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Research Article

Evaluation of Two Different Solvents for *Azolla pinnata* Extracts on Chemical Compositions and Larvicidal Activity against *Aedes albopictus* (Diptera: Culicidae)

Rajiv Ravi,[1] **Nor Shaida Husna Zulkrnin**,[1] **Nurul Nadiah Rozhan**,[1] **Nik Raihan Nik Yusoff**,[1] **Mohd Sukhairi Mat Rasat**,[2] **Muhammad Iqbal Ahmad**,[2] **Zulhazman Hamzah**,[1] **Intan H. Ishak**,[3,4] **and Mohamad Faiz Mohd Amin**[1]

[1]*Faculty of Earth Science, Universiti Malaysia Kelantan, Jeli Campus, Jeli, Kelantan, Malaysia*
[2]*Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan, Jeli Campus, Jeli, Kelantan, Malaysia*
[3]*School of Biological Sciences, Universiti Sains Malaysia, Minden, Penang, Malaysia*
[4]*Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Minden, Penang, Malaysia*

Correspondence should be addressed to Rajiv Ravi; rajiv_ravi86@yahoo.com, Intan H. Ishak; intanishak@usm.my, and Mohamad Faiz Mohd Amin; mohamadfaiz@umk.edu.my

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Limited success for *Aedes* control program has impelled the necessities for new insecticide search. Hence, alternative plant compounds may be competent to overcome the pesticide resistance problem and to lead a chemical-free environment. Following go-green conceptions, larvicidal effects of the *Azolla pinnata* extracts using methanol and acetone solvent against *Aedes albopictus* late 3rd instar larvae were evaluated. The *A. pinnata* fresh plant from Kuala Krai, Kelantan, Malaysia (5° 31′ N 102° 12′ E) was used for crude extraction with Soxhlet apparatus using methanol and acetone solvents. Next, larvicidal test following WHO guidelines was tested against late 3rd instar to early 4th instar larvae of *Ae. albopictus* mosquitoes. Meanwhile, the chemical composition of extracts and their structures have been identified using GCMS-QP2010 Ultra (Shimadzu) fitted with Rtx-5MS capillary column (30 m × 0.25 mm inner diameter, ×0.25 μm film thickness; maximum temperature, 370°C), coupled to QP2010 Ultra (Shimadzu) MS. Results of methanol solvent showed the highest larvicidal activity against late 3rd instar to early 4th instar *Ae. albopictus* larvae with LC_{50} and LC_{95} values of 867 ppm and 1293 ppm at 24 hours, respectively, and 647 ppm and 972 ppm at 48 hours, respectively. Meanwhile, acetone solvent compounds were recorded with LC_{50} and LC_{95} values of 1072 ppm and 1302 ppm at 24 hours, respectively, and 904 ppm and 1126 ppm at 48 hours, respectively. Finally, the chemical composition of *A. pinnata* plant extracts has been characterized for 35 active compounds from methanol solvent and 37 active compounds with acetone solvent. In conclusion, *A. pinnata* plant bioactive molecules are efficient and could be developed as an eco-friendly, "go-green" approach for mosquitoes' larvicidal control programs. Thus, our study suggests that future research can be conducted on *A. pinnata* bioactive ingredients against *Ae. albopictus* larvae in small-scale field trials as botanical insecticide for environmentally friendly approach.

1. Introduction

Mosquitoes cause great health problems in the world because of their predominant role in causing malaria, dengue fever, yellow fever, Zika, and several other disease transmissions [1]. A total of 124 countries were affected by dengue epidemics with approximately 3.61 billion humans at high risk of being infected and yearly 500 million people in denture infection effectively [2]. Meanwhile, Malaysia has recorded 55,744 dengue cases with 131 deaths between January and July 2017 [3].

Vector control programs are requisite part of global strategy for managing mosquito-borne diseases, and commonly used insecticide applications are the most essential components for this sector [4]. In addition to that, the mosquitoes in the juvenile stages can be killed before it emerges into

haematophagous adults, by the larvicidal applications [4]. Since larvae are only bound to their habitats, the control operations will be much easier with larvicides. Although current synthetic chemical control agents are common and effective, their constant repetitive applications have resulted in resistant mosquitoes and environmental pollutions. Hence, the limited success of biocontrol programs on *Aedes* has encouraged the necessity for new insecticide search [5, 6].

