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Molecular identification and bio-control of *Culex quinquefasciatus* from Yanbu region

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Abstract
Each year millions of peoples die to due mosquito's related diseases. Resistance of these mosquitoes towards chemical pesticides is one of serious issue. Bio-control is one of the safe ways to control these vectors. The aim of our study was to evaluate three different plant extract against the 4th instar larvae of *Culex quinquefasciatus* mosquitoes. Two ethanolic plants extract and one plant essential oil (EOs) was used for the larvicidal activity ranging from concentration of 0 to 900 ppm against the 4th instar larvae of *Culex quinquefasciatus.* To determine the LC_{50} and LC_{90} value, data was subjected to probit analysis. Mosquito larvae's used during this study was identified using COI gene sequencing. Among them *Argania spinosa* oil showed moderate larvicidal effect at low concentration but *Thevetia peruviana* extract and *Saueda monoica* extract shows high larvicidal activity at low concentration respectively. The LC_{50} and LC_{90} values for *Thevetia peruviana* extract was 105.95 and 243.36 (ppm). In case of *Saueda monoica* extract the LC_{50} and LC_{90} value was 104. 04 and 282.26 respectively. But the *Argania spinosa* oil shows activity with almost double the concentration of the other two plant extracts. It is concluded from our data that *Thevetia peruviana* and *Saueda monoica* has the potential to control *Culex quinquefasciatus* in an eco-friendly way.

Keywords: *Culex quinquefasciatus,* biocontrol, *Thevetia peruviana, Saueda monoica*

Introduction
Mosquitoes are the most common vector of human diseases and harm millions of people around the world. It is on the record of WHO that mosquitoes are one of human being enemy. More than 100 country of the world is under the threat of mosquitoes, 700 million people around the globe. Some of the common diseased in which mosquito is the vector are dengue fever, chikungunya fever, Malaria, yellow fever, filariasis, encephalitis, west Nile virus infection in all climatic zones of the world [1].

To curtail the prevalence of mosquito-borne diseases and to improve the environment and public health, it is essential to control mosquito. The most prominent way of controlling mosquitoes is through the use of chemical application mostly, organochlorine, and organophosphate compounds [2]. But the control is not much effective due to human, technical, operational, ecological and economic factors. Recently, synthetic chemicals which that were previously used for the control of mosquito have limited, Due to some factors like environmental, health, host-specific, resistance and non-degradable concerns [2, 3]. Therefore, in 1969 environmental protection agency set different rules and regulations to balance the application of chemical control of bio-agents in nature [4]. Hence, it motivated the researchers to develop new technologies with easily adaptable, safe, cheap, environment-friendly and effective against mosquito management strategies. Regarding these points, biological control of vectors become the key focus of the management of pest instead of using chemicals for the purpose.

Under the biological control program one of the most effective alternative techniques is to search for plant biodiversity and explore safe natural biocides which are simple and sustainable method of mosquito control. Conventional insecticides are commonly blended with single active ingredient unlike, natural biocides (plant origin) comprises of many chemical substances which collectively act as behavioral and physiological. Ultimately, have low risk of resistance development of host against these natural biocides. Identifying efficient, suitable and stable natural biocides is commanding tool for effective control management [5]. Insecticidal properties of plants are well known and will be new resource of synthetic

insecticides and will be a suitable alternative source against mosquito borne diseases.

1200 species of plant have effective insecticidal values while 344 plant species have been listed by Sukumar *et al.* that is effective against mosquito [6, 7]. The current scenario of knowledge on larvicidal plant species has been discussed by shallan *et al.* in 2007 along with extraction methodology, phytochemicals inhibiting growth and reproduction, ovicidal effect of plants, synergistic, residual capacity, additive and hostile joint action effects of mixtures, host range, resistance and screening research and some other effective advances in phytochemical research [8, 9]. The performance of different plant products from varieties of edible, aesthetic, woody, shrubs, succulents, grasses and marine plants by extracts developed through eleven various solvent systems and types of activities on mosquito different developmental stages as a source for further studies. The research study aimed to use different plant extracts against indigenous mosquitoes from Yanbu region.

Materials and Methods

Collection of samples

Blackhole trap was used to collect the samples from Yanbu region, Saudi Arabia during November 2018 to August 2019 (24°05′N 38°00′E). The mosquitoes were identified morphologically by using taxonomic keys.

