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Nurhayat Tabanca, Zulfiqar Ali, Ulrich R. Bernier, Nancy Epsky, Ayse Nalbantsoy, Ikhlas A. Khan, Abbas Ali*

Bioassay-guided isolation and identification of *Aedes aegypti* larvicidal and biting deterrent compounds from *Veratrum lobelianum*

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Abstract: An ethanol extract from *Veratrum lobelianum* Bernh. rhizomes showed larvicidal activity with LC_{50} values of 11.79 ppm and 89.9 ppm against 1st and 4th instar larvae, respectively, at 24 h post-treatment. The extract also showed proportion not biting (PNB) value of 0.76 at 100 µg/cm^2 against females of *Ae. aegypti*. Systematic bioassay-guided fractionation of *V. lobelianum* extract resulted in the isolation of five compounds that were identified as ethyl palmitate (**1**), ethyl linoleate (**2**), β-sitosterol (**3**), resveratrol (**4**) and oxyresveratrol (**5**) by GC-MS, ^{1}H-NMR, and ^{13}C-NMR techniques, comparison with literature data, and confirmation with authentic compounds. Compound **2** exhibited larvicidal activity with an LC_{50} value of 24.1 (22.0-26.2) ppm whereas **1** was inactive. β-Sitosterol (**3**) displayed the highest larvicidal activity with LC_{50}= 1.7 (1.3-2.3) ppm and LC_{90}= 5.1 (3.4-13.8) ppm. Compounds **4** and **5** had larvicidal activity with LC_{50} values of 18.5 (15.3-22.3) and 22.6 (19.0-26.8) ppm, respectively, and had an equivalent PNB values of 0.75 at 25 nmol/cm^2. In addition, **4** and **5** were explored for their human-based repellency against *Ae. aegypti*, attractiveness against male medflies *Ceratitis capitata*, and also evaluated against series of human carcinoma cells (A549, HEK293, HeLa, SH-SY5Y); however, no significant activity was found.

Keywords: fatty ethyl esters; phytosterol; stilbenoids; pest control; natural products.

1 Introduction

The mosquito *Aedes aegypti* L. is the vector for major diseases such as yellow fever, dengue fever, Zika and chikungunya viruses [1-7]. A combination of environmental factors such as hurricanes, heavy rainfalls, flooding and climate changes increased more mosquito breeding habitats and created pest resistance along with health and environmental issues [4, 8-12]. Therefore, there is an increasing demand for new environmentally-friendly mosquito control agents. Natural products allow for the discovery of the new and safe products. Under the Deployed War-Fighter Protection (DWFP) Research Program [13], we initiated screening of medicinal and aromatic plant extracts from the National Center for Natural Products Research (NCNPR) repository in order to discover new natural deterrents and larvicidal compounds against *Ae. aegypti*. Based on initial screening results, the ethanol extract of *Veratrum lobelianum* Bernh. rhizomes showed larvicidal activity and also demonstrated biting deterrent activity for *Ae. aegypti*. *Veratrum lobelianum* belongs to the Melanthiaceae family [14], and *Veratrum* has been found to be rich in steroidal alkaloids along with non-alkaloid constituents (stilbenoids, flavonoids, saturated fatty acids, and phytosterols) [15-20]. Toxic effects of *Veratrum* extracts have been reported for a variety of insects, such as *Drosophila melanogaster*, *Leptinotarsa decemlineata, Musca domestica*, *Oncopeltus fasciatus, Pseudococcus longispinus, P. affinis* [18], *Aphis craccivora, Culex pipiens pallens, Mythimna separata*, *Tetranychus cinnabarinus* [19] and *Periplaneta americana* [20]. The objective of this study was to follow the bioassay-guided fractionation of *V. lobelianum* extract to find active larvicidal and biting deterrent compounds against *Ae. aegypti*. Some of the

***Corresponding author: Abbas Ali,** National Center for Natural Products Research, The University of Mississippi, University, MS 38677, USA, E-mail: aali@olemiss.edu
Nurhayat Tabanca, Nancy Epsky: USDA-ARS, Subtropical Horticulture Research Station, Miami, FL 33158, USA
Nurhayat Tabanca, Zulfiqar Ali, Ikhlas A. Khan: National Center for Natural Products Research, The University of Mississippi, University, MS 38677, USA
Ulrich R. Bernier: USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL 32608, USA
Ayse Nalbantsoy: Ege University, Faculty of Engineering, Department of Bioengineering, Bornova, Izmir, 35100, Turkey

active compounds were also evaluated for other biological activities. This is the first report on the activity of *V. lobelianum* extract against *Ae. aegypti*.