Plant products produced positive outcomes as an alternative for synthetic chemical agents for insect biocontrol programs. In this context, the phytochemicals ranging from various classes such as alkaloids, terpenes, steroids, and phenolic constituents were investigated earlier for biocontrol potency, and it has positive results [7–10]. Moreover, the ability to control mosquito larvae and their efficacies of application varies with age, species, part extracted, collection site of plants, and the solvent used [11, 12]. Following this conception, the solvent factor could be compared and examined for its larvicidal efficacies. As an example, Markouk et al. [13] evaluated the differences between ethanolic and aqueous extracts of *Calotropis procera* flowers and leaves at 1,000 ppm, which did not exhibit any activity and the aqueous phase pose activity (with LC_{50} = 28 ppm) against *Anopheles labranchiae*. Rahuman et al. [14] have stated that the highest *Culex quinquefasciatus* larval mortality was found in stembark for acetone and methanol extracts of *Cedrus deodara* (LC_{50} = 141.60 and 95.19 ppm; LC_{95} = 624.19 and 639.99 ppm). Referring to all these studies, we can conclude that different solvents pose different larvicidal efficacies. Moreover, it contributes to the search of new alternative resources for biocontrol applications. The new search would minimize the environmental pollutions.

Azolla pinnata is commonly known as a mosquito weed that has been used for paddy growth nourishments [15]. It provides nitrogen sources for paddy plants and forms a thick mat layer on the water surface, which may prevent the breeding of mosquitoes [15]. Meanwhile, *A. pinnata* field study has reported that the breeding of malaria-transmitting mosquitoes was completely suppressed in pools, wells, and ponds [16]. In paddy fields of Tanzania, Africa, cultivation of *Anabaena azollae* plant has reduced the larvae productivity and larvae densities of *An. gambiae*, *An. funestus*, and *Cx. quinquefasciatus* [17]. Further, suggesting the mosquito productivity is low when the *Azolla* sp. coverage is high (>80%) in paddy fields [17].

Considering the potentials of *A. pinnata* applications, the phytochemical properties have been characterized as alkaloids, flavonoids, phenols, saponins, quinones, tannins, carboxylic acids, proteins, xanthoproteins, coumarins, steroids, and carbohydrates [18]. Till to date, no other studies have been done on the specific chemical compounds and their structures for *A. pinnata* plant. Additionally, no other explicit study has stated the efficacies of either methanol or acetone solvent for *A. pinnata* plant against *Ae. albopictus* larvae. Hence, the purpose of this study is to identify the chemical compounds and their structures from acetone and methanol solvent extraction for *A. pinnata* plant and to test its efficacies compared with both solvents against the late 3rd instar to early 4th instar larvae of *Ae. albopictus*.

2. Materials and Methods

All experimental procedures were approved by animal ethics: USM/IACUC/2018/111/909 from Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Minden, Penang, Malaysia. According to WHO [21] guidelines, the test is only specific to the *Aedes* late 3rd instar to early 4th instar larvae.

2.1. Plant Materials. A total of 50 kg fresh *A. pinnata* (Figure 1) was sampled from Kuala Krai, Kelantan, Malaysia (5°31′N 102°12′E), and its species was identified based on a morphological view of phyllotaxis. Next, *A. pinnata* fresh samples were prepared using a sun-dried (30°C ± 4°C room temperature) for 2 days. Then, the dried samples were powdered electrically using grinding machine, Faber FBG-460 K, and were sieved as fine powder. The fine powders would increase the surface area and, thus, increase the rate of extractions [19]. Finally, methanol and acetone extractions as a solvent were used in this study using Soxhlet extraction procedures.

2.2. Soxhlet Extraction. Following Zuharah et al. [20], Soxhlet extraction apparatus (Favorit, Malaysia) with a total of 40 g of dried plant powder was placed into the paper thimble. Next, some cotton wool was placed on the top part of extraction flask to prevent the sample from overflow into other apparatus parts. One litre of methanol or acetone solvent was placed in a round-bottom flask with the heating mantle underneath. The solvent was repetitively refluxed and heated along with the fine grinded plant materials. It was done in order to extract the desired plant compound into the round bottom flask. The extraction in the Soxhlet apparatus was performed at boiling point 70°C for about 3 hours until the solvent in the siphon arm becomes clear, which indicates the sample has been extracted entirely. Finally, the extracts were evaporated to dryness in the vacuum evaporator.

2.3. Larvae Rearing. Aedes albopictus eggs were obtained from the Vector Control Research Unit (VCRU) at University Sains Malaysia (USM), Penang, Malaysia. We followed the method used by Zuharah et al. [20] in larvae rearing. Then, the eggs were hatched in seasoned water for 24 hours. The larval food with ratio 2 : 1 : 1 : 1 of cat biscuit, beef liver, yeast, and milk powder was used to trigger the hatching process with 0.2 g. The eggs were maintained at 25°C to 30°C (room temperature), a pH of 6.95 to 7.03, relative humidity of 80 ± 10%, and dissolved oxygen from 5.5 to 6.1 mg/L in the laboratory. After 5 to 6 days, the late 3rd instar larvae were used for the bioassay test.

