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Research Article

Larvicidal Activity of Phenylbutanoid Isolated from the Rhizome of *Zingiber montanum* (J. Koenig) against *Aedes albopictus* (Skuse) and *Cx. quinquefasciatus Say*

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I N F O

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A B S T R A C T

Introduction: Mosquito-borne infections are most prevalent in the northern districts of West Bengal, India. The public health administrator in this region relies heavily on synthetic insecticides, which has created more challenges than solving problems. This study attempts to find eco-friendly, biodegradable, and effective botanicals against mosquitoes, especially *Aedes albopictus* and *Culex quinquefasciatus*.

Methods: A preliminary screening study showed that the n-hexane extract of *Zingiber montanum* rhizome possessed potential larvicidal activity against mosquito larvae. Bioassay-guided fractionation led to the isolation of bioactive phenylbutanoid which was characterised using 1H and 13C NMR and HRMS-TOF.

Results: The compound was identified as cis-1,2-Bis-[(E)-3,4-dimethoxystyryl]cyclobutane (DMSCB) by comparing with previously published data. The molecular ion of the compound was found to be 381.261 m/z with a molecular formula of $C_{24}H_{28}O_4$. The compound was found to have LC_{50} values of 39.951 ppm and 58.178 ppm against *Ae. albopictus* and Cx. *quinquefasciatus* larvae respectively.

Conclusions: Phenyl propanoids and terpenoids are known to possess some level of insecticidal activity but our studies show that phenylbutanoid, DMSCB, also possesses insecticidal activity. This implies that plants containing phenylbutanoid affect the overall insecticidal activity of an extract. Therefore, the outcome of our study suggests that the rhizome extract of *Z. montanum* has the potential to be used in field studies.

Keywords: Phenylbutanoid, *Aedes, Culex,* Larvicide, Isolation, Chemical Characterisation

Introduction

Mosquito-borne infections are most prevalent in the northern districts of West Bengal, India. It is home to various mosquito species that are established vectors of many disease pathogens that cause malaria, dengue, filariasis, and other diseases.[1,2] To counteract these vectors, the public health administrator in this region relies heavily on synthetic insecticides, which has created more challenges than solving problems. This is also evident from the previous studies where *Culex quinquefasciatus* Say and *Aedes albopictus* (Skuse) from the Siliguri, Shivmandir area of Darjeeling district were found to have developed insecticidal resistance to synthetic pyrethroids, organophosphates (including temephos), and organochlorines.[3,4] Besides insecticide resistance, the unscientific and exploitative use of synthetic insecticides has also resulted in non-target toxicity and environmental degradation. Several alternative methods, such as mass introduction of sterile males, Bt subsp. israelensis (Bti) toxins and bio-control agents are being studied and applied. However, we are still struggling with challenges to control vector-borne disease incidences. There have been reports of resistance development in mosquitoes against Bti toxins.[5–7] It is not possible to introduce bio-control agents in hard-to-reach mosquito habitats such as water trapped in tyres, plant pockets etc.

Phytochemicals have shown some promising results, targeting not one but several life stages of mosquitoes and also with different modes of action.[8–13] This is basically due to the rich diversity of plant secondary metabolites in different potential plant families.[14,15] Botanicals are cheaper, easier to extract, and usually have less toxicity towards non-target organisms.[14] Therefore, this study attempts to find eco-friendly, biodegradable, and effective botanicals against mosquitoes, especially *Aedes albopictus* and *Culex quinquefasciatus*. Our preliminary study (unpublished data) showed some activity of the *Zingiber montanum* rhizome against mosquito larvae. Therefore, we have focused on the bioassay-guided isolation of phytochemicals from the rhizome extract and their characterisation, if any were found.

Materials and Method

Plant Collection and Sample Preparation

During the months of October(2020) and December(2020), rhizomes of *Z. montanum* were collected from Lebong (27.0615° N, 88.2765° E), Darjeeling District of West Bengal, India. Collected rhizomes were cleaned under tap water and dried in a shed. Then they were chopped into small cubes and kept under shade till they were completely dried (Figure 1). The dried rhizomes were ground using an electric grinder into fine powder and used for extraction.