2 Experimental

2.1 General

^{1}H and ^{13}C NMR experiments were recorded in $CDCl_3$ on a Bruker 400 MHz NMR (Palo Alto, California, USA) spectrometer. Column chromatography was performed using High-Performance Flash Chromatography (HPFC) (SP1, Bioatage Inc., Charlotte, NC, USA) system. Analytical Thin Layer Chromatography (TLC) was performed on silica gel F_{254} aluminum sheet (20 × 20 cm, Sigma-Aldrich Inc., St. Louis, MO, USA). The TLC plates were checked under UV-254 and 366 nm. Visualization was done by spraying with 0.5% vanillin solution in concentrated H_2SO_4-EtOH, followed by drying with a heat gun. Preparative thin layer chromatography (PTLC) was performed using glass-backed plates, pre-coated with silica gel F_{254} (20 cm 20 cm, 500 mm, Uniplate Analtech, Sigma-Aldrich Inc.). Ethyl palmitate (Cas # 628-97-7, Sigma-Aldrich Inc.), ethyl linoleate (Cas # 544-35-4, Sigma-Aldrich Inc.), resveratrol (Cas# 501-36-0, Cayman Chemicals, Ann Arbor, Michigan, USA) and oxyresveratrol (Cas # 29700-22-9, Cayman Chemicals) were purchased.

2.2 Plant material and extraction method

Veratrum lobelianum rhizomes (Figure **1**) were collected by Trish Flaster (Botanical Liaisons, Boulder, CO, USA) from Yundola, Bulgaria and identified by Dr. Vijayasankar Raman, National Center for Natural Products Research (NCNPR), The University of Mississippi, University, MS. The plant material was deposited in the Repository of Botanicals at the NCNPR (accession # 257). The powdered rhizomes of *V. lobelianum* were extracted three times with 95% ethanol using an accelerated solvent extractor to yield crude extract, which was used for subsequent biological bioassays.

2.3 Bioassay-guided fractionation process

Two hundred mg of *V. lobelianum* crude ethanol extract was applied to a HPFC system. The silica gel SI 25M column

Figure 1: *Veratrum lobelianum* rhizomes (Photo credit to Dr. Vijayasankar Raman).

was eluted at a flow rate of 15 mL/min using $CHCl_3$ and $CHCl_3$-MeOH mixtures with the following solvent systems: 100% $CHCl_3$ 500 mL; $CHCl_3$-MeOH (95:5 500 mL MeOH), $CHCl_3$-MeOH (95-5 → 90:10 500 mL; 90:10 → 50:50 400 mL MeOH) and MeOH (100% 100 mL). The aliquots of fractions collected in 12 mL and fractions were combined into six groups (Frs A-F) on the basis of chromatographic profiles on TLC ($CHCl_3$:MeOH 95:0.5:1 drop NH_4OH; $CHCl_3$:MeOH 90:10 and $CHCl_3$:MeOH 80:20, v/v). Fr A (20 mg) was further subjected to HPFC (SI 12M silica gel column), eluted with *n*-hexane (100%) and a gradient of *n*-hexane-ethyl acetate mixtures (90:10 → 50:50). Fractions were combined into two groups [A1 (8 mg) and A2 (6 mg)] on the basis of their TLC profiles. The subfraction A1 was carried out by GC-MS analysis and compounds **1** and **2** were identified. Compound **3** was directly obtained from the subfraction A2 (R_f= 0.6 in *n*-hexane:EtOAc 7:3, v/v). The fractions B (22 mg) and C (15 mg) were cleaned up by preparative TLC ($CHCl_3$:MeOH 9:1, v/v) to afford compounds **4** (9 mg, R_f= 0.31) and **5** (6 mg, R_f= 0.28), respectively.

