	0	1	0	0
50	Caryophyllene	0	0	1	1	0
51	Bergamotene	0	0	1	0	0
52	Gamma-Cadinene	0	0	1	0	0
53	Ledol	0	0	1	0	0
54	Bisabolol	0	0	1	0	0
55	1-Octadecyne	0	0	1	1	1
56	3-Octadecyne	0	0	1	0	0
57	Trans-Stigmasta-5,22-dien-3.bet-ol	0	0	1	0	0
58	4,22-Cholestadien-3-one	0	0	1	0	0
59	Pentalene, octahydro-1-(2-octyldecyl)	0	0	1	0	0
60	Sabinene	0	0	1	0	0
61	Terpilene	0	0	1	0	0
62	Crithmene	0	0	1	0	0
63	Linalool	0	0	1	0	0
64	beta.-Elemene	0	0	1	0	0
65	Zingiberene	0	0	1	0	0
66	beta.-Selinene	0	0	1	0	0
67	Gamma-Cadinene	0	0	1	0	0
68	Alpha.-Copaene	0	0	1	0	0
69	Gamma.-Gurjunene	0	0	1	0	0
70	Nerolidol	0	0	1	0	0
71	Caryophyllene oxide	0	0	1	1	0
72	Farnesene	0	0	1	0	0
73	6-Octadecenoic acid, methyl ester	0	0	1	0	0
74	1-Eicosanol	0	0	1	0	1
75	5-Octadecenoic acid, methyl ester	0	0	0	1	0
76	11,14-Eicosadienoic acid, methyl ester	0	0	0	1	0
77	2-Decenylsuccinic anhydride	0	0	0	1	0
78	1-Undecyne	0	0	0	1	0
79	Germacrene d	0	0	0	1	0
80	1-Decene, 8-methyl-	0	0	0	1	0
81	9-Octadecen-1-ol	0	0	0	1	0
82	9,12,15-Octadecatrienal	0	0	0	1	1
83	Oxirane, dodecyl	0	0	0	1	0
84	Cyclohexane, (2-ethyl-1-methyl-1-butenyl)-	0	0	0	0	1
85	Alloaromadendrene	0	0	0	0	1
86	Patchulane	0	0	0	0	1
87	3,4-(Methylenedioxy)toluene	0	0	0	0	1
88	Gamma.-Tocopherol	0	0	0	0	1
Total number of compounds		37	42	71	71	45

Note: 0: absent, 1: presence

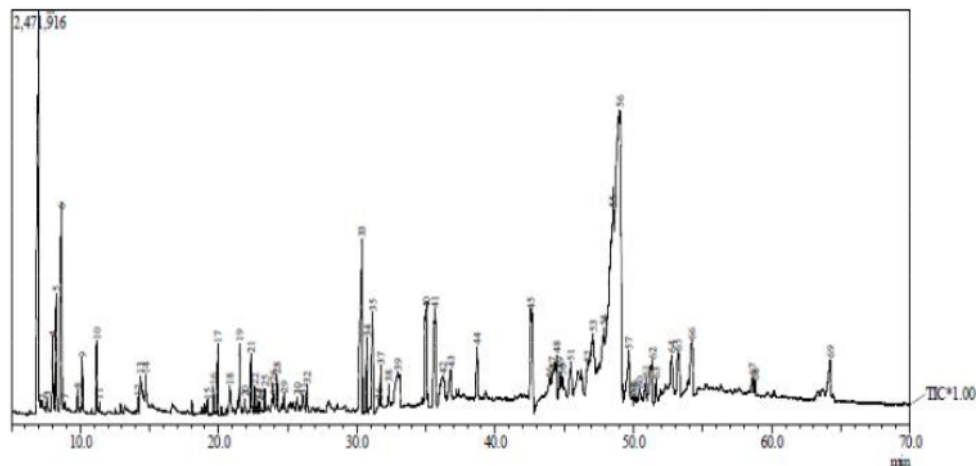


Fig. 5. GC-MS chromatograms of *P. crocatum* chloroform leaves extract

Table 2. Specific compound in the leave of *P. crocatum* which were not found in other four *Piper* spp. detected from GC-MS chromatograms