2.4. Larvicidal Bioassay. Larvicidal bioassays were performed in accordance with the standard WHO [21] larval susceptibility test methods. The bioassay was conducted with 25 of late 3rd instar larvae (homogeneous population consisting of 5 days old 4 to 5 mm in length), in total, four replicates per set for each concentration. Initially, the

Figure 1: Picture of *Azolla pinnata* plant from the field.

mosquito larvae were exposed to a wide range of test concentrations and a control to find out the activity range of the extract solution [22]. After determining the mortality of larvae in this wide range of concentrations, a narrow range (seven concentrations ranging between 10 and 1500 ppm, yielding between 0 and 100% mortality in 24 hours of exposure) was selected as test concentrations for larvicidal bioassays [22]. The control solutions were prepared with distilled water of 1 ml of 10% of the respective solvent for each of the experiment [22]. Solvent was added into the control containers to ensure it was identical to the test solutions [22]. Experiments were conducted at room temperature of 28 ± 2°C. Mortality observations of *Ae. albopictus* larvae were recorded at 24 hours and 48 hours, respectively. Immobilization and total absence from the larvae, even after touch, were the end points of the bioassay [21]. The data were analyzed using a probit analysis, IBM SPSS Statistics 24 [22].

2.5. Photomicrograph View. *Aedes aegypti* late 3rd instar to early 4th instar larvae were observed under an optical microscope (Leica USA), with a magnification of 40–400x [23].

2.6. GC-MS Analysis. The GC-MS analysis of the crude extracts from *Azolla pinnata* was performed on a GCMS-QP2010 Ultra (Shimadzu). We followed the method used by previously published research findings of plant extracts [24]. The GCMS-QP2010 Ultra (Shimadzu) system, fitted with Rtx-5MS capillary column (30 m × 0.25 mm inner diameter, ×0.25 μm film thickness; maximum temperature, 370°C), coupled to a QP2010 Ultra (Shimadzu) MS. Ultra-high-purity helium (99.99%) was used as carrier gas at a constant flow rate of 1.0 mL/min. The injection, transfer line, and ion source temperatures were all 280°C. The oven temperature was programmed from 80°C (hold for 2 min) to 280°C at a rate of 3°C/min. The crude samples were diluted with appropriate solvent (1/100, v/v) and filtered. The particle-free diluted crude extracts (1 μL) were taken in a syringe and injected into an injector with a split ratio of 10 : 1. All data were obtained by collecting the full-scan mass spectra within the scan range of 40–550 amu. The percentage composition of the crude extract constituents was expressed as the percentage by peak area. The identification and characterization of chemical compounds in various crude extracts were based on the GC retention time. The mass spectra were computer matched (>70%) with those of the standards available in the NIST 08 mass spectrum libraries.

3. Results and Discussion

3.1. Larvicidal Bioassay. The bioassay testing from the acetone solvent of *A. pinnata* was tested at 500 ppm, 700 ppm, 1000 ppm, 1100 ppm, 1300 ppm, 1500 ppm, and 1600 ppm; meanwhile, methanol solvent was tested at 300 ppm, 500 ppm, 700 ppm, 1000 ppm, 1300 ppm, 1500 ppm, and 1700 ppm. The entire larvae bioassay test with *A. pinnata* extracts showed a significant increase in the mortality rate with the increase of concentration. Among the plant extracts tested, highest larvicidal activity was observed in the methanol solvent compounds against the late 3rd instar *Ae. albopictus* larvae with LC_{50} and LC_{95} values of 867 and 1293 ppm at 24 hours, 647 and 972 ppm at 48 hours, respectively (Table 1). Meanwhile, the acetone solvent compounds were recorded with LC_{50} and LC_{95} values of 1072 and 1302 ppm at 24 hours, 904 ppm and 1126 ppm at 48 hours, respectively (Table 1). No significant mortality for control assays.

The biological activity of plant-based insecticides against mosquito larvae extensively varies according to the solvent used for its extraction [25]. The findings of current study are inconformity with the past findings whereby methanol solvent for plant extract resulted in higher larvicidal activity against *Ae. albopictus* larvae compared with acetone extract. The reason for choosing methanol and acetone in this study was due to the similar polarity index of 5.1, but the viscosity value varies for acetone and methanol between 0.32 and 0.6, respectively. According to Khayyat and Roselin [24], lower viscosity level on solvents will provide higher coefficient diffusion and yield with active compounds from plants. Additionally, the efficacy of the extracted plant compounds increases with decreasing polarities [26]. According to Ghosh et al. [27], the application of moderate polarity of solvents for plant extraction would produce excellent results on larvicidal bioassays. Hence, following all these conceptions, our current study has selected acetone and methanol solvent as the best test solvent for larvicidal bioassays. Till to date, this would be the first study with *A. pinnata* plant for chemical compound identifications, characterizations, and larvicidal bioassays.