2.4 Identification of compounds

The nonpolar subfraction A1 was analyzed using an HP 5890 series gas chromatograph linked to an HP 5970 mass spectrometer system using a DB-1 capillary column (20 m × 0.18 mm with 0.25 µm film thicknesses). Helium (1 mL/min) was used as carrier gas. The GC oven temperature was held at 50°C for 2 min and then ramped to 280°C at a rate of 5°C/min. The scanned mass range was from *m/z* 40 to 550, and the ion source was operated in electron ionization (EI) mode with an electron energy set to 70 eV.

The components in subfraction of A1 were identified as ethyl palmitate (**1**) and ethyl linoleate (**2**) by comparing their mass spectra with NIST and Wiley libraries and also compared their retention time, retention index and mass spectra with authentic standards (ethyl palmitate and ethyl linoleate) purchased from Sigma-Aldrich Inc. To support the identification of compounds in subfraction of A1, the total *V. lobelianum* crude ethanol extract was performed by headspace solid phase microextraction (HS-SPME) in combination with GC-MS. Compounds adsorbed by SPME fiber coated with 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) were thermally desorbed in the GC injector of GC-MS using an Agilent 5975B (Agilent Technologies) system equipped with a DB5-MS column. The carrier gas was helium, with a flow rate of 1.3 mL min^{-1}. GC oven temperature was kept at 45°C for 1 min and programmed to 94°C at a rate of 4 °C min^{-1}, and increased to 180°C at a rate of 2°C min^{-1}, and lastly increasing at 20°C min^{-1} to 240°C. The PTV injector temperature was 200°C. Mass spectra were recorded at 70 eV. Mass range was *m/z* 35 to 450, ion source temperature was 230°C, and the scan rate was 2.8 sec^{-1}. The identification of compounds in *V. lobelianum* extract was performed on the basis of retention times with those of authentic samples ethyl palmitate and ethyl linoleate (Sigma-Aldrich Inc.), comparing the linear retention indices relative to series of *n*-hydrocarbons and on computer matching against commercial libraries (NIST 2017 and Flavors and Fragrances of Natural and Synthetic Compounds 3 2015).

Structure elucidation of the isolated compounds **3**-**5** was carried out using ^{1}H-NMR and ^{13}C-NMR techniques and data were compared with reported literature values [21-23]. The co-TLC analysis was also carried out for compounds **3**-**5** with authenticated samples deposited in the NCNPR repository [24-26].

2.5 Biological bioassays

2.5.1 Insects

Aedes aegypti L. (Orlando strain) used in larvicidal, biting deterrence and repellent bioassays were supplied from a laboratory colony maintained at the Mosquito and Fly Research Unit at the USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), Gainesville, FL. The detailed mosquito rearing was previously reported [27]. Sterile male *Ceratitis capitata* (Wiedemann) were obtained from the Programa Moscamed mass rearing facility (El Pino, Guatemala), where they were irradiated as pupae 2 d prior to emergence with 95 Gy of gamma radiation from a Co^{60} source. Irradiated pupae were shipped initially to the USDA-APHIS Medfly Project (Sarasota, FL) and then to the USDA-ARS Subtropical Horticulture Research Station (SHRS) in Miami, FL. Holding conditions at SHRS and the rearing procedures were performed as described earlier [28].

2.5.2 Mosquito larvicidal bioassay

The detailed bioassays were conducted using a bioassay system described by Pridgeon et al. [29] to determine the larvicidal activity of plant extracts and individual compounds against *Ae. aegypti*. Samples were diluted in dimethyl sulfoxide (DMSO) to make stock solution of 12.5 µg/µL for extracts and 10 µg/µL for pure compounds. The extract was prescreened at dosages of 125, 62.5, 31.25 and 15.625 ppm (parts-per-million) whereas the pure compounds were tested at 100, 50, 25 and 12.5 ppm. Larval mortality was recorded at 24, 48 and 72 h post-treatment. If pure compounds produced >80% mortality at 25 ppm, dosage mortality bioassays were conducted to determine the LC_{50} values. Data were analyzed using SAS Proc Probit [30]. Permethrin (a combination of 46.1% *cis* and 53.2% *trans* isomers, Chem Service, West Chester, PA, USA) at 0.025 ppm was used as positive control and DMSO served as a negative control.