Figure 1.*Zingiber montanum* Plant; Chopped Rhizome (Inset)

Extraction Methods

The powdered plant samples were macerated using n-hexane for at least a week and then filtered using a clean muslin cloth followed by Whatman filter paper No. 1. This process was repeated thrice with the same extract in n-hexane. Then the residue was subsequently soaked in comparatively polar solvents such as diethyl ether, acetone and methanol in a similar manner. Similar solvent extracts were pooled together and a stock solution of 1,00,000 ppm was prepared in analytical grade acetone using Tween 20 while the surplus was refrigerated for future use.[16]

Mosquito Collection

Both *Ae. albopictus* and *Cx. quinquefasciatus* populations were sampled from their natural habitats in and around the university campus (26.7095° N, 88.3542° E). Collected mosquito larvae were kept inside mosquito cages in the laboratory and fed with crushed fish feed. Larvae were taxonomically characterised using a standard mosquito identification key.[17]

Larvicidal Bioassay-guided Isolation

The WHO 2005 protocol was followed with minor modifications to examine the larvicidal activity of phytochemicals.[18] Thirty larvae of the third and fourth instars were introduced to each aliquot (25, 50, 100, 200, and 500 ppm) of extract in 100 ml of water. Larvae in the control group were treated with a solution of 500 µl carrier solvent and emulsifying agent in 100 ml of water. Temephos was used as a positive control at 1 ppm concentration. Each aliquot had five replicates, and the bioassay was

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repeated three times on different days using the identical experimental setup. Larval mortality was measured after 24 hours, 48 hours, and 72 hours.

The n-hexane rhizome extract was treated to a gravity column (450 x 30 mm, Borosil) to isolate the bioactive compound(s) with mosquitocidal activity. Seventy-five percent of the column was filled with silica gel of 60–120 mesh size (SRL, India). The plant extract was then fed in at a fixed ratio of 1:5 to the stationary phase and eluted with varied ratios of n-hexane and ethyl acetate (100% n-hexane, 1:9, 3:7, 1:1, 3:7, 9:1, 100% ethyl acetate, v/v). Equal volumes of fractions were collected in 15 ml vials, and each fraction was monitored by Thin layer Chromatography (TLC) with TLC Silica gel 60 F_{254} (Merck, Germany) using the same eluent system. The fractions were concentrated using a rotary evaporator before being air-dried. Dried fractions were then screened for larvicidal bioactivity. The experiment was repeated until a sufficient amount of active fraction was generated, which was then subjected to a second column (60–120 Mesh Silica gel, 120 x 10 mm, Borosil) and eluted using a different combination of ethyl acetate and n-hexane. The flow rate was set at 30 drops per minute.

Characterisation

Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) experiments were performed on 2795 Waters HPLC and Waters Micromass Q-T of Micro (Waters Corp., USA) with dual electrospray source having a mass range of up to 20,000 amu. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on an FT NMR Spectrometer model Avance-II (Bruker) (Bruker Inc., Switzerland) with a frequency of 400 MHz for ^{1}H- and 100 MHz for ^{13}C measurements, respectively.

Statistical Analysis

Lethal concentration causing 50% larval mortality or LC_{50} values and lethal concentration causing 90% mortality or LC_{90} was determined by Probit analysis using the IBM SPSS version 21. Results with $p < 0.05$ were considered to be statistically significant. Mortality of 10% or less in the control set (if any) was adjusted using Abbott's correction, given by:

$$\text{Mortality (\%)} = \frac{X - Y}{100 - Y} \times 100$$

Where, X is the percentage mortality in the treated sample and Y is the percentage mortality in the control.

Results

Our previous study (unpublished data) revealed that the n-hexane extract of *Z. montanum* rhizome showed the highest mortality among the four different extracts tested against *Ae. albopictus* larvae. Therefore, the n-hexane extract was subjected to bioassay-guided fractionation.