Plant extracts preparation

The leaves of the three plants were dried at 27-37 ºC for 7- 10 days. The leaves were then powdered using a stainless-steel blender. For two plant, ethanolic extracts were obtained in a soxhlet apparatus (boiling point range 60-80 ºC) for 6 hours. Then Whatman no. 1 filter paper the extracts were filtered through Buchner funnel. For the third plant, the oil extracted educing ethanol. These three plant extracts were transferred to the refrigerator until further experimentation.

Rearing of mosquitoes

The southern house mosquitoes (*Culex quinquefasciatus*) were transferred to dengue mosquitos' station, Jeddah for rearing. During rearing the produced larvae of mosquitoes were took into plastic enamel trays containing tap water. The rearing conditions were set at (27±2 ºC) and 75-78% RH with photoperiod (14:10 light and dark) respectively. Special diet for mosquitoes was prepared from Brewer's yeast, dog biscuits and algae taken from ponds water at a ratio of 3:1:1 respectively [10].

DNA extraction

Mosquito's samples were crushed for DNA extraction in liquid nitrogen. The sample was transferred to a new tube. Firstly, 20 µl of proteinase K was transferred to the tube containing crushed mosquitoes. This was incubated along with of ATL Buffer (180 µl) at 56°C for 60 mins. QIAGEN isolation kit was used for DNA extraction following the protocols with a double final elution step. The extracted DNA were stored at -20 ºC for further experiments.

Gene amplification

Using 'Universal primers, LCO1490 and HCO2198, a smaller 648 bp COI fragment was amplified. The total PCR volume was 25 μL, which contains 15.3 μL 1× bovine serum albumin (BSA), 2.5 μL 10× Reaction Buffer, 2 μL dNTPs (2.5 μM), 1.25 μL of each primer (10 μM/L), 0.2 μL *Taq* DNA Polymerase (1.0 U), and 2.5 μL template DNA**.** The PCR conditions include 94 °C for 2 minutes, 40 cycles (94 °C for 30 seconds, 49 °C for 45 s(Annealing) and 72 °C for 45 s (Extension), then finally at 72 °C for 1 minute. The PCR product was run on 2% agarose gel to validate our experiment.

Larvicidal activity of three plant extract

Amid initial screening with the lab experiment, *Culex quinquefasciatus* mosquito's larvae were collected, identified, from the rearing cage at dengue station, King Abdulaziz University, Jeddah. From the stock solution with the use of dechlorinated tap water 1000 mg/l extract was prepared. This was done according to the World health organization with slight modifications [11, 12, 13].

Results and Discussion

Crude or partially purified plant extracts are proved to be cheap and highly effective for the control of mosquitoes instead of purified compounds or extracts [14-16]. Due to the widespread occurrence of vector resistance to synthetic insecticides, toxicity, non-biodegradability residues contaminating the environment and the undesirable effect on other organisms. Therefore, exploration of new strategies about natural products are necessary [15]. Vectors resistance development against the commercial insecticides motivated new control strategies. Recently, many traditional usage of herbs against insects have been reported all over the world. Plants inhabit insects' growth by producing secondary metabolites. Although different plant species from plant kingdom have been used against mosquito, yet few herbs have been applied in the field. Crude extracts from plants have effectively been used as insecticides from centuries all over the world [16].

Crude extracts of plants are often rich in various active compounds. These complex compounds in plants may act synergistically; they may exhibit effective bioactivity than by using individual constituents and most importantly the resistance development of host is negligible against these compound mixtures [17]. Hence, above discussion support the use of chemically unrefined herbal extracts which has complex compounds rather than pure individual constituents. Leaf extracts mode of action against mosquito is not known yet it has been noted in previous researches that proper functioning of mitochondria is interfered by phytochemicals especially at the proton transferring sites [18]. Furthermore, studies of Rey *et al.* and David *et al.* suggested that phytochemicals in mosquito larvae the mid-gut epithelium is affected and secondarily gastric caeca including the malpighian tubules are affected [19, 20].

Taxonomic status and distribution patterns of *Culex quinquefasciatus* are the first step in the surveillance and control of these mosquitoes and their transmitted diseases. Morphological characteristics have been used as basic for mosquito genus and species identification. Furthermore, these morphological techniques are still not able to distinguish mosquitoes among species complexes [20, 21]. So Molecular technique using CO1gene barcoding help to identify these mosquitoes at sub species level and the possible existence of hybridization between species [21, 22, 23].