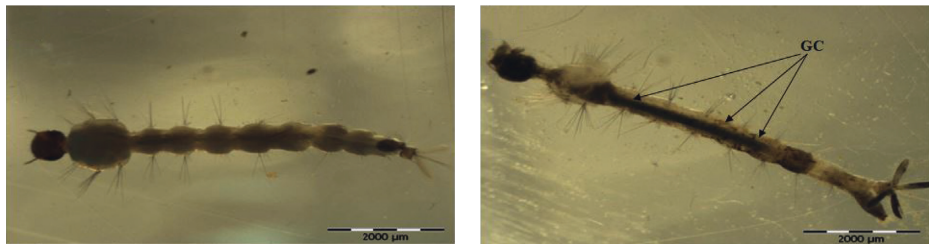
3.2. Photomicrograph View. Photomicrograph view of *Ae. albopictus* larvae shown in Figure 2(b) indicates the presence of *A. pinnata* plant extracts in the midgut content (dark colour) in comparison with the control test (Figure 2(a)). The presence of the midgut content (dark colour) of extracts was indicative of the ingestion mechanisms by larvae towards *A. pinnata* plant extracts. Similarly, Procopio et al. [23] have shown photomicrography for the application of *Moringa oleifera* lectin on the gut content of *Ae. aegypti* larvae.

3.3. GC-MS Analysis and Identification of Compounds. The GC-MS analysis of acetone solvent extracts of *Azolla pinnata* showed 45 peaks, which indicated the presence of 45 phytochemical compounds (Figure 3). In comparison (more than 70% similarity) of the mass spectra on the constituents with NIST 08 library, only 37 compounds were characterized and identified (Table 2). The identified chemical compounds in

Table 1: Larvicidal activity of *Azolla pinnata* extracts against late 3rd instar larvae of *Aedes albopictus*.

Extraction solvent	N^a	LC_{50} (ppm) (95% LCL–UCL)	LC_{95} (ppm) (95% LCL–UCL)	Time (h)
Acetone	700	1072 (994–1146) $Y = -30.339 + 10.011X$	1302 (1214–1435) $Y = -30.339 + 10.011X$	24
Acetone	700	904 (854–952) $Y = -26.159 + 8.848X$	1126 (1068–1198) $Y = -26.159 + 8.848X$	48
Methanol	700	867 (776–958) $Y = -14.264 + 4.854X$	1293 (1257–1498) $Y = -14.264 + 4.854X$	24
Methanol	700	674 (606–733) $Y = -14.975 + 5.293X$	972 (899–1064) $Y = -14.975 + 5.293X$	48

[a]Total number of larvae used in this study; $n = 25$ with 4 replicates per concentration; LC_{50}: lethal concentration with 50% mortality; LC_{95}: lethal concentration with 95% mortality; LCL: lower confidence limits; UCL: upper confidence limits.



(a) (b)

Figure 2: Morphological midgut content induced by *Azolla pinnata* plant extract from acetone and methanol solvent extractions in late 3rd instar *Aedes albopictus* larvae. (a) Control test for midgut content. (b) *A. pinnata* crude extract for midgut content in larvae. Note: arrows indicate the plant extracts (dark colour); GC: gut content (after 24 hours).