Larvicidal Bioassay-guided Fractionation

Around 56 fractions of 15 ml volume each were collected using gravity column chromatography. Out of these fractions, fractions 6, 7, 8, 9, 10 and 11 showed similar analyte profiles on a TLC plate with hexane and ethyl acetate (1:1) as eluent. These fractions were pooled together and tested for larvicidal activity at 100 ppm for both mosquito species. In the second phase of isolation, a light yellow-coloured viscous liquid was yielded in fraction 28. When mosquito larvae were exposed to different concentrations of this liquid, some level of mortality was seen (Figure 2). A hundred percent mortality was observed at 100 ppm against *Aedes albopictus*. *Ae. albopictus* larvae were more susceptible to the fraction 28 as compared to *Cx. quinquefasciatus* larvae (Figures 2d and 2e). Probit analysis predicted an LC_{50} value of 39.951 ppm (Table 1) against *Ae. albopictus* larvae and 58.178 ppm against *Culex quinquefasciatus* larvae (Table 2). This liquid was viscous and produced long threads when stretched. It was

(a) (b) (c)

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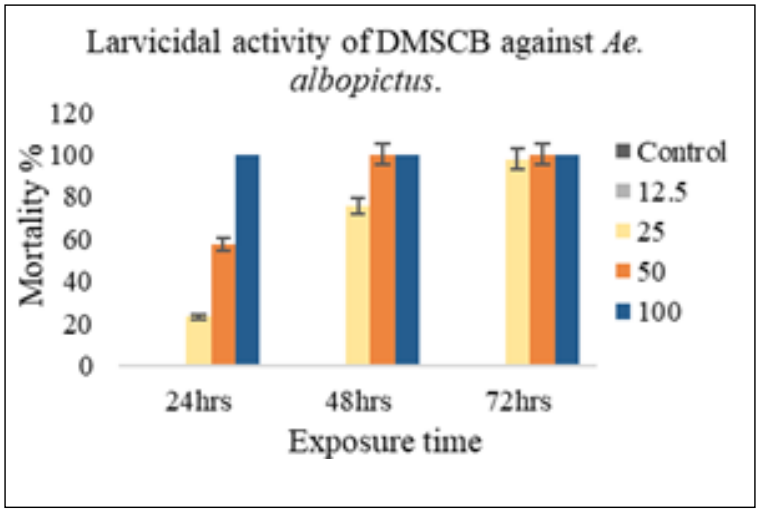
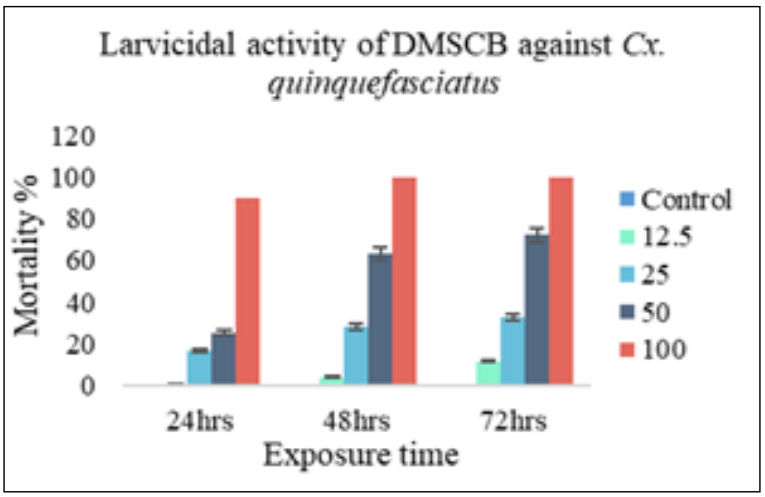


(d)



(e)

Figure 2.Larvicidal Activity of DMSCB isolated from *Zingiber montanum* Rhizome (a).Untreated Larva (b, c).Treated Larvae (d, e).Mortality Percentage of Treated Mosquito Larvae

Table 1.LC50, LC90, LC99 Values of Fraction 28 isolated from *Z. montanum* against *Ae. albopictus* Larvae

95% Confidence Limit for Conc.			Chi-square ($p < 0.05$)	R^2
LC_{50} (ppm) (Lower level–Upper level)	LC_{90} (ppm) (Lower level–Upper level)	LC_{99} (ppm) (Lower level–Upper level)		
39.951 (16.756–110.873)	74.626 (46.591–10653)	124.199 (65.642–719604)	11.524	1.00

Table 1.LC50, LC90, LC99 Values of Fraction 28 isolated from *Z. montanum* against *Cx. quinquefasciatus* Larvae

95% Confidence Limit for Conc.			Chi-square ($p < 0.05$)	R^2
LC_{50} (ppm) (Lower level–Upper level)	LC_{90} (ppm) (Lower level–Upper level)	LC_{99} (ppm) (Lower level–Upper level)		
58.178 (50.813–67.71)	124.93 (101.21–170.75)	232.959 (170.50–377.84)	125.12	0.79

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completely soluble in ethyl acetate.