Recently a diagnostic of specific molecular marker must demonstrate consistent differences between closely related mosquito species [24]. Ajamma *et al.* (2016) [20] investigated that, the usage of post-polymerase chain reaction (PCR)

approach is to determine the differences in nucleic acid sequences, on the basis of which differentiation within '*Anopheles gambiae* and *Culexpipiens'* complexes [20]. Molecular taxonomy mainly depends on DNA sequences, to determine the genetic structure of vector species population, for resolving phylogenetic relationships among or within groups of *Culicidae* [24, 25] and also for the identification of species [26]. Several researchers concluded that mitochondrial genes are considered good markers for mosquito species complexes identification due to lack of introns, limited chance to recombination, and haploid nature of inheritance [22].

Cox 1 gene amplification

DNA was isolated from seven randomly selected mosquito male and female samples. Furthermore the COX1 gene was amplified using polymerase chain reaction. The result for these samples are shown in figure 1.

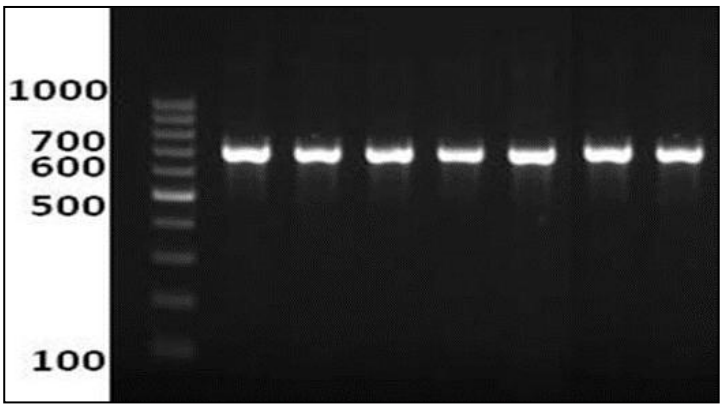

Fig 1: Agarose gel image of the amplified cox-1 gene of the selected samples (100 bp ladder DNA).

Gene sequencing and phylogenetic tree

The amplify COX1 gene was send to macrogen (Korea) for sequencing. The sequence received from macrogen was uploaded to NCBI. Blast result show that all these seven randomly selected mosquitos were identified as *Culex quinquefasciatus*. Phylogenetic tree was constructed using MEGA 10 for seven samples against other closely related mosquitoes as shown in Figure 2. The results of the samples were also analyzed with gene-bank samples, KF406862.1, MK575480.1, MH538709.1, and MH538707.1 and the results showed that the samples were very close to those found in Pakistan, India and Brazil.

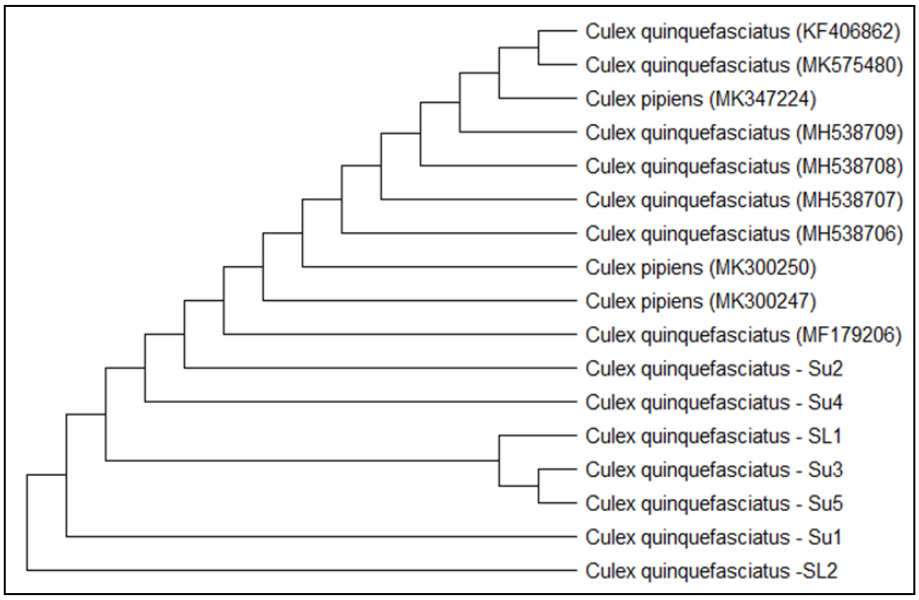

Fig 2: Phylogenetic analysis of seven mosquitoes sample against the closely related mosquitoes using Mega X.

