Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Mosquitocidal activities of *Chenopodium botrys* whole plant n-hexane extract against *Culex quinquefasciatus*

Atividades mosquitocidas do extrato de n-hexano de planta inteira de *Chenopodium botrys* contra *Culex quinquefasciatus*

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Abstract

This research aimed to investigate various mosquitocidal activities of *Chenopodium botrys* whole- plant n-hexane extract against *Culex quinquefasciatus*. The extract showed remarkable larvicidal, pupicidal, adulticidal, oviposition deterrent and adult emergence inhibitory activities against *Cx. quinquefasciatus*. During the larvicidal and pupicidal activities, the 24-hour lethal concentration (LC_{50}) of extract against 2nd instar larvae, 4th instar larvae and pupae were 324.6, 495.6 and 950.8 ppm, respectively. During the CDC (Centers for Disease Control and Prevention) bottle bioassay for adulticidal activity, the median knockdown times (KDT_{50}) at 1.25% concentration was 123.4 minutes. During the filter paper impregnation bioassay for adulticidal activity, the KDT_{50} value at 0.138 mg/cm^2 concentration was 48.6 minutes. The extract was fractionated into 14 fractions through silica gel column chromatography which were then combined into six fractions on the basis of similar retention factor (Rf) value. These fractions were screened for adulticidal activity by applying CDC bottle bioassay. The fraction obtained through 60:40 to 50:50% n-hexanes-chloroform mobile phase with 0.5 Rf value showed 100% adulticidal activity at 0.2% concentration. During oviposition deterrent activity, the highest concentration (1000 ppm) showed 71.3 ± 4.4% effective repellence and 0.6 ± 0.1 oviposition activity index. During adult emergence inhibition activity, the median emergence inhibition (EI_{50}) value was 312.3 ppm. From the outcome of the present investigation, it is concluded that the n-hexane extract of *C. botrys* whole- plant possesses strong larvicidal, pupicidal, adulticidal, oviposition deterrent and adult emergence inhibitory activities against *Cx. quinquefasciatus*.

Keywords: adulticidal, column chromatography, column fractions, larvicidal, pupicidal.

Resumo

Esta pesquisa teve como objetivo investigar várias atividades mosquitocidas do extrato n-hexano de planta inteira de *Chenopodium botrys* contra *Culex quinquefasciatus*. O extrato mostrou atividades larvicida, pupicida, adulticida, dissuasora de oviposição e inibidora da emergência de adultos contra a *Cx. quinquefasciatus*. Durante as atividades larvicida e pupicida, a concentração letal de 24 horas (CL50) do extrato contra larvas de 2º estádio, larvas de 4º estádio e pupa foi de 324,6, 495,6 e 950,8 ppm, respectivamente. Durante o bioensaio com frasco do CDC (Centros para Controle e Prevenção de Doenças) para adulticida, o tempo médio de desativação (KDT50) na concentração de 1,25% foi de 123,4 minutos. Durante o bioensaio de impregnação com papel de filtro para a atividade adulticida do extrato, o valor KDT50 na concentração de 0,138 mg / cm^2 foi de 48,6 minutos. O extrato foi fracionado em 14 frações através de cromatografia em coluna de gel de sílica que foram então combinadas em seis frações com base em um valor de fator de retenção (Rf) semelhante. Essas frações foram selecionadas quanto à atividade adulticida por meio da aplicação do bioensaio com garrafa do CDC. A fração obtida através da fase móvel de n-hexanos-clorofórmio 60:40% a 50:50% com valor de 0,5 Rf apresentou atividade adulticida de 100% na concentração de 0,2%. Durante a atividade de dissuasão da oviposição, a maior concentração de extrato (1000 ppm) apresentou repelência efetiva de 71,3 ± 4,4% e índice de atividade de oviposição de 0,6 ± 0,1. Durante a atividade de inibição da emergência de adultos, o valor médio de inibição da emergência (EI50) foi de 312,3 ppm. A partir do resultado da presente investigação, conclui-se que o extrato de n-hexano da planta inteira de *C. botrys* possui fortes atividades larvicida, pupicida, adulticida, dissuasora da oviposição e inibidora da emergência de adultos contra a *Cx. quinquefasciatus*.

Palavras-chave: adulticida, cromatografia em coluna, frações de coluna, larvicida, pupicida.

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Received: July 11, 2020 – Accepted: December 21, 2020

1. Introduction

Synthetic chemical insecticides play role in pest control. They belong to the carbamate, organophosphate, organochlorine, pyrethroid, and neonicotinoid pesticide (Gullan and Cranston, 2014). Organochlorines were first introduced in the market during 1940s which were then succeeded by organophosphates and carbamates, pyrethroids and most recently by neonicotinoids in 1990s (Casida and Durkin, 2013).

Mosquitoes are small blood sucking insects and transmit several diseases in humans such as filariasis, dengue, yellow fever and malaria (Farajollahi et al., 2011). *Culex quinquefasciatus* (Diptera: Culicidae) is the most wide spread mosquito in tropical and subtropical regions and breeds in wide range of stagnant water bodies such as cemented drains, ditches, pools, rice fields, river margins, marshes, wells and ponds (Kramer et al., 2008; Andreadis, 2012; Ilahi and Suleman, 2013). Its irritating biting creates nuisance and is responsible for transmitting the pathogen of lymphatic filariasis (elephantiasis, *Wuchereria bancrofti*) (Ramaiah et al., 2003). Millions of people suffer from filariasis in different regions of the world (Simonsen, 2009). Few cases of tropical pulmonary eosinophilia in Pakistan have also been reported (Beg et al., 2001). *Cx. quinquefasciatus* is also known for transmitting malaria in birds (Glad and Crampton, 2015).