2.5.3 Mosquito biting bioassay

Bioassays were conducted using a six-celled *in vitro* Klun & Debboun (K & D) module bioassay system developed by Klun et al. [31] for quantitative evaluation of biting deterrent properties of pure compounds and plant extracts. The detailed bioassays conditions were described earlier [32]. Extracts were evaluated at a dose of 100 µg/cm^2 in ethanol. The pure compounds and the positive control DEET (*N*,*N*-diethyl-3-methylbenzamide, Sigma-Aldrich, St. Louis, MO, USA) was tested at 25 nmol/cm^2. Ethanol was used as a negative control. Proportion not biting (PNB) values were calculated using the following formula:

PNB = 1 – (total number of biting females/total number of females)

Proportion not biting data were analyzed using SAS Proc ANOVA [30], and means were separated using Duncan's Multiple Range Test.

2.5.4 Mosquito repellent bioassay

Repellency of compounds **4** and **5** was determined as minimum effective dosage (MED), which is the minimum threshold surface concentration necessary to prevent mosquitoes from biting through the treated surface. MED values were obtained using a cloth patch assay as described in [11,33-34]. There were 3 human volunteers in this study and all 3 provided written informed consent to participate in this study as part of a protocol (636-2005) approved by the University of Florida Human Use Institutional Review Board (IRB-01). DEET was used as the positive control and acetone was used as a negative control.

2.5.5 Short-range attraction

Small cage bioassays were used to quantify the short-range attraction of sterile *C. capitata* males to test substrates [28]. Briefly, compounds **4** and **5** were prepared in 10% in acetone and 5 µL was added to the center of a filter paper disk. The filter paper was placed into the bottom of a Petri dish, which was then placed in the center of the cage with flies. After 5 min, the lid was placed on the dish, and the covered dish was removed from the cage. Percentage of flies responding, which was determined by dividing the number of flies in the dish by the sum total of the number flies in the dish plus the number of flies remaining in the cage at the end of each bioassay, was used for analysis. Flies and Petri dishes were used only once, and cages were pressure washed with hot water between tests to eliminate potential residual chemicals. Acetone only (5 µL added to filter paper) was used as the negative control and 5 µL of 10% tea tree essential oil in acetone (Essential Oil India – SAT Group, Kannauj, India) was added to filter paper as a positive control [28].

2.5.6 Cytotoxicity assay

A549 (human lung carcinoma cells), HeLa (human cervical cancer cells), SH-SY5Y (human neuroblastoma cells) and, as a normal cell line HEK293 (human embryonic kidney cells) were used to test the cytotoxicity for compounds **4** and **5** via modified MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)) assay [35-36]. Doxorubicin (Sigma-Aldrich Inc.) was used as a positive control. The optical density of the dissolved material was measured at 570 nm (reference filter, 620 nm) with an UV-visible spectrophotometer (Thermo Multiskan Spectrum, Thermo Scientific, Waltham, MA, USA). The viability (%) was determined by the following formula:

% Viable cells = [(absorbance of treated cells) – (absorbance of blank)] / [(absorbance of control) – (absorbance of blank)] x 100

The IC_{50} values were reported at ± 95% confidence intervals (± 95% CI). This analysis was performed with Graph Pad Prism 5 (San Diego, CA, USA).
Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

Veratrum lobelianum extract showed LC_{50} values of 11.35 (9.27-13.85) ppm against 1st instar and 89.9 (73.0-109.5) ppm against 4th instar larvae of *Ae. aegypti* at 24 h post-treatment (Table **1**). The extract also showed a proportion not biting (PNB) value of 0.76 at a dose of 100 µg/cm^2 in *in vitro* K&D bioassay against female *Ae. aegypti*. Based on these results, *V. lobelianum* ethanol extract was subjected to bioguided fractionation which yielded six fractions (A-F). All the fractions were evaluated for larvicidal and biting-deterrent activities. Three of the six partitioned fractions (A-C fractions) prompted us to perform isolation and identification of the active compounds whereas the more polar fractions (D-F) were inactive in both the bioassays (Table **2**, Figure **2**). These results suggested that polarity of fractions plays an important role on the activity. In larvicidal bioassays; fractions A, B and C showed 100% mortality at 31.25 ppm. The active subfraction A1 was further analyzed by GC-MS and ethyl palmitate (**1**, LRI: 1993) and ethyl linoleate (**2**, LRI: 2163) were identified and their mass spectra and LRI values were compared with purchased authentic compounds of ethyl palmitate (LRI: 1992) and ethyl linoleate (RRI: 2163). Additional confirmation was also carried out for the detection of these compounds from the *V. lobelianum* crude extract by using HS-SPME in combination with GC-MS, and ethyl palmitate (**1**, *m/z*= 88, 101, 157, 284) and ethyl linoleate (**2**, *m/z*= 67, 81, 95, 109, 308) were found in the total *V. lobelianum* extract (Supplementary material, Figure **S1**). After the confirmation was completed, compounds **1** and **2** were individually evaluated in larvicidal assays. Compound **2** had LC_{50} value of 24.1 (22.0-26.2) ppm and LC_{90} value of 38.2 (34.3-44.3) ppm at 24 h post treatment, whereas compound **1** was inactive at the highest screening dose of 100 ppm; therefore, dose-response bioassays were