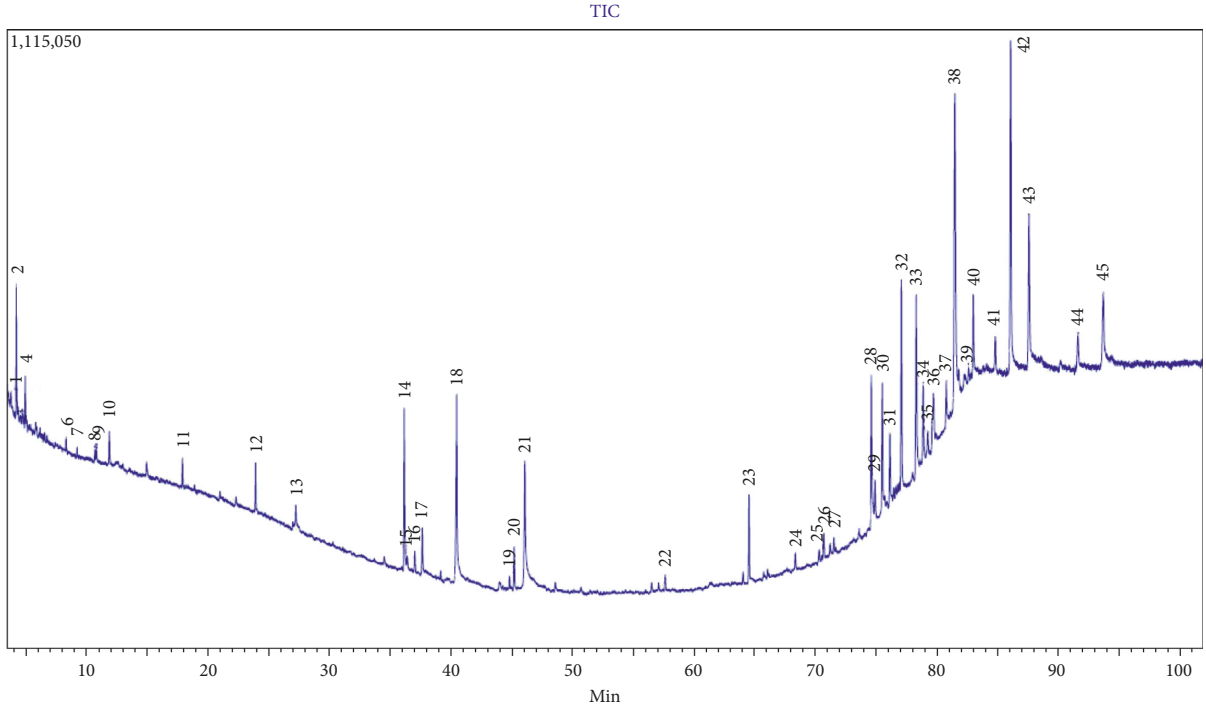


Figure 3: Chromatogram of GC-MS analysis of acetone extract of *Azolla pinnata*.

Table 2: Chemical compositions in acetone extract of *Azolla pinnata*.

Peak no.	Retention time	Area	Area %	Compound name	Activity
2	4.235	246212	8.633	Glycerin	Pesticides and herbicidal
3	4.581	6587	0.231	1,1′-Bicyclooctyl	Herbicidal and insecticidal
4	4.970	57338	2.010	1-Decene, 2,4-dimethyl	Pesticides
5	5.097	5753	0.200	Cyclopentane, 1,2-dimethyl-3-(1-methylethyl)	Pesticides
6	8.326	12614	0.439	2,2,6,6-Tetramethyl-4-piperidone	Antimicrobial
7	9.235	14795	0.519	3-Hexen-2-one	Natural pesticides and insecticidal
8	10.713	11812	0.414	Butanamide, 3-(2-methylpropinonylhydrazono)-N	Insecticidal and herbicidal
9	10.831	17477	0.613	4-Heptanone, 2,3:5,6-diepoxy-2,6-dimethyl	Pesticides
10	11.903	93194	3.268	Benzofuran, 2,3-dihydro	Insecticidal
11	17.921	39904	1.399	1,2-O-Isopropylidene-beta-l-idofuranurono-6,3	Unknown
12	23.947	111946	3.925	Phenol, 2,4-bis(1,1-dimethylethyl)	Pesticides
13	27.262	80732	2.831	Diethyl phthalate	Insecticidal
14	36.194	118203	4.145	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Antimicrobial
15	36.442	10670	0.374	2-Hexadecene, 3,7,11,15-tetramethyl-	Insecticidal, nematicide, and pesticide
18	40.526	184262	6.461	*n*-Hexadecanoic acid	Insecticidal, nematicide, and pesticide
19	44.871	6310	0.221	9-Octadecenoic acid, methyl ester, (E)	Antimicrobial
20	45.258	44798	1.571	Phytol	Insecticidal, fungicide, and miticide
21	46.138	114014	3.998	Oleic acid	Pesticides and insecticidal
22	57.704	32308	1.133	1,2-Benzenedicarboxylic acid, diisooctyl ester	Antimicrobial
23	64.602	153370	5.378	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-h	Antimicrobial
24	68.430	19721	0.691	2H-Pyran-2-one, tetrahydro-6-tridecyl	Antimicrobial
25	70.395	8230	0.289	1-Hentetracontanol	Antimicrobial
26	70.777	20002	0.701	Tetracosyl pentafluoropropionate	Insecticidal
27	71.602	33387	1.171	Vitamin E	Antimicrobial and antioxidant
28	74.685	132335	4.640	1,37-Octatriacontadiene	Antimicrobial
29	74.986	32935	1.155	Octatriacontyl trifluoroacetate	Insecticidal
30	75.599	46184	1.619	Gamma-sitosterol	Antibacterial and antioxidant
31	76.218	26772	0.939	9,19-Cyclo-27-norlanostan-25-one, 3-(acetyloxy)-24	Pesticides
32	77.157	104216	3.654	9,19-Cyclolanost-24-en-3-ol, (3.beta.)	Pesticides
33	78.383	159996	5.610	Stigmast-4-en-3-one	Antimicrobial and antibacterial
34	78.956	54988	1.928	Heptacosyl heptafluorobutyrate	Pesticides
37	80.873	29709	1.042	Oxirane, hexadecyl	Pesticides
38	81.578	216330	7.585	Stigmastane-3,6-dione, (5.alpha.)	Antimicrobial and anti-inflammatory
40	83.088	43183	1.514	Tetracontane-1,40-diol	Antibacterial
41	84.906	25657	0.900	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Insecticidal
42	86.157	380998	13.359	*cis*-11,12-Epoxytetradecen-1-ol	Biopesticides and insecticidal
43	87.677	102881	3.607	43-Tetracontane-1,40-diol	Antimicrobial and antibacterial