Disease transmitting mosquitoes are controlled by the application of chemical insecticides in addition to habitat reduction. Constant and frequent application of insecticides has contaminated the environment, affected the non-target organisms, harmed human health, and developed insecticide resistance in pests (Lee et al., 2001). Application of environment friendly approaches for control of insect pests is gaining attention (Ghosh et al., 2012; Benelli, 2015). Biological control is the common method and ecofriendly approach to pest control. Several living organisms such as microbes (Das et al., 2016), natural predators (Mandal et al., 2008), and plants (Ajaegbu et al., 2016) have been described for their mosquitocidal effects. It is claimed that insecticides derived from medicinal plants are safe for other organisms which are not target (Rawani et al., 2014). Plant extracts on non-target organisms have also been studied and such insecticides have been claimed safe for non-target organisms (Carvalho et al., 2003; Chowdhury et al., 2009; Adhikari et al., 2012).

Insecticides derived from medicinal plants are called botanical insecticides and they have been screened for their mosquitocidal activities (Elango et al., 2012; Prathibha et al., 2014; Reegan et al., 2015; Bekele and Petros, 2017). Mahanta et al. (2019) reported biological effects caused by *Lippia alba* essential oils at various stages of *Cx. quinquefasciatus* and *Aedes aegypti*. Pratti et al. (2015) reported the larvicidal activity caused by *Schinus terebinthifolia* essential oils against *Ae. aegypti*. Fifty-six substances were identified which constituted the 81.67% of the essential oil composition. Bekele and Petros (2017) reported repellent activity on *Anopheles arabiensis* with *Brassica nigra*; and *Aloe pirottae* extracts. Jayapriya and Shoba (2015) studied various mosquitocidal activities of different solvent extracts of *Rhinacanthus nasutus* leaves against *Cx. quinquefasciatus* and *Ae. aegypti*. Prajapati et al. (2005) reported that botanical insecticides also have the ability to deter the mosquitoes from egg laying. Active ingredients contained in plant extracts induced physiological and behavioral changes in the gravid female mosquitoes. The active ingredients of plant-based insecticides act as chemosterilent, growth regulators or they may exert olfactory stimuli acting as repellent (Prathibha et al., 2014). Plants that repel insects, also deter oviposition and inhibit adult emergence (Rajkumar and Jebanesan, 2009; Elango et al., 2012; Prathibha et al., 2014).

Chenopodium botrys Linnaeus (Chenopodiaceae) is a medicinal plant known as Skha Kharawa in Dir district of Malakand division, Khyber Pakhtunkhwa, Pakistan. This plant grows near streams in sandy soil and foot hills up to 1,200 m and its dry leaves decoction is used for the treatment of cough, catarrh, and asthma. The plant is insect repellent and vermifuge for cattle. The parts of *C. botrys* are mixed in soil for killing insect pest larvae (Ilyas, 2015). Plant extracts obtained through non-polar solvent contain more non-polar compounds which can easily penetrate and damage the insect cuticle (Khader, 1999). The aim of the present research was to investigate the oviposition deterrent, larvicidal, pupicidal, adult emergence inhibition and adulticidal activities of non-polar solvent extract of *C. botrys* whole-plant against *Cx. quinquefasciatus*.

2. Materials and methods

2.1. Collection of plant and extract preparation

The plant *C. botrys* was collected during October 2015 in the foothill near the campus of University of Malakand, Khyber Pakhtunkhwa, Pakistan. The plant was authenticated by plant taxonomist at the Department of Botany, University of Malakand. *Chenopodium botrys* whole-plants were rinsed in tap water and then dried in shade. The dried plant materials were ground into powder form by electric chopper and then 400 g powder of *C. botrys* was soaked in 2000 mL n-hexane in a 3-liter plastic jar for 3 days. Extracts obtained through non-polar solvent contain more non-polar compounds which can easily penetrate and damage insect cuticle, therefore during the present study, n-hexane (a non-polar solvent) was used for preparation of extract. The soaked plant material was filtered through Whatman filter paper no.42. Vacuum rotary evaporator was used for evaporation of solvent. Thick solution of extract from rotary evaporator bulb was poured into 500 mL glass beaker which was kept open in the laboratory for 24 h for evaporating the remaining solvent. Brownish extract in paste form was obtained. About 28 g (14.4% yield) of *C. botrys* whole-plants n-hexane extract was obtained. The extract was placed at 4 °C in refrigerator.

2.2. Column fractionation of n-hexane extract and thin layer chromatography

The n-hexane extract of the *C. botrys* whole- plant was subjected to fractionation through column chromatography for getting closure to active principles. First of all, a column of glass was aligned vertically. A cotton plug was tamped

down toward the nozzle of the column. Then the glass column was filled partially with n-hexane. Powder form of silica gel (200 g) was suspended in n-hexane in a 250 mL glass flask to form its slurry. The slurry was poured over the cotton plug in the glass column to make silica gel bed, a stationary phase. Twenty grams of *C. botrys* extract was dissolved in 100 mL n-hexane and then powder form of silica gel was added and stirred. After the extract got dry, the extract was crushed into powder form. The extract powder was then poured into the column (glass) over the bed of silica gel. At the beginning, 100% non-polar solvent (i.e., n-hexane) was passed as the mobile phase through the column. Then mixture of non-polar and polar solvents in different ratios was passed as the mobile phase. Sufficient volume (2 L) of each mobile phase was passed through the column. Various fractions of different polarity were collected and dried. Fourteen fractions were obtained which were then subjected to thin layer chromatography (TLC).