Table 1: Toxicity of *Veratrum lobelianum* extract against *Aedes aegypti* larvae.

Instar	Larvicidal activity against *Ae. aegypti* (ppm)					
	24 h post-treatment		48 h post-treatment		72 h post-treatment	
	LC_{50} (95% CL)	LC_{90} (95% CL)	LC_{50} (95% CL)	LC_{90} (95% CL)	LC_{50} (95% CL)	LC_{90} (95% CL)
1st	11.79 (10.2-13.7)	29.2 (224.0-37.9)	8.9 (7.1-10.9)	25.2 (19.5-36.7)	7.3 (5.7-9.0)	32.4 (24.6-47.9)
4th	89.9 (73.0-109.5)	210.1 (164.6-305.3)	78.4 (63.9-95.1)	176.2 (138.8-255.2)	59.3 (48.4-71.5)	123.3 (98.5-178.0

Table 2: Larvicidal and biting-deterrent activity of *V. lobelianum* extract, fractions and isolated compounds (1-5).

Samples	Larvicidal activity ppm	Biting-deterrent activity PNB values
V. lobelianum EtOH extract	100% mortality at 31.25 ppm	0.76 ± 0.027 at 100 µg/cm^2
Fractions		
Fr A	100% mortality at 31.25 ppm	0.52 ± 0.09 µg/cm^2 at 100 µg/cm^2
Fr A1 (subfraction)		
Compound **1**	Not active at 100 ppm	-
Compound **2**	LC_{50}= 24.1 (22.0-26.2) ppm LC_{90}= 38.2 (34.3-44.3) ppm	-
Fr A1 (subfraction)		
Compound **3**	LC_{50}= 1.7 (1.3-2.3) ppm LC_{90}= 5.1 (3.4-13.8) ppm	-
Fr B	100% mortality at 31.25 ppm	0.76 ± 0.085 µg/cm^2 at 100 µg/cm^2
Compound **4**	LC_{50}= 18.5 (15.3-22.3) ppm LC_{90}= 41.6 (32.8-60.2) ppm	0.75 ± 0.05 µg/cm^2 at 25 nmol/cm^2
Fr C	100% mortality at 31.25 ppm	0.80 ± 0.065 mg/cm^2 at 100 µg/cm^2
Compound **5**	LC_{50}= 22.6 (19.0-26.8) ppm LC_{90}= 49.4 (39.7-68.4) ppm	0.75 ± 0.05 µg/cm^2 at 25 nmol/cm^2
Fr D	0 mortality at 31.25 ppm	0.4 ± 0.09 mg/cm^2 at 100 µg/cm^2
Fr E	0 mortality at 31.25 ppm	0.4 ± 0.09 mg/cm^2 at 100 µg/cm^2
Fr F	0 mortality at 31.25 ppm	0.4 ± 0.09 mg/cm^2 at 100 µg/cm^2
Positive and negative controls		
DEET	-	0.84 µg/cm^2 at 25 nmol/cm^2
Ethanol	-	0.24-0.26
Permethrin	100% mortality at 0.025 ppm	-
Dimethyl sulfoxide	0	-

not performed. Compound **3** (β-sitosterol) was directly isolated from subfraction A2 and showed an LC_{50} value of 1.7 (1.3-2.3) ppm and LC_{90} value of 5.1 (3.4-13.8) ppm at 24 h post treatment. Compounds **4** (*trans*-resveratrol) and **5** (oxyresveratrol) were isolated from Frs B and C, respectively. In dose-response bioassays, compound **4** had LC_{50} and LC_{90} values of 18.5 (15.3-22.3) and 41.6 (32.8-60.2) ppm, respectively. Compound **5** had LC_{50} and LC_{90} values of 22.6 (19.0-26.8) and 49.4 (39.7-68.4) ppm, respectively, at 24 h post treatments.

