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RESEARCH ARTICLE

Molecular Identification and Bio-Control of Mosquitoes using Black Seeds Extract in Jeddah

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ABSTRACT

With rational water management programs, Saudi Arabia should not be an ideal place for mosquito problems due to its geographical location, weather conditions and, a small amount of exposed water for mosquito population buildup. The aimed to obtain an overview of the biodiversity of Mosquito strains and their bio-control. COI gene-based analyses were used to identify the mosquitoes. The mosquitoes were treated with different concentrations of black seed methanolic extract. About 20 species of mosquito vectors belonging to two genera, based on the morphology one *Aedes* and one *culex* was selected for further studies. *Aedes* and *Culex* were identified using COI gene sequencing. The partial COI sequence of *Aedes aegypti* and *Culex pipien*s was submitted for the accession number. Our results showed remarkable larval activity as well as inhibition of adult emerging percent in the treated groups compared to the control group. This study adds basic knowledge to the molecular evolution of mosquito vectors of medical and veterinary importance and may be useful to improve biotechnological tools employed in the *Culicidae* control program. Furthermore, this study reveals that black seeds extract can be used for the bio-control of these vectors.

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INTRODUCTION

Mosquitoes have created great concern due to the reported cases of mosquito-borne disease that sometimes lead to human fatalities. Generally, the type of mosquito population builds up to higher levels causing nuisance, bites and infecting human diseases is generally sporadic which is geared most of the time with the weather and environmental conditions. Although vigilant care has been exercised by the government take care of this sporadic infestation not to reach epidemiological level (Alhaeli *et al*., 2016; Selim *et al*., 2019).

The incidence of Dengue fever is not a day to day problem that arrives from an unaccounted for break from unidentified foci of manifestations that could have been missed by homeowners or not so vigilant eyes of public health and technical scouting team due to mismanagement of water that was left stagnant for an appreciable amount of time, therefore with the presence of inoculum potential carried by mosquito vector, these cases will be pronounced (Abbas *et al*., 2014; Alhaeli *et al*., 2016).

Depending on the geographical situation of western Saudi Arabia, coupled with the realization of weather conditions and ecological orientation, mosquito problem is not a day to day acute and critical problem if compared with Florida, equatorial Africa ,or the rich savanna of the tropics and the subtropics regions of the world, where an abundance of fresh surface water in rivers, lakes, ponds together with heavy vegetation cover and heavy torrential rains pouring all the year round in a lush thick ecosystem create a suitable utopia for mosquito population build up in skyrocketing numbers reaching astronomical levels (Bhatt *et al*., 2013; Khan *et al*., 2014).

The western Saudi Arabia is characterized by dry hot weather mostly all the year round, scarce rainfall and low vegetation covering most areas except in valleys and pockets of wadi enclaves. Therefore, it can be described as a precarious and delicate ecosystem. With regard to

Dengue fever and its vector *Aedes aegypti* and the great precautionary measures and concerns from the government and the citizens alike there are updated data released to show or give an indication about the exact extent of the infestation (Al-Azraqi *et al*., 2013: Khan *et al*., 2014: Alahmed *et al*., 2019).

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For species identification DNA barcoding is used as powerful tool. This involves sequencing of short DNA, having very little variation among species. Some studies are available for Saudi mosquito's identification using DNA barcoding (Abbas *et al*., 2013; Ehab *et al*., 2018; Hamdy *et al*., 2018). These studies elaborate DNA barcoding as molecular approach to identify species. DNA barcoding is a standard marker that contains a small segment of mitochondrial gene (COI). Barcode of life Database choose this region as a standard Marker. The barcode of life database (BOLD) is plateform to collect the barcoding sequence data throughout the world (Ratnasingham and Hebert 2007). Some studies also showed that nuclear genes are used as marker for DNA barcoding of mosquitoes (Lin and Danforth 2004). These includes internal transcribed spacer subunit 2 (ITS2), acetylcholinesterase 2 (ace-2), alpha-amylase, elongation factor-1 alpha (EF-1a) and zinc finger (Foley *et al*., 2007; Hasan *et al*., 2009; Hemmerter *et al*., 2009; Puslednik *et al*., 2012).