During this experiment, small (5×10 cm) TLC cards of Merck Darmstadt, Germany were cut with the sharp blade. Solutions of fractions were spotted with the help of capillary tubes on the base of TLC cards. Solvent system of 70% n-hexane and 30% chloroform was arranged in a glass tank (14×10×10 cm). TLC cards were then placed in the glass tank for sometimes till the TLC runs up to solvent front slightly lower than top. TLC spots on TLC cards were observed by using ultraviolet UV254, UV365 and visible light. TLC cards were sprayed with ceric sulphate solution and then placed on hot plate for few seconds for increasing visibility. Retention factor (Rf) values were calculated (Equation 1). Fractions with similar Rf values were combined.

$$\mathrm{Rf} = \frac{\text{Distance from the starting line to the center of spot}}{\text{Distance from starting line to the solvent front}} \quad (1)$$

The Rf values of all the fourteen fractions were calculated. The fractions with similar Rf value were recombined.

2.3. Establishment of laboratory colony of Cx. quinquefasciatus

Culex quinquefasciatus larvae were collected from the stagnant water bodies at the campus of University of Malakand during May 2016 and reared in the laboratory in 500 mL plastic containers inside mosquito cage in the laboratory for establishing mosquito colony. Maximum temperature inside the laboratory was 27 °C to 31 °C. Yeast powder and dog biscuit in 2:3 was supplied as food for the larvae. The larvae developed into pupae from which adults emerged. Adults were initially fed with 10% sucrose solution in cotton pad. After emergence, mosquito adults were blood fed for eggs development during evening time by restraining mice inside mosquito cage. The use of mice in this study was approved by animal ethical committee at University of Malakand. The gravid female mosquito adults laid eggs in plastic jars containing inside the mosquito cages. The eggs hatched into 1st instar larvae which after few days developed into pupae and adults. All stages of mosquito were available for mosquitocidal experiments.

2.4. Larvicidal and pupicidal activities

For conduction of larvicidal bioassay, standard procedures were followed (Larvicides, 2005). During this bioassay, 4000 mg of *C. botrys* whole-plant extract was emulsified in 16 mL acetone, 5 mL Tween-80 and some non-chlorinated tap water in 250 mL glass flask. This emulsified solution was then poured into 2500 mL plastic bottles to which further water was added till the volume reached to 2000 mL. Thus 2000 mL extract solution of 2000 ppm extract solution containing 0.8% acetone and 0.025% tween 80 was prepared. Then, 100 mL solutions of 1000, 500, 250, 125, 62 and 30 ppm concentrations were prepared in 200 mL plastic containers for assessing the larvicidal activity against 2nd and 4th instar larvae of *Cx. quinquefasciatus*. Extract solutions of the same concentrations were prepared for assessing the pupicidal activity against *Cx. quinquefasciatus*. Control solutions which was consisting of tap water, acetone (0.8%) and Tween-80 (0.025%) was also prepared. Second instar larvae (n=20) of *Cx. quinquefasciatus* were transferred from established colony to each concentration of extract solutions arranged for larvicidal activity against 2nd instar larvae. Twenty 2nd instar larvae were transferred to the control solution jar. Similarly, 20 *Cx. quinquefasciatus* larvae each of 4th instar were transferred to each concentration of extract solution arranged for larvicidal activity against 4th instar larvae. Twenty 4th instar larvae were transferred to control containers containing non-chlorinated tap water with 0.8% acetone and 0.025% Tween-80. Twenty pupae of *Cx. quinquefasciatus* were transferred to each concentration of extract solutions arranged for pupicidal activity. Twenty pupae were transferred to control solution container. The experiment was conducted in four replicates and percentage mortalities (larvae and pupae) were calculated after 24 h of exposure period. The criterion for death was lack of response to prodding. The lethal concentration (LC_{50}) value of extract was determined.

2.5. Adulticidal activity

During the present study, the adulticidal activity of *C. botrys* whole plant n-hexane extract against the adults of female of *Cx. quinquefasciatus* was conducted through CDC (centers for disease control and prevention) bottle and filter paper impregnation bioassays. In addition to the adulticidal activity of *C. botrys* whole plant n-hexane extract, the adulticidal activity of six fractions (F1-F6) of the extract was also studied through CDC bottle bioassay. The following are the details:

2.5.1. CDC bottle bioassay

During this bioassay, CDC (2010) protocol was followed. Ten milliliter extract solution of 1.25% was prepared in 25 mL glass flask. This solution was then sequentially diluted by factor of two into dilutions of 0.625%, 0.31% and 0.15% concentrations. Four 250 mL CDC glass bottles were labelled for four concentrations of extract solution (1.25%, 0.625%, 0.31% and 0.15%). One milliliter (1 mL) solution of each concentration was poured into the bottle for respective concentration. The bottles were placed side by side. A control bottle was also placed into which

1 mL of acetone was poured. All the bottles were rotated gently for swirling the solutions and thus the inside of each bottle became coated with the solution. The bottles were rolled continuously after removing their lids for making the inside of bottles dry. Aluminum foil was raped around the bottles to protect them from the effect of light and then placed in horizontal position for 24 h, and subsequently, the solvent evaporated completely. Then, 20 glucose-fed and blood-starved female mosquito adults were introduced into each CDC bottle, including the control bottle with the help of mouth aspirator. The opening of each bottle was closed with their lids after introduction of mosquito adults. The percentage of knock-down of mosquito adults was noted after every 15 min for 90 min. A mosquito was considered as dead or knocked down if it was unable to move or stand within 60 min of exposure (WHO, 2016). The median knock-down time (KDT_{50}) value of each concentration was determined by subjecting the knockdown data to probit test of Finney (1971). After 90 min, the lid was removed from each bottle and the mouth was covered by fine white cloth mesh. A small cotton pad soaked in 10% glucose solution was placed on the mesh. Percent mortality was noted after 24 h exposure in CDC bottles (Ajaegbu et al., 2016).

