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Insecticidal activities of *Cymbopogon nordus* (Linn.) wholeplant ethyl acetate extract against lymphatic filariasis vector with study on non-target organisms

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Abstract

This study aimed to evaluate the insecticidal activities of *Cymbopogon nardus*-whole plant ethyl acetate extract (CNEAE) against the lymphatic filariasis vector, *Culex quinquefasciatus*. The extract caused insignificantly higher (P>0.05) mortality of early instar larvae as compared to late instar larvae. The 24-hour LC_{50} values of CNEAE against *Cx. quinquefasciatus* 2nd, 3rd and 4th instar larvae were 439.1ppm, 451.8ppm and 665.4ppm, respectively. The 24-hour LC_{50} value of CNEAE against pupae (LC50= 2740.4ppm) was several times higher than the LC_{50} values of this extract against larvae. During adulticidal activity, the KDT_{50} value at the highest concentration (1.25%) of CNEAE during CDC bottle bioassay was 286.4 minutes. The KDT_{50} value at the highest concentration (0.138mg/cm^2) of CNEAE during filter paper impregnation bioassay was 218.2 minutes. During the study on non-target insect, *Libellula fulva,* CNEAE caused no mortality up to concentration of 500ppm, however at highest concentration (1000ppm), CNEAE caused 4.6±1.6 mortality in *L. fulva* nymphs. During this study, grass carp fish, *Ctenopharingodon idella* was exposed to the LC_{50} of CNEAE, estimated against *Cx. quinquefasciatus* 4th instar larvae. The CNEAE caused no behavioral changes or mortality in *C. idella*. During this study, high dose (2000mg/kg. b.w/oral) of CNEAE was orally administered to male rabbits. The CNEAE caused no increase in the serum levels of ALT, AS, ALP and creatinine. Similarly, the CNEAE caused no abnormal change in the blood cells counts and haemoglobin concentration.

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Introduction

Human lymphatic filariasis is a mosquito-borne disabling and disfiguring disease characterized by impaired lymphatic system, swelling and pain in the groin area and legs. This disease is caused by infection of human with filarial nematode worm. This disease create social stigma in the patients which is associated with mental health loss, employment loss and ultimately poverty (Simonsen, 2009). Worldwide, lymphatic filariasis affects about 70 million people (WHO, 2017). Lymphoedema induced by filarial worm is the main cause of disability across the world and according to a report two million people across the globe have become disabled in lymphatic filariasis (Simonsen, 2009). Cases of tropical pulmonary eosinophilia have been reported in in Pakistan (Beg *et al.*, 2001). Three species of filarial nematode viz; *Brugia malayi, Brugia timori* and *Wuchereria bancrofti* are responsible for this dreadful infection (Simonsen 2009). Most of the human lymphatic filariasis is due to infection with *W. bancrofti* (Simonsen and Mwakitalu, 2013). The wide spread vector of lymphatic filariasis is *Cx. quinquefasciatus* (Ramaiah *et al.*, 2003), which is a culicine mosquito and abundant mosquito species 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). *Cx. quinquefasciatus* is also a serious cause of nuisance due to its irritating biting. *Cx. quinquefasciatus* is also known for transmitting malaria in birds (Glad and Crampton, 2015).

The most important strategy for the control of mosquito-borne diseases is to control mosquitoes. Generally, disease transmitting mosquitoes are controlled by the application of chemical insecticides in addition to habitat reduction. Usually, the chemical insecticides belong to four classes of pesticides i.e., carbamate (e.g. carbofuran, methomyl etc.), organochlorine (e.g. dieldrin, DDT etc.), organophosphates (e.g. dichlorvos, chlorpyrifos etc.), pyrethroids (lambda cyhalothrin, deltamethrin etc.) and neonicotinoids (acetamiprid, imidacloprid etc.) (Gullan and Cranston, 2005). Constant application of these synthetic insecticides has contaminated the environment, destroyed the non-target organisms, harmed human health, and resulted in insecticide resistance development in insect pests (Lee *et al.*, 2001; Antwi *et al.*, 2015). Application of alternative approaches for the mosquito-borne diseases control is gaining attention because such approaches are environment friendly (Ghosh *et al.*, 2012; Benelli *et al.*, 2016).