Plants, microbes and algae provides active compound used to control mosquitoes. This can not only reduce the chance of resistance of these mosquitoes toward pesticides but also environment friendly (Rahbar *et al*., 2012). Most of oil from plants contain bioactive compounds extracted from leaves and seeds using steam distillation. Plant families such as Myrtaceae, Asteraceae, Rutaceae Lamiaceae and Umbelliferae are rich with essential oils (vivekraj *et al*., 2015). The oil from these plant have insecticidal, bactericidal, fungicidal and anti-feeding activity. The oil from these plant have insecticidal, bactericidal, fungicidal and anti-feeding activity (Soliman and sheriff, 1995; Batish *et al*., 2008). Some of plant extract and oil have strong ovicidal activity against mosquito eggs (Govindarajan *et al*., 2011) and larvicidal activity (Benelli *et al*., 2013; Chellappandian *et al*., 2017; Vivekanandhan *et al*., 2017). In the South Asia and Middle East countries the *Nigella sativa* is used as treatment for variety of diseases. The oil of Nigella sativa has the ability to work in different types of diseases including kidney disorders, cardiovascular related diseases, Cancer, inflammation and diabetes. Earlier studies showed that Nigella sativa seed extract has the ability to kill *Culex pipens* (khater, 2018) The aim of the study was to establish a vibrant hub of excellence in the mismanagement of mosquito populations to reduce extensive nuisance, inconvenience, and biting habits. Also, use black seeds extract in order to control it in an eco-friendly way.

MATERIALS AND METHODS

Mosquitoes and seeds collection: Using insect baiting surveillance traps mosquitoes were collected from five different sites within three regions around Jeddah, Saudi Arabia (Fig. 1). Most of the mosquitoes were collected during the 2017/2018.

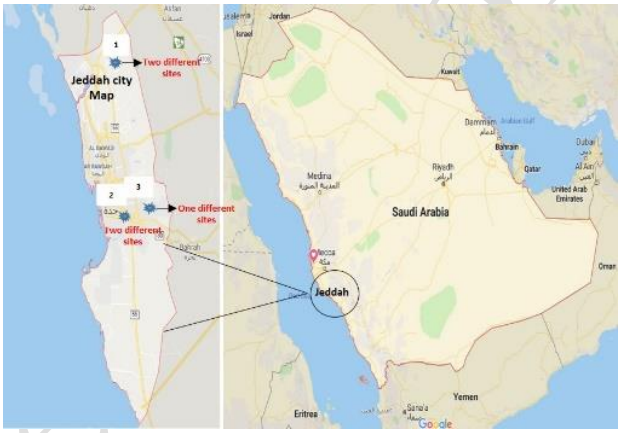

Fig. 1: Map of mosquito trapping locations around Jeddah, Saudi Arabia. Al Hamadaniyyah (Two sites); 2. Al Jamiah (Two sites); 3. Al Hazarat (One site).

Mosquito Rearing: The larvae were reared on artificial food (add company name or protocol), added in containers (25 × 35 × 6 cm) filled with distilled water. The colonies were maintained at 27°C±2°C temperature and 70±5% R.H. Some were used for rearing and others remained preserve in −20°C freezer. Black Seeds were collected from the supermarket of Jeddah Saudi Arabia and were stored in the freezer prior to extraction.

Black seeds extraction: Plant extraction Fresh seeds were washed and crushed in the presence of liquid nitrogen. Forty to sixty grams of seeds were finely ground and transfer to a bottle containing 500 ml of 70% Methanol for 48 hrs. Methanol was evaporated from the extract using a rotary evaporator to make it semi-dry form. The evaporated extract was transferred to -20°C refrigerator until be used for testing against selected insect stages.

DNA isolation: For the extraction of DNA legs of mosquitoes were homogenized individually in a sterilized condition. About 20 µL of proteinase K to the tube containing crushed mosquito sample. Furthermore, the sample was incubated at 56°C for 60 mins in 180 µL of ATL Buffer. QIAGEN kit was used for the extraction of DNA, according to the manufacturer's instructions. For each sample, a total of 100 µL of DNA was extracted. The

extracted DNA was transferred immediately to the -20°C refrigerator.