A. pinnata acetone extracts were *cis*-11,12-epoxytetradecen-1-ol (13.359%), glycerin (8.633%), stigmastane-3,6-dione, (5. alpha.) (7.585%), *n*-hexadecanoic acid (6.461%), stigmast-4-en-3-one(5.610%), 2,6,10,14,18,22-tetracosahexaene,2,6,10,15,19, 23-h (5.378%), 1,37-octatriacontadiene (4.640%), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (4.145%), oleic acid (3.998%), phenol, 2,4-bis(1,1-dimethylethyl) (3.925%), tetracontane-1,40-diol (3.607%), 9,19-cyclolanost-24-en-3-ol (3.beta.) (3.654%), benzofuran, 2,3-dihydro (3.268%), diethyl phthalate (2.831%), 1-decene, 2,4-dimethyl (2.01%), heptacosyl heptafluorobutyrate (1.928%), gamma-sitosterol (1.619%), phytol (1.571%), tetracontane-1,40-diol (1.514%), 1,2-*O*-isopropylidene-beta-l-idofuranurono-6,3 (1.399%), vitamin E (1.171%), octatriacontyl trifluoroacetate (1.155%), 1,2-benzenedicarboxylic acid, diisooctyl ester (1.133%), oxirane, hexadecyl (1.042%), 9,19-cyclo-27-norlanostan-25-one,3-(acetyloxy)-24(0.939%), 3,7,11, 15-tetramethyl-2-hexadecen-1-ol (0.9%), tetracosyl pentafluoropropionate (0.701%), 4-heptanone, 2,3:5,6-diepoxy-2,6-dimethyl-(0.613%), 2H-pyran-2-one, tetrahydro-6-tridecyl (0.691%), 3-hexen-2-one (0.519%), 2,2,6,6-tetramethyl-4-piperidone (0.439%), butanamide 3-(2-methylpropinonylhydrazono)-N (0.414%), 2-hexadecene 3,7,11,15-tetramethyl (0.374%), 1-hentetracontanol (0.289%), 1,1′-bicyclooctyl (0.231%), 9-octadecenoic acid, methyl ester (E) (0.221%), cyclopentane, and 1,2-dimethyl-3-(1-methylethyl) (0.2%). The attached supplementary pdf file contains the NIST 08 library search for chemical compound structures and details.

The GC-MS analysis of methanol solvent extracts using maceration extraction of *A. pinnata* showed 44 peaks, which indicated the presence of 44 phytochemical compounds (Figure 4). In the comparison (more than 70% similarity) of the mass spectra on the constituents with the NIST 08 library, only 35 compounds were characterized and identified (Table 3). The identified chemical compounds in methanol solvent extracts were stigmastane-3,6-dione, (5.alpha.) (11.933%), *n*-hexadecanoic acid (11.909%), stigmast-4-en-3-one (10.892%), glycerin (9.375%), DL-proline, 5-oxo-, methyl ester (5.992%), 9,19-cyclolanost-