In *in vitro* K&D biting-deterrent bioassays, the crude extract showed PNB value of 0.76 at a dose of 100 µg/cm^2. Out of the six fractions, fraction A demonstrated weak biting deterrent activity with PNB value of 0.52 ± 0.09 µg/cm^2 and frs B and C exhibited biting deterrent activity with PNB values of 0.76 ± 0.085 and 0.80 ± 0.065 µg/cm^2 at 100 µg/cm^2, respectively. Both compounds **4** and **5** had the PNB value of 0.75 at 25 nmol/cm^2. PNB values for DEET was 0.84 µg/cm^2 at 25 nmol/cm^2 (Table **1**). To determine their repellency level, compounds **4** and **5** were evaluated in *in vivo* cloth patch repellent bioassays; however, **4** and **5** did not effectively repel adult *Ae. aegypti* with a minimum effective dosage (MED) of 0.75 mg/cm^2 which was a very high dose as compared to DEET (0.006 ± 0.000 mg/cm^2).

To test for potential attraction of plant-derived natural compounds to male Mediterranean fruit fly

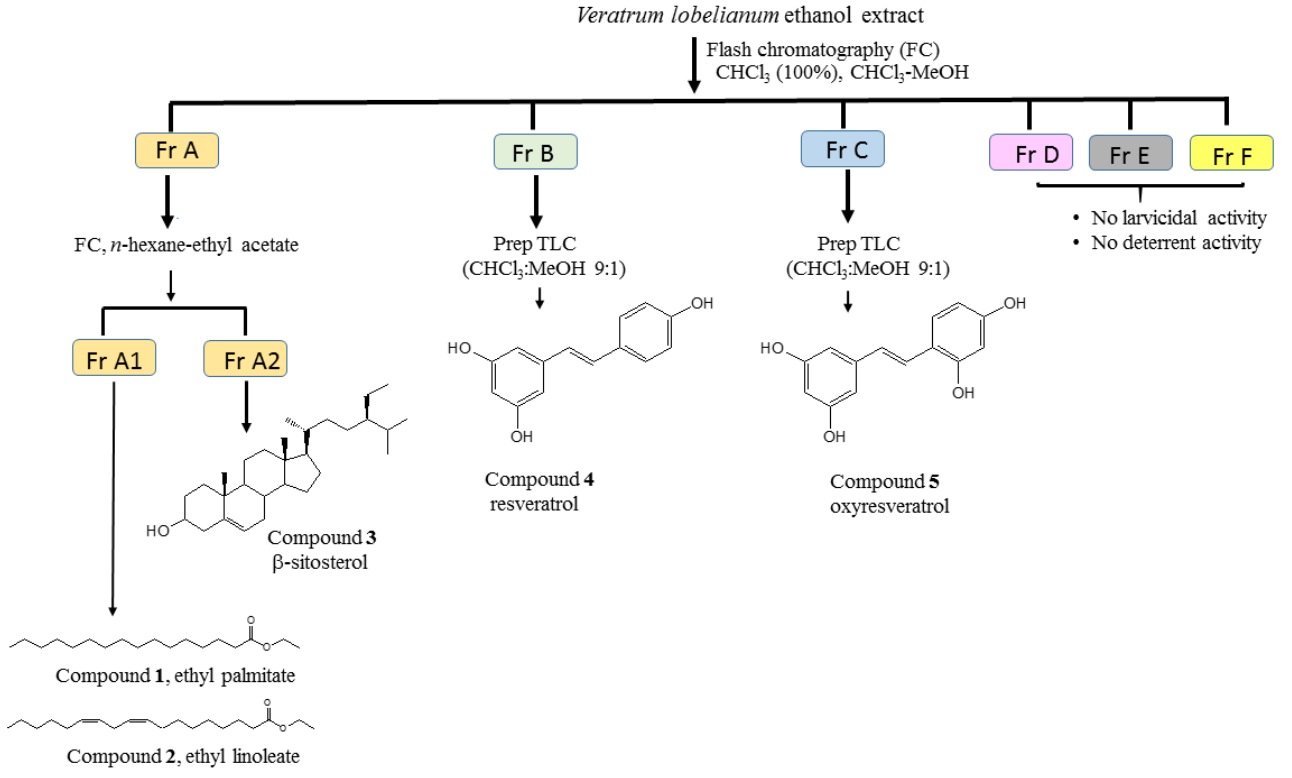

Figure 2: Schematic procedure of bioassay-guided isolation of active components from *Veratrum lobelianum* extract.