Barcoding gene Amplification: Universal primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAA TCA) were used for the Amplification of barcoding gene COI (Mitochondrial cytochrome oxidase subunit 1) (Folmer *et al.* 1994). Amplification of the gene was performed in 25 µl that contains Template DNA (1 µl), Primer Forward (1 µl), Primer Reverse (1 µl), 5x Go Taq Reaction buffer (5 µl) and 17 µl of syringe water. The tube to transfer to PCR Machine. Which was programmed as: denaturation for 2 mins at 94°C and 40 cycles for 30 sec at 94°C. Annealing at 49°C for 45 seconds and for 45 seconds at 72°C followed with final extension for 10 mins at 72°C. The PCR product was run to check the size of gene with that of Marker.

Sequencing: The COI gene product was commercially sequenced by Macrogen (Korea). Both forward and reverse gene was sequenced. The sequences were edited and assembled using Geneious version 8.1 (Kearse *et al.* 2012). MEGA X version 10 was used for the alignment and phylogenetic tree generation (Tamura *et al.* 2007). COI gene sequence for *Aedes* and *Culex pipien*s were submitted to NCBI genbank. The accession number for these two mosquitoes were provided as shown in Table 1.

Table 1: Accession numbers provided by NCBI

Sample ID	Names	Accession number
J2	*Aedes aegypti*	MK729109
J7	*Culex pipiens*	MK729108

Larvicidal activity of *Nigella sativa:* Larval susceptibility tests were conducted according to the method of (WHO. 2005). The Identified mosquitoes were treated with different concentration of black seeds extract. About 20 larvae per concentration were used in this experiment for both treated and control. Larvae of *Aedes* and *Culex* species were fed during the test. Larval and pupal mortalities as well as the adult emergences were recorded.

Data analysis: Our data was analyze using ANOVA (One-way analysis of variance). SPSS software (version 19) was use in order to compare the data by Duncan's multiple range test.

RESULTS

Molecular identification: Our research out puts directly related to the further development of a comprehensive and consistent mosquito through DNA barcoding. After DNA isolation COI gene was amplified as shown in Fig. 2. The gene product was sent to macrogen company for sequencing. The received sequences were submitted to NCBI for accession number. Neighbor-joining tree was constructed for both *Aedes* and *Culex* against present mosquito's species in the database as shown in Fig. in Fig. 3A and 3B. Similar research work is on the record regarding identification of mosquitoes through gene sequencing (Batovska *et al* 2016; Gupta *et al*., 2016; Weeraratne *et al*., 2017; Weeraratne *et al*., 2018). These studies indicate the importance of DNA barcoding for identification of mosquitoes.

Larvicidal effect: The mortality rate of the 4th instar larvae of *Aedes aegypti* and treated with different concentration of the *Nigella sativa* extract is shown in Table 2. The effective concentration was between 5-40 ppm, and the corresponding mortality rate was between 23.5–96.9%. While the Table 3 showed that the mortality rate of the 4th instar larvae of *Culex pipien*s and its treatment with a different concentrations of the *Nigella sativa* extract. The effective concentrations were between 5-40 ppm, and the corresponding mortality was between 23.5–96.9%.

Oviposition deterrence: The effects of treatment of *Nigella sativa* extract against *Aedes aegypti* and *Culex pipiens* larvae on the behavior of eggs laid by female mosquitoes in the treated water is shown in Table 4. The results showed that the treatment of *Nigella sativa* extract has given a repellent effect to female mosquitoes of *Aedes aegypti* to lay eggs in cups containing the treated water (289 eggs) compared to the control egg cups (870 eggs).

Table 2: Dose dependent effect of *Nigella sativa* extract against 4th instar of *Aedes aegypti*

Conc./ppm	Dose * I	Log (Dose * I)	Treated	Observed response %	Linear response %	Linear probit
5	5	0.699	100	23.469	20.4301	4.1735
10	10	I	100	45.918	47.1733	4.9291
20	20	1.301	100	68.367	75.3163	5.6846
30	30	1.4771	100	86.735	87.0034	6.1266
40	40	1.6021	100	96.939	92.4981	6.4403

Dose -I. Log (Dose Ratio - I).