Characterisation

The active fraction of the rhizome extract displayed a single spot on the TLC plate with a solvent system of hexane and ethyl acetate (1:1). This fraction was subjected to ^{1}H NMR, ^{13}C NMR and LC-HRMS.

The fragmentation ion at m/z of the bioactive compound (HRMS m/z, Figure 3) was as follows: 151.1007, 191.136, 242.3208, 243.1749, 381.2617, 403.2462, 404.2489. It showed the molecular ion at m/z 381.267 g/mol (M+1) and the calculated mass of the isolated pure compound was found to be 380.456 g/mol. This is very close to the molecular mass of (+/-)-cis-1,2-bis-[(e)-3,4-dimethoxystyryl]-cyclobutane (DMSCB) ($C_{24}H_{28}O_4$) i.e., 380.198 g/mol.

The chemical shift values of the bioactive compound (^{1}H NMR δ (ppm), Figures 4a and 4c) were as follows: 3.898 (-OCH3), 6.507 (2´H), 5.33(1´H), 6.823 (5´´H), 6.7 (6´´H),

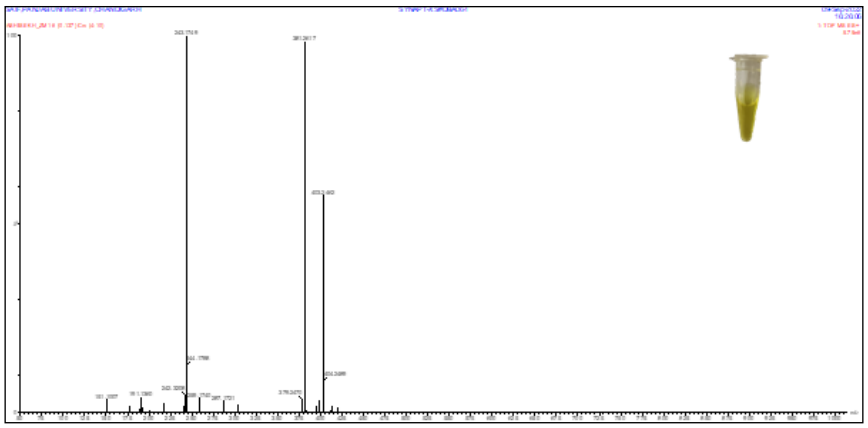
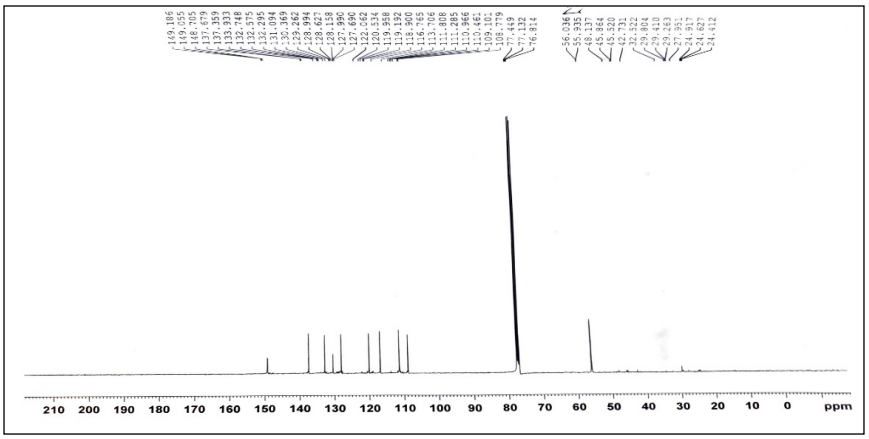
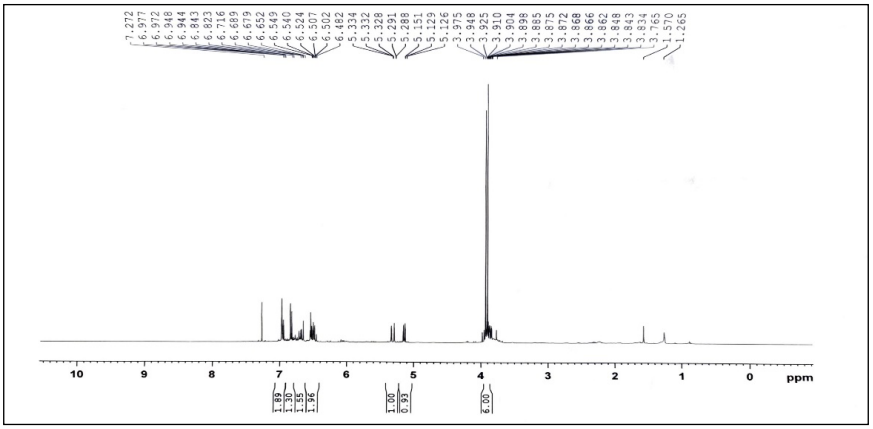