Bioassay against mosquito larvae (*Culex quinquefasciatus*)

The results of the *Thevetia peruviana* extract and *Saueda monoica* extract showed high larvicidal activity at low concentration as shown in Table 1 and 2. The relation between different concentration and larvae death of these two plants is shown in Figure 3 and 4. In case of *Argania spinosa* oil did no Larvicidal activity was found with the same concentration used for other two plants extract. The *Argania spinosa* oil activity but when high concentration of the *Argania spinosa* oil was used against Table 3 and Figure 5. The *Thevetia peruviana* extract and *Saueda monoica* extract show high mortality rate of mosquito larvae at a concentration of 250 ppm. But in case of *Argania spinosa* oil there was no Larvicidal activity at this concentration. The *Argania spinosa* oil show highest activity at a concentration of 900 ppm as shown in Table 5. This shows *Argania spinosa* oil has very week activity compare to that of other extracts against the mosquito larvae's.

The LC_{50} value for the *Thevetia peruviana* extract was 105.95 (ppm) and LC_{90} was 243.36 (ppm) as shown in Table 2. For

Saueda monoica extract the LC_{50} value was 104. 04 (ppm) and LC_{90} was 282.26 as shown in Table 4. But in case of *Argania spinosa* oil the LC_{50} was 520.20 and LC_{90} value was 874.25 as shown in Table 6. The results show that *Thevetia peruviana* extract and *Saueda monoica* extract has the ability to kill the mosquitoes at low concentration. But in case of *Argania spinosa* extract the larvae survive at the low concentration. Similar studies are also done using plant extract showing Larvicidal activity against *Culex quinquefasciatus* [27, 28, 29].

Table 1: Level of sensitivity of *Culex quinquefasciatus* larvae against the *Thevetia peruviana* extract

Con.(ppm)	0	50	100	150	200	250
Mortality %	4	19	45	68	81	97

(Five repeaters per concentration, 20 larvae per duplicate).

Table 2: LC 50 and LC 90 values, of *Thevetia peruviana* extract against larvae of *Culex quinquefasciatus*

Slope		Chi		LC_{50}(ppm)	LC_{90}(ppm)
3.549	+/- 0.2904	7.620	tabulated 7.8	105.9574	243.3672
Lower limit				96.4498	216.2034
Upper limit				115.2704	283.4536

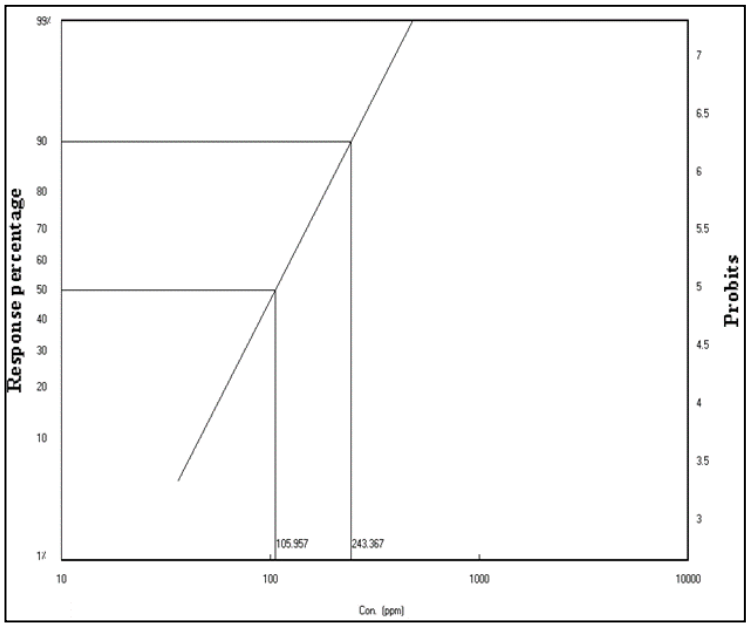

Fig 3: Relationship between concentrations of *Thevetia peruviana* extract and the percentage of death of *Culex quinquefasciatus* larvae

Table 3: Level of sensitivity of *Culex quinquefasciatus* larvae for *Saueda monoica* extract

Con.(ppm)	0	50	100	150	200	250
Mortality %	4	25	45	63	81	93

(Five repeaters per concentration, 20 larvae per duplicate).