A popular ecofriendly approach for the control of insect pest is the biological control. Several living organisms such microbes (Rozendaal, 1997; Phillips, 2001; Das *et al.*, 2016), naturally occurring predators (Chatterjee *et al.*, 2007; Walton, 2007) and plants (Tonk *et al.*, 2006; Ajaegbu *et al.*, 2016) have been described for their mosquitocidal potential. Plant based insecticides are also called botanical insecticides. Different solvent extracts of medicinal plants have been screened for mosquitocidal activities (Elango *et al.*, 2012; Prathibha *et al.*, 2014; Reegan *et al.*, 2015; Bekele and Petros, 2017). *Cymbopogon nardus* (Linn.) is a medicinal plant of the family *Poaceae* and is known as Sargarai in Malakand division, Khyber Pakhtunkhwa, Pakistan. This plant is known for repelling insects (Silva *et al.*, 2011). Recently, Ilahi and Yousafzai (2017) reported the insecticidal activities of its n-hexane extract against *Cx. quinquefasciatus* mosquito immature stages and adult stage. The present study aimed to investigate the insecticidal potential of ethyl acetate extract of *C. nardus* whole-plant against *Cx. quinquefasciatus* mosquito in order to explore an alternative botanical product, which can be effectively applied for the control *Cx. quinquefasciatus* position.

Synthetic insecticides not only eradicate insect pests but also kill non-pest organisms (Morrissey *et al.*, 2015). On the contrary, it is claimed that plant based insecticides are safe non-pest organisms including insects (Carvalho *et al.*, 2003; Chowdhury *et al.*, 2009; Adhikari *et al.*, 2012; Rawani *et al.*, 2014). It is commonly known that ingestion of synthetic chemical insecticides even in small amount causes the damage of organs in the body in mammals (Soni *et al.*, 2011). Therefore, this study also aimed to study the effect of *C. nardus* whole-plant ethyl acetate extract on non-target insect i.e., dragonfly nymph (*Libellula fulva*), grass carp fish (*Ctenopharingodon idella*) and rabbit (*Oryctolagus cuniculus*).

Materials and methods

Collection of plant

The plant *C. nordus* was collected during September 2016 in the foothills of Chakdara, Dir lower, Khyber Pakhtunkhwa, Pakistan. The plant was identified by expert in taxonomy of plants.

Preparation of extract

Cymbopogon nordus whole-plants were rinsed in tap water (non-chlorinated) and then placed in shade for seven days in well ventilated room. The dried plant materials were crushed into powder form by using electric chopper and then its 200 gram powder was soaked in 1000ml ethyl acetate (EA) in a 3 liter plastic jar for four days. The soaked material was filtered by using Whatman filter paper no.42. Vacuum rotary evaporator was used for evaporating the solvent and thick extract solution was then poured from rotary evaporator bulb into 250ml glass beaker which was kept open in the laboratory for 24 hours for evaporating the remaining solvent. Brownish extract in paste form was obtained. About 15 gram (7.5% yield) of *C. nordus* whole-plants EA extract was obtained. The yield of extract was calculated by applying the following equation of Anokwuru *et al.* (2011):

$$\% \text{ Yield} = \frac{W2 - W1}{W0} X\, 100$$

Where W2 is the weight of the extract and the container, W1 represents the weight of the container only and W0 stands for the weight of the dried plant powder.

Establishment mosquito colony

Culex quinquefasciatus immature stages were collected from the stagnant water bodies near University of Malakand campus during May 2016. Larvae were reared in the laboratory in 500ml plastic containers inside mosquito cages in the laboratory for establishing mosquito colony. Day time temperature inside the laboratory was 27°C to 31°C. Dry yeast powder and Dog biscuit in 2:3 was supplied as larval food. Pupae and then adults emerged from the larvae. Adults initially received 10% sucrose solution in cotton pad. After 3 days of emergence, the mosquito adults were blood fed for eggs development during evening time by restraining mice inside mosquito cage. The gravid female mosquito adults laid eggs in plastic jars containing inside the mosquito cages. First instar larvae hatched out from the eggs which after few days developed into pupae and adults. Adults and immature stages of *Cx. quinquefasciatus* were available for different mosquitocidal experiments. The species of mosquito adults and larvae was confirmed by using literature (Harbach, 1988).