Figure 3.Mass-spectra of Bioactive Phenylbutanoid isolated from Z. montanum Rhizome in LC-HRMS Q-Tof Micro



(a)



(b)

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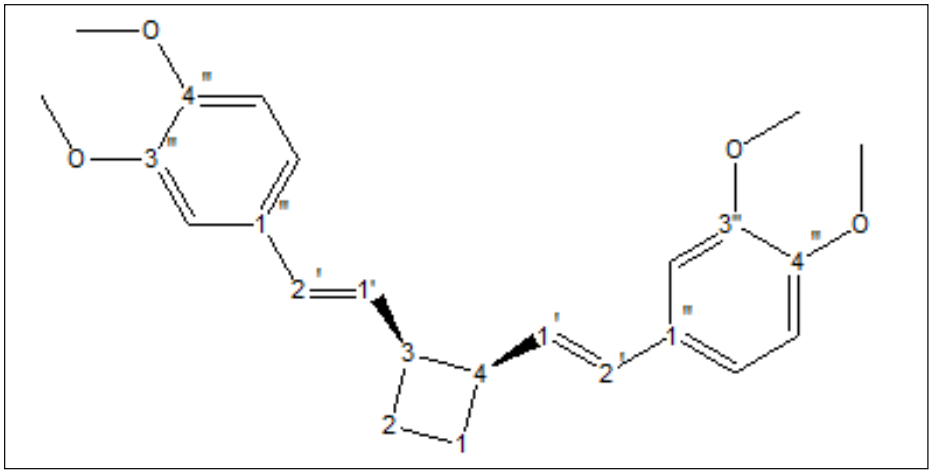

(c)

Figure 4(a).1H NMR Spectra (b).13C NMR Spectra (c).Structure of Bioactive Phenylbutanoid, cis-1,2-Bis-[(E)-3,4-dimethoxystyryl]cyclobutane, isolated from the Rhizome of *Z. montanum*

6.944 (2´´H), 1.26 (1H, 2H), 1.57 (1H, 2H), 3.872 (3H, 4H). Compared to the earlier report.[19]

The chemical shift values of the bioactive compound (^{13}C NMR δ (ppm), Figures 4b and 4c) were as follows: 56.036 (-OCH3), 109.1 (2´´C), 111.808 (5´´C), 119.958 (6´´C), 128.158 (1´´C), 131.094 (2´C), 27.951(1C, 2C), 137.679 (1´C), 149.186 (3´´C, 4´´C), 42.731 (3C, 4C). The peak at 77.4 m/z represents the solvent impurity peak of $CDCl_4$.[19]

Discussion

The anautogenous nature of mosquitoes has led to the transmission of several lethal mosquito-borne diseases which have become unmanageable even after advancement in the field of vector biology and epidemiology. The limitations in modern vector control techniques and the failure of synthetic insecticides have revived the study of screening botanicals in controlling mosquitoes. In terms of botanical insecticides, plants belonging to the families Asteraceae, Rutaceae, Zingiberaceae, Apiaceae etc. need special attention as they were reported to possess promising mosquitocidal properties.[14,15] *Z. montanum* is one of the few medicinal plants that were screened for their mosquitocidal activity. A preliminary screening study showed that the n-hexane extract of its rhizome possessed potential larvicidal activity. Therefore, in order to find the major bioactive insecticidal compound, this study was conducted.