No.	Compounds	Groups	Retention time (minute)	% Area
1	Sabinene	Monoterpene	7.258	0.26
2	Terpilene	Monoterpene	7.622	0.31
3	Crithmene	Monoterpene	8.892	0.1
4	Myrcene	Monoterpene	6.984	8.6
5	Limonene	Monoterpene	8.006	1.06
6	Ocimene	Monoterpene	8.654	1.56
7	Terpinolene	Terpene	9.817	0.22
8	Linalool	Monoterpene	10.147	0.62
9	Myrcenol	Monoterpene	11.375	0.17
10	Geraniol	Monoterpene	14.164	0.17
11	4-Tolualdehyde	Aldehyde	14.353	1.14
12	1,6-Octadiene, 3,5-dimethyl-	Hydrocarbon	14.732	0.53
13	Beta.-Elemene	Sesquiterpene	19.188	0.16
14	Zingiberene	Sesquiterpene	19.642	0.26
15	Beta.-Selinene	Sesquiterpene	20.81	0.49
16	Bergamotene	Sesquiterpene	22.343	0.84
17	Gamma-Cadinene	Sesquiterpene	22.597	0.37
18	Alpha.-Copaene	Sesquiterpene	22.825	0.11
19	Gamma.-Gurjunene	Sesquiterpene	22.975	0.1
20	Nerolidol	Sesquiterpene	23.342	0.3
21	Caryophyllene oxide	Sesquiterpene	23.937	0.43
23	Farnesene	Sesquiterpene	24.042	0.18
24	Ledol	Sesquiterpene	25.656	0.66
25	Bisabolol	Sesquiterpene	26.374	0.28
26	3-Octadecyne	Hydrocarbon	31.016	0.79
27	6-Octadecenoic acid, methyl ester	Fatty acid	31.575	0.15
28	1-Eicosanol	Fatty alcohol	38.674	0.83

Table 3. Essential oil of *P. crocatum* chloroform leaves extract with insecticidal potency

No.	Compounds	Groups	Biological properties	Target insects	References
1	Myrcene	Monoterpene	Repellent activity	<i>Tribolium castaneum</i> Herbst	[18]
2	Limonene	Monoterpene	Repellent activity	<i>Tribolium castaneum</i> Herbst	[18,19]
3	Ocimene	Monoterpene	Repellent activity	<i>Anopheles arabiensis</i> , <i>Aedes aegypti</i>	[20]
4	Terpinolene	Terpene	Fumigant, Repellent activity	<i>Tribolium castaneum</i> , <i>Liposcelis bostrychophila</i>	[21]
5	Geraniol	Monoterpene	Repellent activity, antifeedant effect	<i>Tyrophagus putrescentiae</i> , <i>Otodectes cynotis</i> , <i>Periplaneta americana</i> , <i>Blaberus discoidalis</i>	[19]
6	Bergamotene	Sesquiterpene	Systemic defense from herbivory	<i>Andruca sexta</i>	[22]
7	Ledol	Sesquiterpene	Aphidicidal activities	<i>Aphis gossypii</i> Glover, <i>Aphis spiraeicola</i> Patch, <i>Myzus persicae</i> (Sulzer)	[23]
8	Bisabolol	Sesquiterpene	Insecticidal activity, larvacidal	<i>Bemisia argentifolii</i> , <i>Aedes aegypti</i>	[24,25]
9	Sabinene	Monoterpene	Repellent activity	<i>Bemisia tabaci</i>	[26]
10	Linalool	Monoterpene	Repellent activity	<i>Tribolium castaneum</i> , <i>Lasioderma serricornis</i> , <i>Liposcelis bostrychophila</i>	[27]
11	Beta elemene	Sesquiterpene	Repellent activity	<i>Bemisia tabaci</i>	[26]
12	Gamma cadinene	Sesquiterpene	Insecticidal, Repellent activity	<i>Sitotroga cerealella</i>	[28]
13	Caryophyllene oxide	Sesquiterpene	Toxic effects, repellency and respiration rate	<i>Sitophilus granarius</i> L.	[29]
14	Zingiberene	Sesquiterpene	Repellent activity	<i>Tribolium castaneum</i>	[30]
15	Farnesene	Sesquiterpene	Repellent pheromone	<i>Myzus persicae</i>	[31]
16	1-Eicosanol	Fatty alcohol	Attractiveness, Repellent activity	<i>Apis andreniformis</i> , <i>Apis florea</i>	[32]

Tholl [34] said that terpenes are produced by plants to control pests and as abiotic stress defense. In this study, compounds from the terpene, monoterpene and sesquiterpene groups were found in the leaves of *Piper spp.*, especially in *P. crocatum*. This further reinforces that the terpene group has the potential as a biopesticide to repel rice ear bugs.

4. CONCLUSION

Chloroform leaves extract of *P. crocatum* was the most potent material as the repellent of rice ear bug than those of four others. Based on GC-MS studies, the extract had 28 specific compounds which were not found in the other species. Literature studies showed that most of the compounds had repellent activities from monoterpene and sesquiterpene groups.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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