Larvicidal and pupicidal activities

For conduction of larvicidal bioassay, guidance was taken from WHO (2005) standard procedures. During this bioassay, 4000mg of *C. nardus* whole-plant EA extract (CNEAE) was emulsified in 32ml acetone, 1ml tween 80 and some non-chlorinated tap water in 250ml flask of glass. This emulsified solution was then poured into 2500ml plastic bottles to which further water was added till the volume reached to 2000ml. Thus 2000ml CNEAE solution of 2000ppm extract solution with acetone (1.6%) and tween 80 (0.05%) was prepared. Then, 100ml solutions of 1000, 500, 250, 125, 62 and 30ppm concentrations were prepared in 200ml 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 containing only non-chlorinated tap water with acetone (1.6%) and tween 80 (0.05%) was also prepared. Twenty 2nd instar larvae of *Cx. quinquefasciatus* from mosquito colony were transferred to each concentration of extract solutions arranged for larvicidal activity against 2nd instar larvae. Twenty 2nd instar larvae were transferred to the control solution container. Similarly, 20 *Cx. quinquefasciatus* larvae each of 3rd and 4th instar were transferred to each concentration of extract solution arranged for larvicidal activity against 3rd and 4th instar larvae, respectively. Twenty 3rd and 4th instar larvae were transferred to control containers. 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. The percentage mortalities of larvae and pupae were calculated after exposure period of 24 hours. Lack of response to prodding was the criteria for considering a larva or pupa dead.

Adulticidal activity

CDC (Centers for Disease Control and Prevention) bottle and filter paper impregnation bioassays were conducted for adulticidal activity. The detail of each bioassay is as under:

CDC bottle bioassay

During this bioassay, CDC (2010) protocol was followed. Ten milliliter *C. nardus* whole-plant EA extract solution of 1.25% (12.5mg/ml) was prepared in 25ml glass flask. This solution was then sequentially diluted by factor of two into dilutions of 0.625% (6.25mg/ml) and 0.31% (3.1mg/ml) concentrations. Three 250ml transparent CDC glass bottles were labelled for three concentrations of extract solution (1.25%, 0.625% and 0.31%). One milliliter 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 one milliliter 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 hours. After 24 hours, the solvent evaporated completely. Then, 20 glucose-fed and blood-starved fem mosquito adults were introduced into each CDC bottle, including the control bottle with the help of mouth aspirator. The opening of all bottles were closed with their lids after introduction mosquito adults. The percentage of knock-down of mosquito adults was noted after every 15 minutes for 90 minutes. A mosquito was considered as dead or knocked down if it was unable to move or stand within 60 minutes of exposure (WHO, 2016).

Paper impregnation bioassay

During this bioassay, WHO protocol (WHO, 1981b) was followed. Ten milliliter *C. nardus* whole-plant EA extract solution of 1.25% (12.5mg/ml) was prepared in 25ml glass flask. This solution was then sequentially diluted by factor of two into dilutions of 0.625% (6.25mg/ml) and 0.31% (3.1mg/ml) concentrations. For the three different concentration solutions, papers of 12 x15cm size (area=180cm^2) 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, 2ml was applied on the respective Whatman no.1 filter papers of 12 x15cm. Thus three impregnated filter papers with 0.138, 0.069 and 0.034mg extract/cm^2, respectively, were prepared. A control 12×15cm filter was also arranged onto which only 2ml of acetone was applied. The extract treated, 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 minutes. Percentage of knock-down of mosquito adults was noted after every 15 minutes for 90 minutes.

Effect of CNEAE on non-target organisms

The effect of CNEAE on non-target insect, fish and mammal was also studied during this study.

Effect on non-target insect

During this research, the effect of CNEAE on survival of non-target insect i.e., dragonfly nymph (*Libellula fulva*) which is the predator of mosquito immature stages was studied. Dragonfly nymphs (7th instar) were collected from stagnant water near River Swat close to the University of Malakand campus during August 2016. Maximum temperature was 31-34^{o}C. Most of the nymphs were belonging to *Libellula fulva* species. Therefore experiment was conducted on this insect.