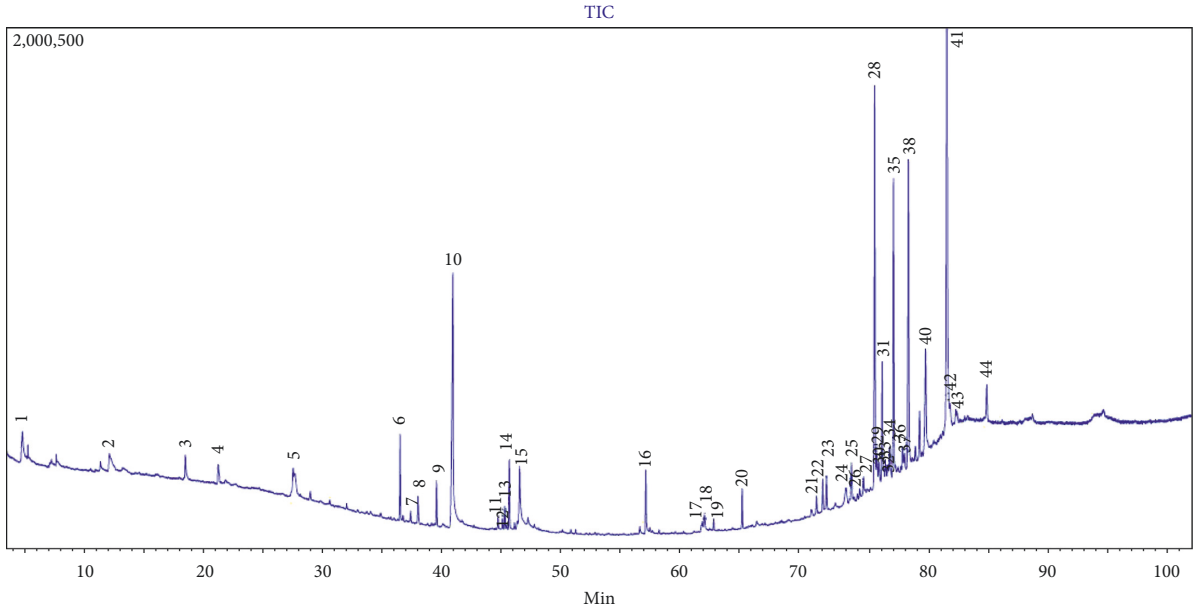


Figure 4: Chromatogram of GC-MS analysis of methanol extract of *Azolla pinnata*.

Table 3: Chemical compositions in methanol extract of *Azolla pinnata*.

Peak no.	Retention time	Area	Area %	Compound name	Activity
1	4.827	504294	9.375	Glycerin	Pesticides and herbicides
3	18.362	322274	5.992	DL-Proline, 5-oxo-, methyl ester	Antibacterial and antifungal
4	21.099	168354	3.130	Benzaldehyde, 2-hydroxy-4-methyl-	Pesticides
5	27.310	212453	3.950	Benzoic acid, 2-4-(4-hydroxy-4-methylpentyl)	Pesticides
6	36.206	108402	2.015	Neophytadiene	Larvicidal, insecticidal, and antimicrobial
8	37.695	47152	0.877	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Insecticidal
9	39.234	148583	2.762	Hexadecanoic acid, methyl ester	Insecticidal, nematicide, and pesticide
10	40.590	640591	11.909	Hexadecanoic acid <n->	Insecticidal, nematicide, and pesticide
11	44.331	15262	0.284	*n*-Nonadecanol-1	Pesticides
12	44.703	12360	0.230	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Antifeedant and insecticidal
13	44.903	26198	0.487	9-Octadecenoic acid (Z)-, methyl ester	Antifeedant and insecticidal
14	45.280	173301	3.222	Phytol	Insecticidal, fungicide, and miticide
15	46.132	101905	1.895	Oleic acid	Pesticides and insecticidal
16	56.629	77698	1.445	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)eth	Antimicrobial
17	61.312	14017	0.261	9-Octadecenoic acid, 1,2,3-propanetriyl ester	Antimicrobial
18	61.490	26880	0.500	7-Tetradecenal, (Z)	Pesticides
19	62.247	27895	0.519	Tetracosanoate methyl	Pesticides
20	64.625	144155	2.680	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-h	Antimicrobial
21	70.800	30142	0.560	17-Pentatriacontene	Pesticides
22	71.325	18144	0.337	Cholesterol	Unknown
23	71.624	156179	2.904	Alpha-tocopherol, beta-D-mannoside	Antimicrobial
24	73.259	17620	0.328	Ergosterol	Unknown
25	73.704	34527	0.642	Ergost-5-en-3-ol, (3.beta.)	Antimicrobial
26	74.399	10666	0.198	Stigmasterol	Antimicrobial
27	74.707	26693	0.496	Oxirane, 2-decyl-3-(5-methylhexyl)-, *cis*-(.+/-.)	Pesticides
28	75.627	287315	5.342	Gamma-sitosterol	Antibacterial and antioxidant
29	75.820	22998	0.428	Stigmastanol	Antimicrobial
31	76.249	100498	1.868	9,19-Cyclo-27-norlanostan-25-one, 3-(acetyloxy)-24	Pesticides
32	76.560	43084	0.801	Cholest-4-en-3-one	Insecticidal
33	76.725	25375	0.472	Cholestan-3-one	Insecticidal
35	77.196	294724	5.479	9,19-Cyclolanost-24-en-3-ol, (3.beta.)	Pesticides
36	77.942	44894	0.835	Vitamin E	Antimicrobial and antioxidant
38	78.423	107567	10.892	Stigmast-4-en-3-one	Antimicrobial and anti-inflammatory
39	79.365	7507	0.732	Lup-20(29)-en-3-ol, acetate, (3.beta.)	Insecticidal
41	81.635	641850	11.933	Stigmastane-3,6-dione, (5.alpha.)	Antimicrobial and anti-inflammatory