2.5.2. Paper impregnation bioassay

During this bioassay, WHO (1981) protocol was followed. Ten milliliter extract solution of 12.5 mg/mL was prepared in 25 mL glass flask. This solution was then sequentially diluted by factor of two into dilutions of 6.25 mg/mL, 3.1 mg/mL and 1.56 mg/mL concentrations. For four different concentration solutions (12.5, 6.25, 3.1 and 1.5 mg/mL), papers (12x15 cm) were cut from the sheet of Whatman no.1 filter paper. A control paper of the same size was also cut. From each concentration solution, 2 mL was applied on the respective filter papers. Thus, four impregnated papers with four extract concentrations of 0.138, 0.069, 0.034 and 0.017 mg /cm^2, respectively, were prepared. A control (12×15 cm) paper was also arranged onto which only 2 mL of acetone was applied. The extract impregnated papers and control papers were placed in exposure tubes in WHO kits for adulticidal activity. Twenty glucose-fed and blood starved female mosquito adults were introduced into each holding tube. The mosquito adults were then exposed to test papers in exposure tubes for 90 min. Percentage of knock-down mosquito adults was noted after every 15 min for 90 min. The median knock-down time (KDT_{50}) value of each concentration was determined by subjecting the knockdown data to probit test of Finney (1971). Mosquitoes were then transferred back to the holding tube and kept for a recovery period of 24 h. A cotton pad soaked in 10% glucose solution was placed on the mesh screen. After 24 h of recovery period, mortality of mosquito was recorded.

2.5.3. Adulticidal activity of column chromatographic fractions

Six fractions (F1-F6) of *C. botrys* whole-plants n-hexane extract was tested for adulticidal activity against *Cx. quinquefasciatus* female adults though CDC bottle bioassay (Ajaegbu et al., 2016). The concentrations of each fraction tested were 0.2%, 0.1%, 0.05% and 0.025%. During this bioassay, mortality of adult mosquitoes was noted for each fraction after 24 h. The detail of CDC bottles coating with extract for the adulticidal activity was the same as described earlier in CDC bottle bioassay.

2.6. Oviposition deterrent bioassay

The method of Xue et al. (2001) was followed for oviposition deterrent bioassay. Extract solutions of 31.25, 250 and 1000 ppm showed the lowest, moderate and highest larvicidal activity, therefore these concentrations were selected for conducting oviposition deterrent activity. During this bioassay, stock solution of extract was prepared in tap water by the procedure as practiced for stock solution preparation during larvicidal and pupicidal bioassay. As in larvicidal and pupicidal activity, this solution was also containing 0.05% of Tween-80 and 1.6% of acetone. Three mosquito cages labeled as cage A, cage B and cage C were arranged. From the stock solution, three extract solutions of 31.5, 250 and 1000 ppm, 200 mL each were prepared in three polyethylene containers, 400 mL each, which were then placed inside cage A, cage B and cage C, respectively. The volume of each solution was 200 mL. Inside each cage, 200 mL tap water having 1.6% acetone and 0.05% Tween-80 was also placed in 400 mL polyethylene container as control container. The experiment was run in triplicates. Hundred gravid female adults of *Cx. quinquefasciatus* were caught from established colonies through mouth aspirator and then introduced into each mosquito cage. After 2 or 3 d of introduction, egg rafts were observed in the containers. Under dissecting microscope, the total number of egg rafts and eggs were counted for each container. The oviposition deterrence data was presented as % ER (effective repellence) and mean OAI (oviposition activity indices). According to Govindarajan et al. (2011), negative OAI values represent repellents activity. The % ER was calculated by applying the Rajkumar and Jebanesan (2009) method (Equation 2).

$$\% \text{ ER} = \frac{\text{NC} - \text{NT}}{\text{NC}} \text{X100} \quad (2)$$

In the above formula, NC stands for eggs number in control container, and NT stands for eggs number in extract solution container. The OAI value for each treatment container was calculated by using Kramer and Mulla (1979) method (Equation 3).

$$\text{QAI} = \frac{\text{NT} - \text{NC}}{\text{NT} + \text{NC}} \quad (3)$$

In the above formula, Nt stands for total eggs number in extract solution container and Nc stands for the total eggs number in the container labeled as control.