Libellula fulva nymphs were exposed to different concentrations of CNEAE in 500ml plastic containers. The concentrations were those to which mosquito larvae and pupae were exposed i.e., 31.25, 62.5, 125, 250, 500 and 1000ppm. The volume of each concentration of testing solution was 250ml. Control container containing 250ml non-chlorinated tap water was also arranged. To avoid cannibalism, seven nymphs were exposed for 24 hours individually in 7 containers (6 CNEAE concentrations and 1 control) (Hardersen and Wratten, 1996). During the 24 hours exposure period, the nymphs were not fed (ASTM standard E47, 2008). At the end of exposure period, nymphs were checked for observing mortality. Lack of response to prodding was the criterion for death.

Effect on fish

Same size (6.2± 1.3cm) healthy grass carp fish, of *Ctenopharingodon idella* species were brought to the laboratory from local fish hatchery in 5L water cooler with water of collection pond. No fish was sluggish or died when brought to the laboratory. Small fish aquaria, each of 45×40×40cm volume were used for maintaining the fish in laboratory. Air pumps were provided to the aquaria for aeration. The laboratory was well ventilated and receiving solar illumination through windows. They were exposed to the LC_{50} of CNEAE estimated against *Cx. quinquefasciatus* 4^{th} instar larvae that was 665.4ppm. Twenty liters solution of 665.4ppm CNEAE in aquarium with acetone (1.5%) and tween 80 (0.05%) was prepared. Control aquarium containing 20 L non-chlorinated tap water with acetone (1.5%) and tween 80 (0.05%) was also arranged. Ten *C. idella* fish were introduced to the aquarium. The experiment was run in triplicate. The fish were regularly checked for 24 hours to observe mortality or abnormality in behavior. After 24 hours, the fish were transferred from CNEAE solutions to aquaria containing tap water and mortality was checked after another 24 hours. During Experiment, maximum temperature was 34^{o}C).

Effect on rabbit

Eight male domestic rabbits of *Oryctolagus cuniculus* of 600-700 grams weight and 6 months age were divided into group A and group B, four in each. Group A rabbits orally received CNEAE at a dose of 2000mg per kg body weight per oral, dissolved in 3ml vegetable oil. Group B rabbits represented control group and received only 3ml vegetable oil per kg body weight per oral. Rabbits were regularly observed for 72 hours for behavioural changes and mortality. The study on animals was approved by University of Malakand Animal Ethics Committee. After 72 hours, the rabbits were anesthetized through diethyl ether inhalation. The rabbits were dissected and blood collected from the heart ventricle through 21 gauge needle syringes put into EDTA coated tubes for the study of blood cells count and hemoglobin level. Some blood from each rabbit was also put into sterile tubes with coagulant for the study of liver function markers i.e., alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) and kidney function marker i.e., creatinine. These biochemical parameters were assayed using commercially available kits.

Analysis of data

Abbott's formula (Abbott, 1925) was applied for correcting mortality percentages in extract solutions if 5–20% mortality was observed in control solution (WHO, 2005). If there occurred more than 20% mortality in control then the experiment was discarded and repeated again. Linear regression test was applied for determining correlation between increase in extract concentration and mortality. LC_{50} (Lethal concentration that cause 50% mortality in a given period of exposure) values were estimated by applying log probit test of Finney (1971). The LC_{50} values of extract against 2^{nd}, 3^{rd} and 4^{th} instar larvae were compared by 95% confidence limits overlap method of Wheeler *et al.* (2006). The liver and kidney related biochemical parameters were analyzed by independent sample–T test for comparison between the CNEAE treated and control group of rabbits. SPSS 16 software was used for statistical analysis.

Results

Larvicidal activity

The 24-hour larvicidal activity of different concentrations (1000 to 31.2ppm) of CNEAE against 2^{nd}, 3^{rd} and 4^{th} instar larvae of *Cx. quinquefasciatus* is shown in table 1. The highest concentration (1000ppm) of CNEAE caused 76.25 ± 7.5%, 77.5 ±11.9% and 65.0 ± 2.04% mortality of *Cx. quinquefasciatus* 2^{nd}, 3^{rd} and 4^{th} instar larvae, respectively. The lowest concentration (31.2ppm) of CNEAE caused 2.5 ± 2.9% and 3.8 ± 4.8% mortality of *Cx. quinquefasciatus* 2nd and 3rd instar larvae, respectively. This concentration of CNEAE caused no mortality of 4th instar larvae. There was a highly positive correlation between CNEAE concentration and larval mortality (R square value > 90). The 24-hour LC_{50} values of CNEAE against *Cx. quinquefasciatus* 2nd, 3rd and 4th instar larvae were 439.1ppm, 451.8ppm and 665.4ppm, respectively. The LC_{50} value of CNEAE against 4th instar larvae was insignificantly higher than the LC_{50} values of extract against lower instar larvae (i.e., 2nd and 3rd instars).