Table 3: Shows effect of *Nigella sativa* extract against 4th instar of *Culex pipiens*

Conc./ppm	Dose * I	Log (Dose * I)	Treated	Observed response %	Linear response %	Linear probit
5	5	0.699	100	19.588	16.6473	4.0316
10	10	I	100	43.299	43.9893	4.8487
20	20	1.301	100	65.979	74.723	5.6659
30	30	1.4771	100	88.66	87.3606	6.1439
40	40	1.6021	100	97.938	93.0945	6.4833

Table 4: Ovipositional behavior of *Aedes aegypti* and *Culex pipiens* against *Nigella sativa* Methanolic extract

		%IE	Total No. of laid eggs	OAI**	Total No. of hatched eggs	Hatchability*** (%)
Aedes aegypti	*Nigella sativa*	99	289	-0.5	230	79.6
	Control		870		845	97.1
Culex pipiens	*Nigella sativa*	99	349	-0.42	272	77.9
	Control		863		847	98.1

* 3 replicates, 10 engorged mosquito females. ** Oviposition activity index. *** All newly hatched larvae were died within 1-2 days.

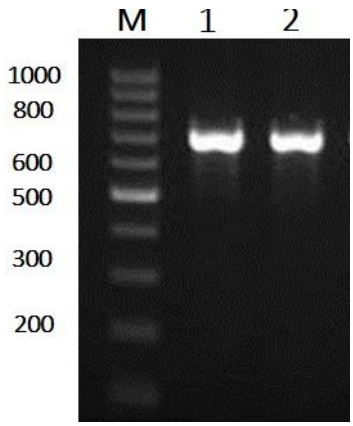

Fig. 2: Image of Agarose gel electrophoresis of COI gene (~ 700 bp) against the Marker

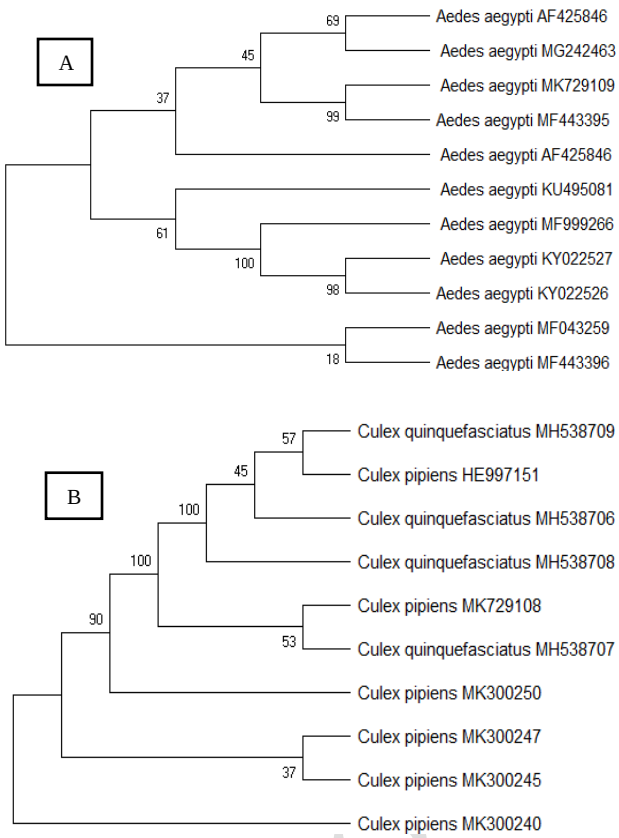

Fig. 3: A). Phylogenetic analysis of *Aedes aegypti* in Jeddah, B). Phylogenetic analysis of *Culex pipiens* in Jedda

The treatment of *Nigella sativa* has also given a repellent effect to female mosquitoes of *Culex pipiens* to lay eggs in cups containing the treated water (349 eggs) compared to the control egg cups (863 eggs). The ovipositional behavior of mosquito females with reference to egg hatchability for female mosquitoes in pond water was (-0.42) as shown in Table 4. The results also showed that the treatment of *Nigella* sativa was affecting the egg hatching rate in the treated water (79.6%) and (77.9) respectively. On the other hand, the results of the treatment of *Nigella* sativa showed a significant decrease in the percentage of egg hatching in the treated water. The percentage of egg hatching in treated water cups was 79.6-77.9%, while 97.1-98.1% in the control. Overall, all newly hatched larvae died within 1-2 days.