(medfly, *C. capitata*), compounds **4** and **5** were evaluated. For both compounds, 5 µL of 10% solution (w/v in acetone) were applied separately to filter paper disks. After the application, the filter paper was air dried to allow evaporation of acetone before placing into the cage bioassays; however, no significant attraction was observed compared to positive control 10% tea tree essential oil.

Compounds **4** and **5** belong to stilbenoids which are receiving much attention for their potential therapeutic agents for human health [37-41]. In the current study, we expanded our biological activity to evaluate compounds **4** and **5** for their effect on different human tumor cells (A549, HEK293, HeLa, SH-SY5Y). Compounds **4** and **5** were initially screened at 50 µg/mL and both compounds demonstrated approximately 40% inhibition of cell growth versus A549 and SH-SY5Y cells (Figure **3**); however, compounds **4** and **5** did not induce detectable toxicity. Therefore, their IC_{50} determination could not be performed. With doxorubicin, the positive control, IC_{50} values for A549, HEK293, HeLa, and SH-SY5Y cells were 2.87 ± 0.07, 1.03 ± 0.01, 1.37 ± 0.17 and 1.11 ± 0.03 µg/mL, respectively, after 48 h exposure.

In the present study, *V. lobelianum* extract exhibited high larvicidal activity against 1st instar *Ae. aegypti* larvae and also showed biting deterrent activity against females of *Ae. aegypti*. This investigation led to the isolation and identification of two esters of fatty acids (**1** and **2**), one phytosterol compound β-sitosterol (**3**) and two stilbenoids (**4** and **5**). Since natural products are complex mixtures and minor compounds may contribute to the improvement of the biological activity, therefore importance of these interactions should not be ignored. In the current study, the unsaturated fatty ethyl ester (**2**) was toxic to 1st instar *Ae. aegypti* larvae, whereas the saturated fatty ethyl ester (**1**) was not active. This selectivity might be coming from the ester chain length in fatty ethyl esters or position of the double bonds. In a previous study, unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid, and elaidic acid) were more toxic than the saturated fatty acids (arachidic acid, behenic acid, palmitic acid, and stearic acid) against third instar larvae of *Ae. aegypti*, *Ae. albopictus* and *Culex pipiens pallens* [42]. More detailed studies on unsaturated and saturated fatty ethyl esters are needed to understand their structure-activity relationships against mosquito larvae. Among the isolated compounds, β-sitosterol (**3**) showed the highest larvicidal activity, with LC_{50} value of 1.7 (1.3-2.3) ppm. A previous study also reported β-sitosterol as a potential larvicidal compound against three mosquito species: *Ae. aegypti*, *Anopheles stephensi* and *C. quinquefasciatus* [43]. β-Sitoterol or phytosterol type of compounds might be useful as larvicides in the control