Table 1. The 24-hour larvicidal activity of CNEAE against *Cx. quinquefasciatus*.

Instar	Conc	% Mortality	R^2 and Y Equation	LC_{50} with 95% CL (ppm)
2nd	1000	76.25 ± 7.5	R2 = 0.95	
	500	52.5 ± 5		
	250	28.8 ± 11.1	0.0749x+6.4858	439.1 (371.7-531.8)[a]
	125	18.8 ± 4.8		
	62.5	7.5 ± 5		
	31.2	2.5 ± 2.9		
3rd	1000	77.5 ± 11.9	$R^2 = 0.96$	
	500	46.3 ± 6.3		
	250	33.8 ± 6.3	y= 0.0746x+6.1782	451.8 (380.3-598.6)[a]
	125	16.3 ± 2.5		
	62.5	6.3 ± 2.5		
	31.2	3.8 ± 4.8		
4th	1000	65.0 ± 2.04	$R^2 = 0.95$	
	500	36.3 ± 3.8		
	250	22.5 ± 2.5	0.067x+0.672	665.4 (574.8-772.8)[a]
	125	8.8 ± 2.4		
	62.5	2.5 ± 1.4		
	31.2	0		

a.- represents that LC50 values of extract against different instar larvae are not different significantly (at $P<0.05$ significance level) on the basis of 95% confidence limit overlap.

Pupicidal activity

The 24-hour pupicidal activity of different concentrations (1000 to 31.2ppm) of CNEAE against *Cx. quinquefasciatus* pupae is shown in table 2. The highest concentration of CNEAE caused 30 ± 8.2% mortality of *Cx. quinquefasciatus* pupae. The lowest concentration of CNEAE caused no mortality of *Cx. quinquefasciatus* pupae. The 24-hour LC_{50} value of CNEAE against *Cx. quinquefasciatus* pupae was 2740.4ppm which was several times higher than the LC_{50} values of this extract against *Cx. quinquefasciatus* larvae.

Table 2. The 24-hour pupicidal activity of CNEAE against *Cx. quinquefasciatus*.

Concentration	% Mortality	R^2 and Y Equation	LC_{50} with 95% CL (ppm)
1000	30 ± 8.2	$R^2 = 0.96$	
500	20 ± 8.2		
250	8.8 ± 2.5	y= 0.030x+1.953	2740.4 (1631.4–6565.2)
125	7.5 ± 5		
62.5	3.8 ± 4.7		
31.2	0		

CL.- 95% confidence limits

Adulticidal activity

Centers for Disease Control and Prevention (CDC) bottle bioassay and filter paper impregnation bioassay were used for assessing the adulticidal activity of CNEAE against *Cx. quinquefasciatus* female adults. The results of CDC bottle bioassay and filter paper impregnation bioassay are shown in table 3. In case of CDC bottle bioassay, the KDT_{50} values of 1.25%, 0.625% and 0.31% solutions of CNEAE were 286.4, 480.8 and 510.3 minutes, respectively.

During paper impregnation bioassay, the KDT_{50} values of 0.138mg/cm², 0.069mg/cm² and 0.034mg /cm²of CNEAE were 218.2, 347.3 and 461.6 minutes, respectively.

Table 3. Adulticidal activity of CNEAE against *Cx. quinquefasciatus*.

Bioassay	Concentration	KDT_{50} (L-U) (Minutes)
CDC bottle	1.25%	286.4 (162.1–1707.2)
	0.625%	480.8 (247.8- 2971.7)
	0.31%	510.3 (377.2–3306.3)
Filter paper	0.138mg/cm²	218.2 (175.4 – 300.3
	0.069mg/cm²	347.3 (215.4–908.03)
	0.034mg/cm²	461.6 (266.9–1482.5)

L-U.- lower and upper limits of 95% confidence

Effect on non-target insect

The *C. nardus* whole- plant EA extract appeared safe for dragonfly (*L. fulva*) nymphs. During exposure up to the concentration of 500ppm, no *L. fulva* nymph died. However the highest concentration (1000ppm) of CNEAE caused mortality of small percentage of nymphs (4.6±1.6%) (Table 4).