2.7. Adult emergence inhibition bioassay

The adult emergence inhibitory activity of extract was studied in the laboratory at 125, 250, 500 and 1000 ppm in the laboratory by following the method of Elimam et al. (2009). During this bioassay, stock solution

of extract was prepared in tap water by the procedure as practiced for stock solution preparation during larvicidal and pupicidal bioassay. This solution was also containing 0.05% of Tween-80 and 1.6% of acetone. From this stock solution, 300 mL extract solutions of 125 ppm, 250 ppm, 500 ppm and 1000 ppm were prepared in polyethylene containers, 600 mL each. Control solution consisting of tap water with acetone (0.8%) and Tween-80 (0.025%) was also prepared in 600 mL polyethylene containers and each control container was also placed along each concentration. Then, from the laboratory colony, one hundred 3rd instar *Cx. quinquefasciatus* larvae were transferred to each container. This experiment was run in four replicates. Yeast was provided as larval food to each container at two days interval. Each jar was capped with gauze for preventing emerged adult mosquito escape. The jars were checked time to time for noting the pupal and adult appearance. The emerged adults were caught with the help of mouth aspirator and then transferred into reagent bottles, labeled accordingly. Cotton swabs soaked with diethyl ether were applied to the bottles for anaesthetizing the mosquito adults. The timing of this experiment was 14-25 August 2016. Temperature in the laboratory was 30 ± 4 °C. When no live larva or pupa was left in the control container then the observations were stopped. The number of emerged adults was noted for each container. The effect was expressed as percentage of emergence inhibition (% EI). The % EI was calculated by applying the method of Elimam (2007) (Equation 4).

$$\% \text{ EI} = 100 - \left[\frac{\text{Tx100}}{\text{C}}\right] \quad (4)$$

In the above formula, T stands for the percentage of emerged adults in the container having extract solution and C stands for the percentage of emerged adults in the container labeled as control. The median emergence inhibition (EI_{50}) value of the extract was determined.

2.8. Analysis of data

Abbott's formula (Abbott, 1987) was applied for correcting mortality percentages in extract solutions if mortality was observed in control (WHO, 2005). Regression analyses were carried out by subjecting the data to linear regression test. Linear regression equation Y= A + BX was calculated, where 'Y' represents dependent variable on Y axis, 'A' the Y-intercept, 'B' the slope and 'X' represents independent variable on X axis. To determine the values of LC_{50}, KDT_{50} and EI_{50}, the data was subjected to Probit test of Finney (1971). The LC_{50} values were compared by 95% confidence limits overlap method of Wheeler et al. (2006). The means of percent mortality were compared by Tukey's test in one-way Anova. Computer software SPSS 16 was used for all these analyses.

3. Results

28 g (14.4% yield) extract was obtained from 400 g powder of *C. botrys* whole-plants. The n-hexane extract was fractionated into several fractions through column chromatography. Fourteen fractions were collected and then subjected to thin layer chromatography (TLC). During TLC, Rf values were in the range of 0.2-0.8. On the basis of similar Rf values, all the 14 fractions were recombined into six fractions (F1-F6) (Table 1).

3.1. Larvicidal and pupicidal activities

The 24 h larvicidal and pupicidal activities of different concentrations (31.2 to 1000 ppm) of *C. botrys* n-hexane extract against the 2nd and 4th instar larvae and pupae of *Cx. quinquefasciatus* mosquito are shown in Table 2.

Table 1. Detail of column chromatographic fractionation of *C. botrys* whole-plant n-hexane extract.

Mobile phase	Solvents ratio	Rf value	TLC based fractions	Yield (gram)
n-Hexane	100	0.2	F1	1.3 (6.5% w/w)
n-Hexane: chloroform	90:10	0.2		
n-Hexane: chloroform	80:20	0.4	F2	1.7 (8.5% w/w)
n-Hexane: chloroform	70:30	0.4		
n-Hexane: chloroform	60:40	0.5	F3	2.7 (13.5% w/w)
n-Hexane: chloroform	50:50	0.5		
n-Hexane: chloroform	60:40	0.6	F4	3.5 (17.4% w/w)
n-Hexane: chloroform	30:70	0.6		
Chloroform	100	0.7	F5	3.7 (18.5% w/w)
Chloroform: methanol	80:20	0.7		
Chloroform: methanol	70:30	0.7		
Chloroform: methanol	60:40	0.8	F6	4.4 (22% w/w)
Chloroform: methanol	50:50	0.8		
Chloroform: methanol	40:60	0.8		

The highest concentration (1000 ppm) of *C. botrys extract* caused 72.4 ± 6.4%, 55.1 ± 2.9 and 38.3 ± 2.1% mortality of 2nd and 4th instar larvae and pupae, respectively. Lowest concentration (31.2 ppm) of *C. botrys* extract caused 7.4 ± 1.4% and 3.8 ± 1.3% mortality of 2nd and 4th instar larvae, respectively. This concentration (31.2 ppm) of extract caused no mortality of pupae during 24 h of exposure. Table 3 shows regression analyses of mean mortality of mosquito larvae and pupae after 24 h of exposure to extract. There was a strong positive correlation between extract concentration and larval and pupal mortality (R square value 0.80 to 0.84). Table 3 also shows the probit analyses of mean mortality of mosquito larvae and pupae after 24 h of exposure to extract. During these analyses, the 24 h LC_{50} values of *C. botrys* n-hexane extract against 2nd and 4th instar larvae were 324.6 ppm and 495.6 ppm, respectively. The LC_{50} value of extract against 4th instar larvae was significantly higher than the LC_{50} value of extract against 2nd instar larvae. The 24-hour LC_{50} value of extract against pupae was 950.8 ppm which was significantly ($P < 0.05$) higher than the LC_{50} values of this extract against *Cx. quinquefasciatus* larvae.