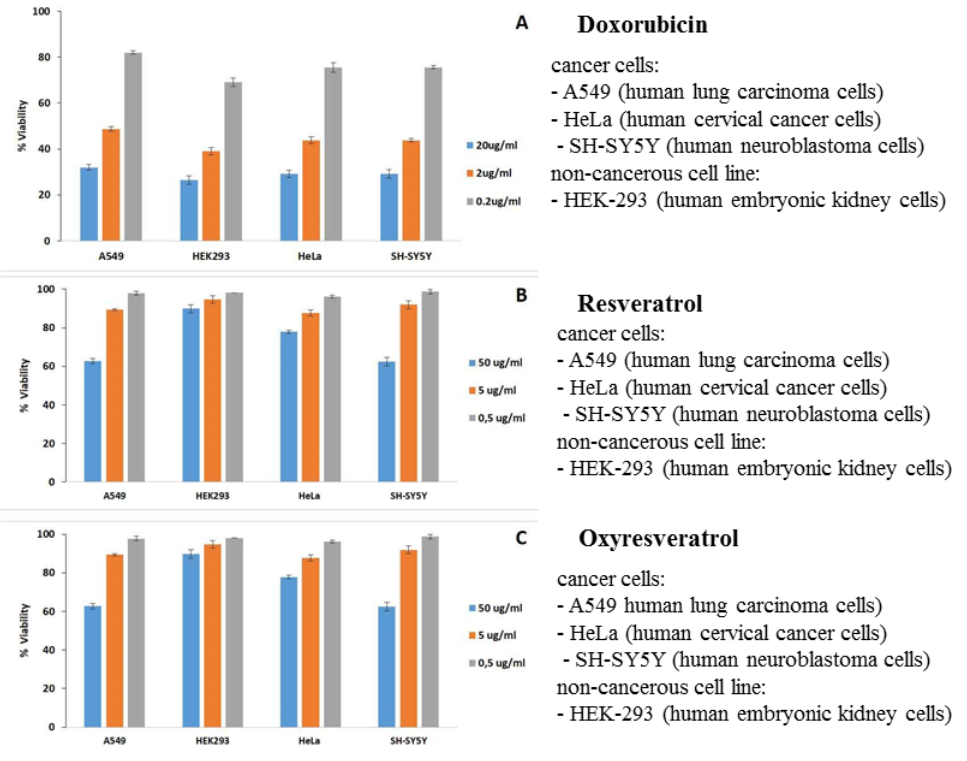

Figure 3: Viability of cancer and non-cancerous cell lines after compounds **4** (resveratrol) and **5** (oxyresveratrol) treatments for 48 h. Cell viability was determined by MTT assay, control was exposed to vehicle only which was taken as 100% viability.

of mosquito populations. Compounds **4** (resveratrol) and **5** (oxyresveratrol) are stilbenoids and several stilbenoid derivatives were previously evaluated by our research team for toxicity in larvae and adult stages of *Ae. aegypti* and the results demonstrated that olefinic configuration, number, and position of hydroxyl groups in stilbenoids tend to be important factors in determining biological activities [37]. However, high toxicity has been reported for the insecticides used against mosquitoes than the natural products. For example, permethrin (LC_{50} = 0.0031 ppm) [44] and chlorfenapyr (LC_{50} = 0.0019 ppm) [45] showed very high toxicity as compared to the natural products tested in this study.

Resveratrol has also been studied in other invertebrates. It was reported that lifespan was extended in *Caenorhabditis elegans* and *Drosophila melanogaster* with resveratrol [46]. Insect nutrition is important and can influence lifespan and fecundity in insects, especially for gamma-ray sterilized insects. Resveratrol has antioxidant and radioprotective properties [47]; therefore, the addition of resveratrol to the diet for fruits flies *Bactrocera dorsalis* and *B. cucurbitae* was investigated [45]. In irradiated *B. dorsalis*, adult emergence improved from 12 to 29% with a 100 µM resveratrol enriched diet, but not with a diet supplemented with 50 or 200 µM concentrations. In irradiated *B. cucurbitae*, 49-53% of adults could fly when 50, 100 or 200 µM resveratrol was added to the diet compared with 32% in flies reared without resveratrol [48]. Efficacy of resveratrol treatments may be dose-dependent. More studies are needed with irradiated pupae and resveratrol (and its analogs) to develop rearing protocols which improve adult performance while maintaining sterility in tephritid fruit flies.

4 Conclusions

Bioassay-guided fractionation was an effective technique to identify chemicals of the ethanol extract of *Veratrum lobelianum*, and use of this technique will aid in the discovery of new biologically active natural products from plants. Overuse of conventional insecticides has led to widespread pesticide resistance and created serious concerns to human health and the environment. There have been great research efforts in academic and industrial laboratories to discover new biologically active natural biopesticides from plants and microorganisms. Development and application studies on natural products

for pest control can enhance their acceptance and registration by the EPA (U.S. Environmental Protection Agency). A combination of natural products with commercial insecticide studies are promising to evaluate for delaying pesticide resistance and the results might help minimizing the side effects to human health and environment, however, further research could clarify these issues.

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