Table 4. Effect of CNEAE on non-target insect, dragonfly nymph of *L. fulva* species.

Concentration (ppm)	Mortality (Mean ± SE)
1000	4.6±1.6
500	0
250	0
125	0
62.5	0
31.25	0
Control	0

Effect on fish

Fish of *C. idella* species were exposed to 665.4ppm concentration of extract. Fish exposed to extract in aquarium were regularly checked for mortality or behavioral changes during 24 hours. No fish died and no change in behavior of fish was observed.

Effect on rabbit

The effect of oral administration of CNEAE high dose on the serum levels of liver and kidney related biochemical parameters and haematological parameters of male rabbits was studied. The serum levels of liver parameters i.e., ALT, AST, ALP and kidney related parameter i.e., creatinine of extract treated group of rabbits were statistically homogeneous (P>0.05) to those of control group of rabbits (Table 5). Similarly, the RBCs count, WBCs count and platelets count of extract treated group of rabbits were statistically homogeneous (P>0.05) to those of control group of rabbits (Table 6).

Table 5. Effect CNEAE on some biochemical parameters of normal rabbits. N=4 Values are mean and standard error of mean.

Plants	ALT (U/L)	AST (U/L)	ALP (U/L)	Creatinine mg/dl
C. nardus	36.0±4.5	34.6±4.3	103.7±11.7	0.4±0.13
Control	40.0±2.2	41.3±6.2	98.0±17.4	0.5± 0.07
T value	0.18	0.2	0.01	0.05
DF	8	8	8	8
Significance	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Table 6. Effect of CNEAE on some haematological parameters of normal rabbits. N=4 Values are mean and standard error of mean.

Plants	RBCs (X 10⁶/µl)	WBCs (X 10³/µl)	Platelets (X 10³/µl)	Hb (g/dl)
C. nardus	6.6±0.2	10.3±0.8	269.3±4.1	12.7±0.9
Control	5.9±0.3	11.5±0.4	265.4±12.3	13.1±0.5
T value	0.3	0.2	0.4	0.8
DF within groups	8	8	8	8
Significance	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Discussion

Control of mosquitoes is very essential for the control of mosquito borne diseases. Synthetic pyrethroids are expensive while organochlorine and organophosphate are not safe for the environment, therefore researchers are now looking for plant based insecticides to control mosquitoes (Shaalan *et al.*, 2005). The insecticidal potential of CNEAE was investigated against *Cx. quinquefasciatus* during the present study. The CNEAE showed efficient larvicidal and pupicidal activity against *Cx. quinquefasciatus* (Table 1 to 2). The percentage of mosquito larval and pupal mortality was strongly correlated with increase in extract concentration as reflected by high R square value (P>0.90). Such correlations have been observed in the studies of Adhikari *et al.* (2012) and Rawani *et al.* (2013). The plant *C. nardus* is known for its insect repellent or insecticidal property. For example, Doumbia *et al.* (2014) and Nyamador *et al.* (2017) reported the insecticidal role of *C. nardus* essential oils against insect pests of stored food products. Silva *et al.* (2011) reported the insect repellent activity of *C. nardus* essential oil against mosquito.

Ríos *et al.* (2017) reported the larvicidal action of essential oils of *C. nardus* against *Ae. Aegypti*. The results of the present research show that CNEAE also possesses larvicidal and insecticidal ability against *Cx. quinquefasciatus*.

During the present research, the extract caused insignificantly higher mortality of early instar larvae as compared to the mortality of late instar larvae (4th instar). The LC_{50} value of extract for 2nd instar larvae was insignificantly lower ($P<0.05$) when compared to its LC_{50} value for 4th instar larvae. The higher sensitivity of early instar larvae to larvicidal extracts during the present study may be due to higher filtration rate in early instars than in late instar larvae (de Andrande and Modolo, 1991). Chowdhury *et al.* (2009) and Kovendan *et al.* (2012) also reported higher susceptibility of early instars larvae to insecticides. During the present research, it was also observed that pupae are not as susceptible to extract solution as the larvae. The possible reason is the presence of much heavier cuticle in pupae than larvae (Christophers, 1960; Beloti). Such trend can be seen in the results of research work of other researchers (Jayanthi *et al.*, 2012; Kovendan *et al.*, 2012).