24-en-3-ol (3.beta.) (5.479%), gamma-sitosterol (5.342%), benzoic acid 2-4-(4-hydroxy-4-methylpentyl) (3.950%), benzaldehyde, 2-hydroxy-4-methyl (3.130%), phytol (3.222%), alpha-tocopherol-beta-D-mannoside (2.904%), hexadecanoic acid, methyl ester (2.762%), 2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-h (2.680%), neophytadiene (2.015%), 9,19-cyclo-27-norlanostan-25-one, oleic acid (1.895%), 3-(acetyloxy)-24 (1.868%), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)eth (1.445%), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (0.877%), vitamin E (0.835%), cholest-4-en-3-one (0.801%), Lup-20(29)-en-3-ol, acetate (3.beta.) (0.732%), ergost-5-en-3-ol (3.beta.) (0.642%), 17-pentatriacontene (0.560%), tetracosanoate methyl (0.519%), 7-tetradecenal, (Z)- (0.500%), oxirane, 2-decyl-3-(5-methylhexyl), *cis* (0.496%), 9-octadecenoic acid (Z)-methyl ester (0.487%), cholestan-3-one (0.472%), stigmastanol (0.428%), cholesterol (0.337%), ergosterol (0.328%), *n*-nonadecanol-1 (0.284%), 9-octadecenoic acid, 1,2,3-propanetriyl ester (0.261%), 9,12-octadecadienoic acid (Z,Z)-, methyl ester (0.230%), and stigmasterol (0.198%). The attached supplementary pdf file contains the NIST 08 library search for chemical compound structures and details.

Furthermore, as discussed previously, the conception of lower viscosity values gave more yields of active compounds using acetone solvent compared to methanol, which was evidentially proven from this study. *Azolla pinnata* plant yields 37 chemical compounds from acetone solvent extract compared to 35 compounds from methanol solvent. As stated in Table 1, acetone solvent extracts produced highest chemical composition of *cis*-11,12-epoxytetradecen-1-ol (13.359%), glycerin (8.633%), stigmastane-3,6-dione (5.alpha.) (7.585%), *n*-hexadecanoic acid (6.461%), stigmast-4-en-3-one (5.610%), 2,6,10,14,18,22 tetracosahexaene, 2,6,10,15,19,23-h (5.378%), which were extensively used for insecticidal, pesticidal, and antimicrobial properties [28–30]. Besides that, methanol solvent (Table 2) extracts have yielded highest chemical compositions of stigmastane-3,6-dione (5.alpha.) (11.933%), hexadecanoic acid (11.909%), stigmast-4-en-3-one (10.892%), glycerin (9.375%), DL-proline, 5-oxo-, methyl ester (5.992%), and 9,19-cyclolanost-24-en-3-ol (3.beta.) (5.479%), which were used for antimicrobial, anti-inflammatory, insecticidal, nematicidal, and pesticidal applications [31–36].

According to the chemical composition results in our study, it showed that less number of active compounds have been extracted for methanol solvent on *A. pinnata* plant compared to acetone solvent. However, the efficacy of extracts for larvicidal was superior for methanol solvent compared to acetone solvent. These can be further discussed as the total composition (34.734%) of compounds from stigmastane-3,6-dione, (5.alpha.), hexadecanoic acid, and stigmast-4-en-3-one has the ability of antimicrobial properties when compared to major components of acetone solvent extracts. According to Minard et al. [37], there was an interaction from all the midgut bacterial diversity for *Ae. albopictus* mosquitoes in their life cycle. Meanwhile, a recent study has stated that *Ae. aegypti* larvae require live gut bacteria for its development and that they rely on multiple bacterial diversity [38]. According to previous microbial findings, our recent results on methanol-extracted chemical compounds may be more active in its antimicrobial properties on its gut microbial interference within *Ae. albopictus* larvae compared to acetone extracts.

4. Conclusion

In conclusion, the findings of this study have shown the effectiveness of *A. pinnata* extracts by acetone and methanol solvents against one major mosquito species in the late 3rd instar to early 4th instar larvae stages. Moreover, our findings showed that the *A. pinnata* bioactive molecules can be effective as larvicides for *Ae. albopictus* mosquito vector control programs. Finally, this study suggests that future research work can be conducted on the field evaluation of its larvicidal effectiveness against *Ae. albopictus* species for environmentally safer botanical insecticide inventions.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared that no conflicts of interest exist.

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Supplementary Materials

The NIST 08 library search for chemical compound structures and details. (*Supplementary Materials*)

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