Table 4: LC 50 and LC 90 values, of *Saueda monoica* extract against larvae of *Culex quinquefasciatus*

Slope		Chi		LC_{50}(ppm)	LC_{90}(ppm)
2.9568	+/- 0.2689	7.4877	tabulated 7.8	104.045	282.2678
Lower limit				93.164	243.0985
Upper limit				114.6576	345.3032

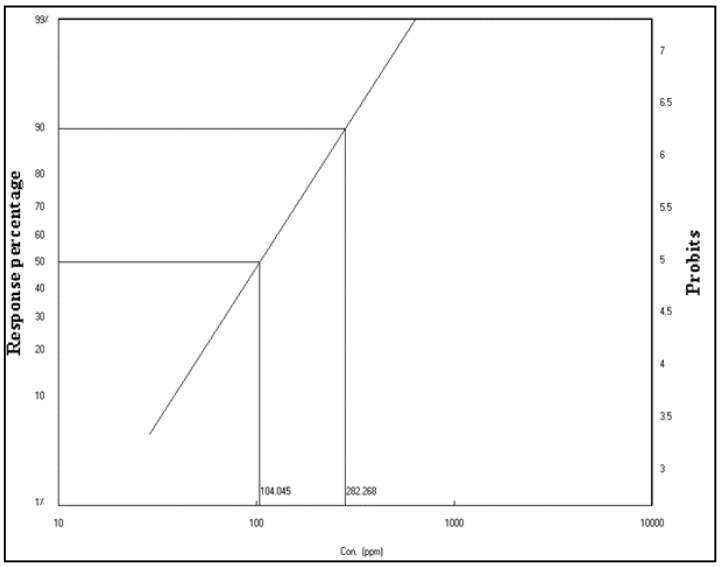

Fig 4: Relationship between concentrations of Saueda monoica extract and the percentage of death of Culex quinquefasciatus larvae

Table 5: The sensitivity level of *Culex quinquefasciatus* larvae against *Argania spinosa* oil extract

Con.(ppm)	0	300	450	600	750	900
Mortality %	2	14	34	59	81	96

(Five repeaters per concentration, 20 larvae per duplicate).

Table 6: LC $_{50}$ and LC $_{90}$ values, of *Argania spinosa* oil against larvae of *Culex quinquefasciatus*

Slope		Chi		LC_{50}(ppm)	LC_{90}(ppm)
5.6843	+/- 0.4458	6.3146	tabulated 7.8	520.2012	874.25
Lower limit				491.9609	809.4832
Upper limit				548.0996	964.7741

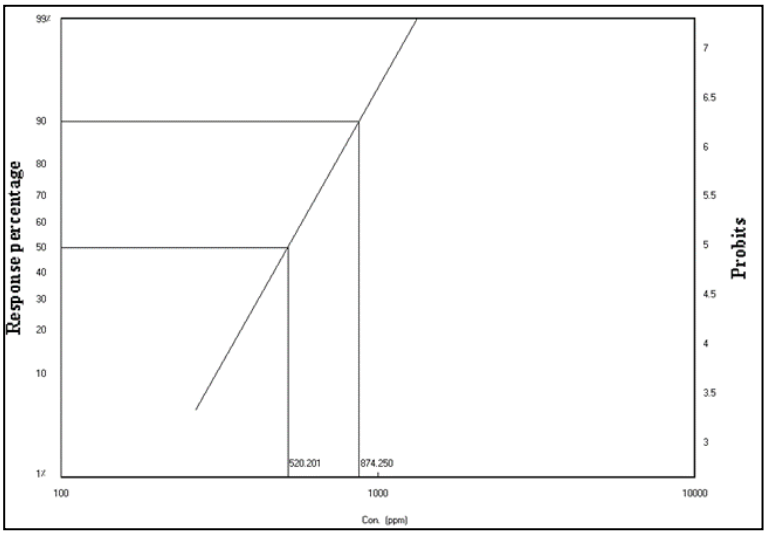

Fig 5: Relationship between Argania spinosa oil concentrations and the percentage of death of Culex quinquefasciatus larvae

Conclusion

Three of the plant extracts were used for the bio-control of mosquitoes. Among them, *Thevetia peruviana* and *Saueda monoica* extract shows high larvicidal activity against *Culex quinquefasciatus*. The IC_{50} and IC_{90} values showed that both of these plant extracts has the ability to control this mosquito in the larvae stage. The activity may be attributed to cannogenin, digitoxigenin, thevetin and phenol compounds present in these plant extract. Further studies are need to purify and identify the compound showing Larvicidal activity.

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