3.2. Adulticidal activity

Table 4 shows the results of probit analysis of adulticidal activity of *C. botrys* whole-plant n-hexane extract during CDC bottle and filter paper impregnation bioassays. During CDC bottle bioassay, the KTD_{50} values at the lowest concentration (0.15%) and highest concentration (1.25%) were 413.2 and 123.4 minutes, respectively. The 24-hour mortality during exposure in CDC bottles at 0.15% and 1.25% concentrations were 16.7 ± 3.2 and 52.4 ± 2.2%, respectively. During filter paper impregnation bioassay, the KDT_{50} values of extract at the lowest concentration (0.017 mg/cm²) and highest concentration (0.138 mg/cm²) were 291.1 and 48.6 minutes, respectively. The 24-hour mortality during recovery period at extract concentration 0.017 mg/cm² and 0.138 mg/cm² were 25.3 ± 3.8% and 81.2 ± 1.2%, respectively.

3.2.1. Adulticidal activity of column chromatographic fractions

The mortality of female *Cx. quinquefasciatus* mosquito adults during 24-hour exposure to six column chromatographic fractions (F1-F6) of *C. botrys* whole-plants

Table 2. Mean mortality of *Cx. quinquefasciatus* larvae and pupae after 24 hours of exposure to *C. botrys* extract.

Stages	Concentration (ppm)/ % Mortality (Mean ± SE)					
	1000	500	250	125	62.5	31.25
2nd	72.4 ± 6.4	58.5 ± 2.9	33.7 ± 3.9	22.3 ± 2.7	8.8 ± 2.8	7.1 ± 1.8
4th	55.1 ± 2.9	52.4 ± 1.8	28.5 ± 5.8	17.4 ± 3.6	8.3 ± 2.7	2.9 ± 1.3
Pupae	38.3 ± 2.1	18.3 ± 2.5	11.7 ± 2.2	5.2 ± 1.8	1.3 ± 1.3	0

Table 3. Regression analyses of *Cx. quinquefasciatus* larvae and pupae mean mortality after 24 hours of exposure to *C. botrys* extract.

Stages	N	R square	Slope	Intercept	LC50	L-U	Chi-square	P value
2nd	24	0.84	0.069	12.6	342.6	321.3-400.1	18.4	0.000
4th	24	0.80	0.042	6.45	495.6	461.9-547.3	26.7	0.000
Pupae	24	0.84	0.037	−1.206	950.8	899.4-1047.6	31.8	0.000

L-U. = Lower and upper limits of 95% confidence intervals.

Table 4. Probit analysis of adulticidal activity of *C. botrys* whole-plant n-hexane extract.

Concentration (%)	KDT_{50}	95% confidence limits (L-U)	Chi-Square	% Mortality (Mean ± SE)
CDC bottle				
1.25%	123.4	108.6-148.6	18.2	52.4 ± 2.2
0.625%	171.5	154.9- 215.3	19.7	35.3 ± 3.7
0.31%	209.3	196.3-233.4	12.8	31.8 ± 3.7
0.15%	413.2	374.4-613.9	32.5	16.7 ± 3.2
Paper impregnation				
0.138 mg/cm²	48.6	34.7-63.3	22.1	81.2 ± 1.2
0.069 mg/cm²	85.7	61.6- 121.5	16.3	68.5 ± 6.2
0.034 mg/cm²	129.2	95.7-168.9	27.4	58.9 ± 5.1
0.017 mg/cm²	291.1	256.2-348	37.5	25.3 ± 2.3

L-U. = Lower and upper limits of 95% confidence intervals.

Table 5. Results of 24-hour adulticidal activity of column chromatographic fractions of *C. botrys* whole-plant n-hexane extract against *Cx. quinquefasciatus*.

Concentration (%)	Fractions / Mortality (%)					
	F1	F2	F3	F4	F5	F6
0.2	50 ± 9.1	65.0 ± 8.7	100 ± 0	67.5 ± 9.4	52.5 ± 8.5	30.0 ± 9.1
0.1	36.3 ± 5.5	40.0 ± 10.6	80.0 ± 4.1	47.5 ± 9.5	30.0 ± 9.1	20.0 ± 5.8
0.05	11.3 ± 3.1	27.5 ± 6.6	50.0 ± 7.4	31.3 ± 6.6	16.3 ± 5.5	6.3 ± 2.3
0.025	1.3 ± 1.3	5.0 ± 2.9	30.0 ± 5.4	17.5 ± 4.8	6.3 ± 2.4	1.3 ± 1.3
Control	0	0	0	0	0	0

Table 6. Comparison of adulticidal activity of six column chromatographic fractions at highest (0.2%) concentration.

Fractions	Mortality (%)	Statistics
F1	50 ± 9.1[b]	
F2	65.0 ± 8.7[c]	DF within groups= 18
F3	100[d]	
F4	67.5 ± 9.4[c]	F= 69.9
F5	52.5 ± 8.5[b]	P= 0.00
F6	30.0 ± 9.1[a]	

Mean values with different letters represent significant difference (at P<0.05 significance level in Tukey's test), alphabetical order is according to increasing mean values.

n-hexane extract is shown in Tables 5 and 6. The F3 fraction (obtained through mobile phase consisting of n-hexane and chloroform in 60:40 or 50:50% ratio, Rf=0.5) showed significantly higher (P < 0.05) mortality against female mosquito adults when compared to other fractions. Its lowest (0.02%) and highest concentration (0.2%) concentrations caused 30.0 ± 5.4% and 100% mortality, respectively. The rest of fractions caused lower mortality of female mosquito adults. Lowest mortality was observed for fraction F6 (obtained through mobile phase consisting of chloroform and methanol in 60:40 to 40:60% ratio, Rf= 0.8). Its 0.025% and 0.2% concentration caused 1.3 ± 1.3% and 30.0 ± 9.1% mortality, respectively.