The rhizome of *Z. montanum* is safer for human consumption and it is eaten raw in small amounts.[20–22] *Z. montanum* consists of aldehyde compounds, terpenoids, curcuminoids, phenylpropanoids and phenylbutanoids, and has beneficial properties like antimicrobial activity, anti-oxidative activity, anti-inflammatory activity etc.[23–25] So far, *Zingiber* plants are divided into two chemotypes, one with a high content of terpenoids and the other with a high content of hydrocarbons and phenylpropanoids. *Z. ottensi*, *Z. montanum* and *Z. zerumbet* contain high amounts of terpenoids, whereas *Z. junceum, Z. pyroglossum* and *Z. niveum* contain high amounts of hydrocarbons and phenyl propanoids.[27] Terpinen-4-ol, ɤ-terpinene, α-pipene, sabinene and β-caryophyllene are some of the terpenoids that are common and are found in *Zingiber* plants.[25–27] The presence of terpinen-4-ol imparts insecticidal properties against *Aedes albopictus*[28], whereas phenylbutanoids like (E)-1-(3,4-Dimethoxyphenyl)butadiene and (Z)-1-(2,4,5-Trimethoxyphenyl)butadiene have been reported to possess insecticidal property against neonate larvae of *Spodoptera littoralis*.[29] Terpenoids present in Zingiberaceae are well known to have insecticidal activity and our study shows that the phenylbutanoid dimers isolated from *Z. montanum* rhizome also possess potential insecticidal properties against mosquitoes.

Bioassay-guided fractionation of *Zingiber montanum* rhizome led to the isolation of larvicidal phenylbutanoid which was characterised using ^{1}H and ^{13}C NMR and HRMS-TOF. NMR spectral data and mass spectral data identified the compound as DMSCB by comparing it with previously published data (Figures 4a, 4b and 4c).[19,24] The molecular ion of the compound was found to be 381.261 m/z which implies a molecular weight of 380 with a molecular formula of $C_{24}H_{28}O_4$. Fragment 243.1749 m/z is characteristic of phenylbutanoids which is achieved by fragmentation of 1,2 dimethoxybenzene (138Da). Fragment 151.1007 m/z suggests the presence of 3,4-dimethoxysteryl group (136Da).[24] It is also known to possess apoptogenic properties in T-acute lymphoblastic leukaemia cells and anti-inflammatory activity through inhibition of nitric oxide.[30,31]

The LC_{50} values of this compound were estimated to be 39.95 ppm and 58.178 ppm against *Ae. albopictus* and *Cx.*

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quinquefasciatus larvae, respectively, which is comparable with the toxicity of *Zingiber cassumunar* essential oils against first instar *Ae. albopictus* larvae and sabinene as reported by other researchers.[28,32] The level of lethality of DMSCB against *Cx. quinquefasciatus* can also be compared with that of rhizome essential oil of *Zingiber collinsii* (Mood & Theilade).[31,33] However, both studies concluded that the terpenoid content of the essential oil was responsible for the mosquito larvicidal activity. In another study, three isolates of petroleum ether extract of *Z. officinale* Roscoe i.e., 4-gingerol, 6-Dehydrogingerdione and 6-Dihydrogingerdione, were found to be more toxic to *Ae. aegypti* and *Cx. quinquefasciatus* than DMSCB.[34] DMSCB was found to be more toxic than Zerumbone isolated from *Z. zerumbet*, limonene and p-cymene.[32,35]

Conclusions

The bioassay-guided fraction of *Z. montanum* rhizome extract led to the isolation of phenylbutanoid dimer, DMSCB, which showed larvicidal activity against *Aedes albopictus* and *Culex quinquefasciatus*. Phenyl butanoids, phenyl propanoids and terpenoids are some of the major compounds in two chemotypes of Zingiber spp. Phenyl propanoids and terpenoids are known to possess some level of insecticidal activity but our study showed that phenylbutanoid, DMSCB, also possesses insecticidal activity. This implies that plants containing phenylbutanoids affect the overall insecticidal activity of an extract. Therefore, the outcome of our study suggests that the rhizome extract of *Z. montanum* has the potential to be used in field studies.

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Conflict of Interest: None

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