During the study of adulticidal activity, the knockdown of *Cx. quinquefasciatus* adults exposed to various concentration of CNEAE was studied by CDC bottle and filter paper impregnation bioassays. The CNEAE caused knock down of female mosquito adults during both, CDC bottle and filter paper impregnation bioassays. In each of these bioassays, low KDT50 value was observed for high CNEAE concentration, while for low extract concentration, high KDT_{50} value was observed (Table 3). The essential oil of this plant has insect repellent property (Silva *et al.*, 2011). The adulticidal activity of n-hexane extract of this plant against mosquito has been reported (Ilahi and Yousafzai, 2017). The present study showed that CNEAE also possesses adulticidal property against mosquitoes. The adulticidal activity of essential oils or extracts of other medicinal plants against mosquitoes have also been reported (Dua *et al.*, 2010; Karaborklu *et al.*, 2011; Ajaegbu *et al.*, 2016).

The insecticidal properties of plant extracts are due to the presence of secondary metabolites. For example, Harraz *et al.* (2015) identified twelve metabolites in *Chenopodium ambrosioides* essential oil in which α-terpinene and o-cymene were the major metabolites. In the essential oil of *Chenopodium botrys*, several secondary metabolites have been identified 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 (Monzote *et al.* (2014). In the essential oil of *C. nordus,* the plant studied for mosquitocidal activities during the present research, contain limonene, elemol, geraniol, citronellol and Citronellal (Karaborklu *et al.*, 2011).

The effect of CNEAE on non-target insect i.e., dragonfly nymph of *L. fulva* species, fresh water grass carp fish i.e., *C. idella* species and domestic male rabbits of *O. cuniculus* was also studies during the present research. During the study on *L. fulva* species, the *C. nordus* whole- plant EA extract appeared safe. No mortality of *L. fulva* nymphs was observed when exposed to extract solution of 500ppm concentration. However, its highest concentration (1000ppm) caused mortality of 4.6±1.6% nymphs (Table 4). During the study on *C. idella* fish, the CNEAE caused no mortality or behavioural changes during 24-hour exposure. The effect of larvicidal plant extract on non-target organisms have been reported. For example, during a study the larvicidal effect of extract from the leaves of *Solanum villosum* was studied against *Anopheles* with study on *Chironomus circumdatus* larval stage (Chowdhury *et al.*, 2009). During their study, the extract was not toxic against *Chironomus circumdatus*. In another study, *Swietenia mahagoni* extract repelled mosquito adults and killed the larvae *Cx. quinquefasciatus* but caused no mortality of *Gambusia affinis* fish, *Bufo* tadpoles and larvae of *Chironomus* (Adhikari *et al.*, 2012). During their study, the extract was not harmful against non-pest organisms.

During this study, the effect of oral ingestion (2000mg/kg. b.w/oral) of CNEAE in male rabbits on the serum levels of some liver and kidney related biomolecules i.e., AST, ALT, ALP and creatinine was studied (Table 5). There occurred no significant alteration (P>0.05) in the serum levels of these parameters from control rabbit group. The effect of oral ingestion of CNEAE on blood cells counts and haemoglobin concentration of male rabbit was also studied. There occurred no significant alteration in these haematological parameters of extract treated rabbits group from the control rabbits group (Table 6). Similar studies have been reported for other plants in other mammal models. For example, during a study, the insecticidal activity of *Lippia sidoides* essential oils against *Aedes aegypti* with effect on mice (Carvalho *et al.*, 2003). They injected pure and diluted hydrolate (30ml/ kg. b. w. into the mice intraperitoneum. During their study, the essential oil showed no toxicity in mice.

Conclusion and recommendation

The CNEAE is larvicidal, pupicidal and adulticidal against the lymphatic filariasis vector, *Cx. quinquefasciatus*. The CNEAE is not toxic for non-target insects, fresh water fish and mammals. There is a need for screening of native medicinal plants to find more effective herbal pesticides against mosquitoes and other insect pests.

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