3.3. Oviposition deterrence

The percentage of effective repellency (% ER) of plant extract against the adult female gravid mosquito is shown in Table 7. Highest effective repellency (% ER=71.3 ± 4.4) against the adult female gravid mosquito was noted for the container that was containing *C. botrys* extract solution of highest concentration (1000 ppm). Lowest percentage of effective repellency (% ER= 37.1 ± 8.7) was noted for the container that was containing *C. botrys* extract solution of lowest concentration (31.25 ppm). The number of eggs in control and extract solution containers is also shown in Table 7. The range of eggs in control container, lowest extract solution container and highest extract solution container was 715-1399, 411-800 and 209-408, respectively. The oviposition activity indices (OAI) of extract solutions are also shown in Table 7. The solution of highest concentration of extract (1000 ppm) showed lowest mean OAI value (– 0.6 ± 0.1). The solution of lowest concentration of extract (31.25 ppm) showed highest mean OAI value (0.1 ± 0).

3.4. Adult emergence inhibition

The adult emergence inhibition activity of n-hexane extract of *C. botrys* whole plant against *Cx. quinquefasciatus* is shown in Table 8. Maximum percentage (≥ 90%) of adults emerged from the control containers. Adult emergence was restricted in the extract solution containers. There occurred lowest emergence (17.5 ± 11.4%) of adults from the containers which were containing extract solution of highest concentration (1000 ppm). There occurred highest emergence (76.8 ± 10.2%) of adults from the containers which were containing extract solution of lowest concentration (125 ppm). The percentage of emergence inhibition (% EI) of each concentration of extract solution was calculated. The % EI due to the lowest concentration and highest concentration of extract solution was 18 ± 11.7and 82.7 ± 14.4, respectively. There was a positive correlation between the concentration of extract solution and % EI (R square= 0.94). The EI_{50} value was 312.3ppm.

4. Discussion

The control of immature (aquatic) stages of mosquitoes through source reduction, application of insecticides or through combination of both approaches is a preferred strategy for controlling mosquito population (Mulla et al., 2001). Synthetic insecticides are not environment friendly; therefore researchers are now looking for plant-based insecticides to control mosquitoes (Shaalan et al., 2005). The insecticidal potential of *C. botrys* whole- plant n-hexane extract was investigated against *Cx. quinquefasciatus*. The *C. botrys* non-polar extract showed remarkable larvicidal and pupicidal activity against *Cx. quinquefasciatus* (Table 2). The larvicidal and pupicidal activity of *C. botrys* whole- plant n-hexane extract was positively correlated with increase in extract concentration. Such correlations have also been reported by other researchers (Adhikari et al., 2012; Rawani et al., 2013). In this research, the extract resulted in higher mortality of 2nd instar larvae (LC_{50}=324.6 ppm) as compared to the mortality of 4th instar

Table 7. Oviposition deterrent activity of *C. botrys* whole-plant against *Cx. quinquefasciatus*.

Concentration (ppm)	No. of eggs (range)	% ER	OAI
1000 (cage 1)	209-408	71.3 ± 4.4	0.6 ± 0.1
250 (cage 2)	400-597	56.8 ± 0.9	0.4 ± 0.02
31.2 (cage 3)	411-800	37.1 ± 8.7	0.3 ± 0.1
Control (cage 4)	715-1399	----------	----------

Table 8. Adult emergence inhibition activity of *C. botrys* whole-plant n-hexane extract against *Cx. quinquefasciatus*.

Concentration (ppm)	% E in treatment	% E in control	% EI	R Square	EI_{50} (ppm)	Chi-square	DF
1000	17.5 ± 11.4	94.8 ± 4.1	82.7 ± 14.4	0.94	312.3	0.5	2
500	38.8 ± 17.5	95 ± 4.4	58.5 ± 20.4				
250	59 ± 20.4	95.3 ± 4.1	38.4 ± 20.5				
125	76.8 ± 10.2	93.8 ± 4.3	18 ± 11.7				

% E = percentage of adults emerged, DF=Degree of Freedom.

larvae (LC_{50}=495.6 ppm). According to de Andrande and Modolo (1991), the higher susceptibility of early instar larvae to the extract solution is due to higher filtration rate in early instars. Chowdhury et al. (2009) and Kovendan et al. (2012) also reported that early instars larvae are more susceptible to insecticidal agents than late instar larvae when exposed. During this study, it was also observed that mosquito pupae are less susceptible to *C. botrys* n-hexane extract than larvae. The extract LC_{50} value against pupae (LC_{50}=950.8 ppm) was higher than its LC_{50} values against larvae (LC_{50} against 4th instar larvae=495.6 ppm) (Table 3). Similar trend has also been reported by Panneerselvam et al. (2012). They reported the *Artemisia nilagiric* LC_{50} values of 311.4, 442.51 and 477.23 ppm against *Anopheles stephensi* 2nd and 4th instar larvae and pupae, respectively. Pupae possess much thick cuticle therefore they are less sensitive to the insecticidal agents (Beloti et al., 2015).

During the present study, the *C. botrys* whole- plant n-hexane extract also showed adulticidal activity against *Cx. quinquefasciatus* (Table 4). Panneerselvam et al. (2012) reported the insecticidal potential of *Artemisia nilagiric* methanol extract against the adults of *Anopheles stephensi* and *Aedes aegypti*. Soonwera and Phasomkusolsil (2017)) reported the adulticidal activities of *Zanthoxylum limonella* fruit essential oil against the adults of *Cx. quinquefasciatus* and *Ae. aegypti*. The insecticidal activities of plant extracts are due to the presence of secondary metabolites. For example, Karabörklü et al. (2011) identified several secondary metabolites in *C. botrys* essential oil in which Veridiflorol, Juniper camphor, Elemol, Caryophyllene oxide, 2-(4a.8-Dimethyl-1, 2, 3, 4, 4a, 5, 6, 7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol and β-Eudesmol were the major metabolites. Harraz et al. (2015) identified twelve metabolites in *Chenopodium ambrosioides* essential oil in which α-terpinene and o-cymene were the major metabolites. Monzote et al. (2014) identified a peroxide known as ascaridole as the major constituent of *C. ambrosioides* essential oil. Doumbia et al. (2014) identified several secondary metabolites such as limonene, elemol, geraniol, citronellol and citronellal in *C. nordus* essential oil.

The *C. botrys* whole-plant n-hexane extract was further fractionated through column chromatography into several fractions for getting closure to the active principles. Non-polar and polar solvents in different ratios were passed through the glass column as the mobile phases. Fourteen fractions were collected and then subjected to TLC. On the basis of similar Rf values, all the fractions were recombined into six fractions (F1-F6). The Rf value is a number in the range of 0-1. It is estimated by dividing the distance a compound traveled from the starting point by the distance a solvent traveled from the same starting position. Rf value characterizes the behavior of compounds in thin layer chromatography (TLC) (Balammal and Kumar, 2014).

Fractions obtained were evaluated for adulticidal activity against *Cx. quinquefasciatus* female mosquito adults by applying the CDC bottle bioassay method of Ajaegbu et al. (2016) (Tables 5 and 6). The fraction F3 (obtained through mobile phase consisting of 60:40 to 50:50% n-hexane and chloroform, Rf= 0.5) showed strongest adulticidal activity. Its 0.02% concentration caused 30.0 ± 5.4% mortality and its 0.2% concentration caused 100% mortality of female adults of *Cx. quinquefasciatus*. The strongest adulticidal activity of fraction F3 shows that it contains strong insecticidal compounds. These findings can provide a basis for the isolation of insect repellent and insecticidal compounds of plant origin. Kramer et al. (2008) also performed such insecticidal activity-oriented fractionation of *Spondias mombin* leaf methanol extract and its fractions against *Aedes aegypti*. The most effective fraction (dichloromethane fraction) was further fractionated and the active principles were identified. Sharma et al. (2014) also performed insecticidal activity-oriented fractionation of *Artemisia annua* leaf chloroform extract against *Anopheles stephensi*.

The n-hexane extract of *C. botrys* whole-plant is also oviposition deterrent against mosquitoes. The gravid female adults of *Cx. quinquefasciatus* mosquito were mostly avoiding

the containers for egg laying which were containing *C. botrys* whole-plant n-hexane extract solution (Table 7). Highest concentration of extract solution showed maximum oviposition deterrence. The deterrence of oviposition was presented in percent effective repellence (% ER) and oviposition activity index (OAI). Highest % ER was 71.3 ± 4.4, which was noted for the container containing 1000 ppm of *C. botrys* whole-plant n-hexane extract solution. Negative OAI values were noted for the containers containing extract solution. According to Govindarajan et al. (2011), negative OAIs values indicate oviposition deterrence. Few studies have been conducted on the oviposition deterrent activity of medicinal plants against mosquitoes (Elimam et al., 2009; Kamaraj et al., 2009; Prathibha et al., 2014; Reegan et al., 2015). The alterations induced in the behavior and physiology of the adult female gravid mosquitoes by the plant extracts may be the main reason of oviposition deterrence (Raghavendra et al., 2014). Some active principles contained in plant extracts act as chemosterilent or growth regulators while some create olfactory stimuli that cause repellence (Prathibha et al., 2014). Most of the insect repellent plants are oviposition deterrent (Mehra and Hiradhar, 2002; Rajkumar and Jebanesan, 2009).

The adult emergence inhibition activity of *C. botrys* whole-plant n-hexane extract was also studied during the present research (Table 8). This was expressed in percentage of emergence inhibition (% EI). The extract solutions in the containers restricted the emergence of adults. The EI_{50} value of *C. botrys* whole-plant n-hexane extract was 312.3 ppm. Medicinal plants contain active principles which may be responsible for inhibition of adult emergence (Arivoli and Tennyson, 2011). They may inhibit the development of mosquito immature stages (Arivoli and Tennyson, 2011). According to Shaalan et al. (2005), plant extracts have the potential to inhibit moulting, delay development of larval stages, increase pupal duration, cause melanization processes, mortality and structural abnormalities during moulting. Few studies have been conducted on the adult emergence inhibition activity of medicinal plants against mosquitoes (Wiesman and Chapagain, 2006; Howard et al., 2009; Arivoli and Tennyson, 2011; Elango, Rahuman et al., 2012).

5. Conclusion

The n-hexane extract of *C. botrys* whole- plant possesses strong larvicidal, adulticidal, oviposition deterrent and adult emergence inhibitory activities against *Cx. quinquefasciatus*. Its fractions obtained after elution of non-polar and polar solvents in different ratio through silica gel column show strong adulticidal activity against female *Cx. quinquefasciatus* even at very low concentrations. Hence, these fractions could be effective insecticides against *Cx. quinquefasciatus,* the most common and abundant nuisance mosquito species.

Acknowledgements

The authors are thankful to Mr. Mirza Gul for his help in plant collection from foothill areas, and Mr. Afzal Hussain for his help in larval collection from cemented drains near the campus of University of Malakand.

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