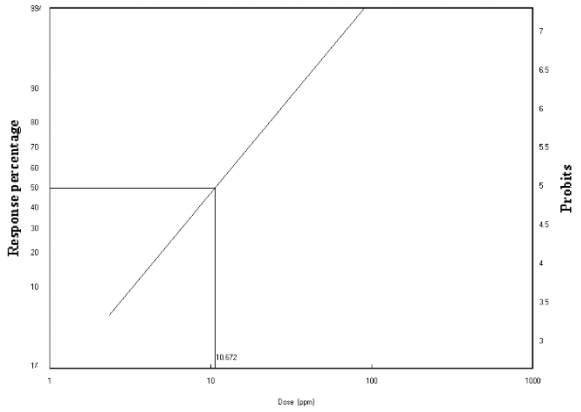

Fig. 4: Laboratory toxicity line of the *Nigella sativa* extract with the fixed statistics in relation to the determination of the level of efficacy against the larvae of *A. aegypti* mosquito.

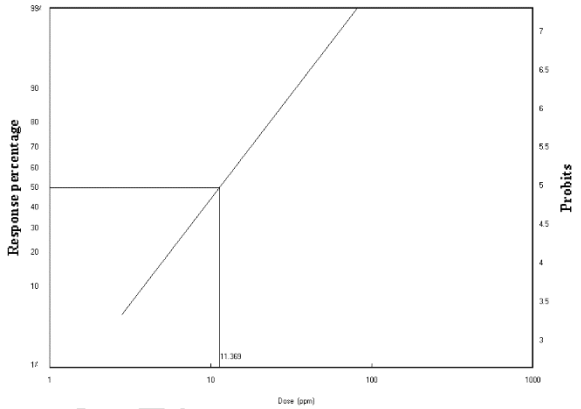

Fig. 5: Laboratory toxicity line of the *Nigella sativa* extract with the fixed statistics in relation to the determination of the level of efficacy against the larvae of *Culex pipiens* mosquito.

The *Nigella sativa* extracts showed a clear variation in the level of sensitivity of the mosquito larvae under the effect of the prepared formulations. Moreover, from the toxicity lines Ld–p line and the recovery of the values of the mortality concentrations for 50% and 90% of the larvae that were subjected to the different concentrations of the extracts under investigation are shown in (Figs. 4, 5 & Table 2,3). The concentrations of *Nigella sativa* extract needed to kill 50% and 90% of the treated *Aedes aegypti* larvae 24 hrs post-treatment for this extract were 10.67 and 34.58 ppm consecutively, while the extract concentrations of the *Nigella sativa* that needed to produce a killing effect of 50% and 90% of the *Culex pipiens* larvae post-treatment were 11.369 and 34.58 ppm consecutively. These results proved that the *Nigella sativa* extract gave the highest efficacy against the 4th instar larvae of the *Aedes aegypti* and *Culex pipiens* mosquito. The values of the indicator of resistance ratio (RR) also showed that the larvae of *Ae. aegypti* and *Culex pipiens* were more sensitive to the *Nigella sativa* extracts under investigation were (0.975-0.97) respectively. Also, this study showed the Chi-square of *Aedes aegypti* and *Culex pipiens* larvae were (6.086-7.46). in addition, there were deformations on body segments especially on the larval abdomens which might be due to an imbalance in the secretion and distribution of the body pigmentation, therefore the affected larvae were totally black or it appeared as black patches and spots as pigmented areas on all body parts with clear evident elongation of the neck region when compared with the untreated larvae (Fig. 6 & 7).

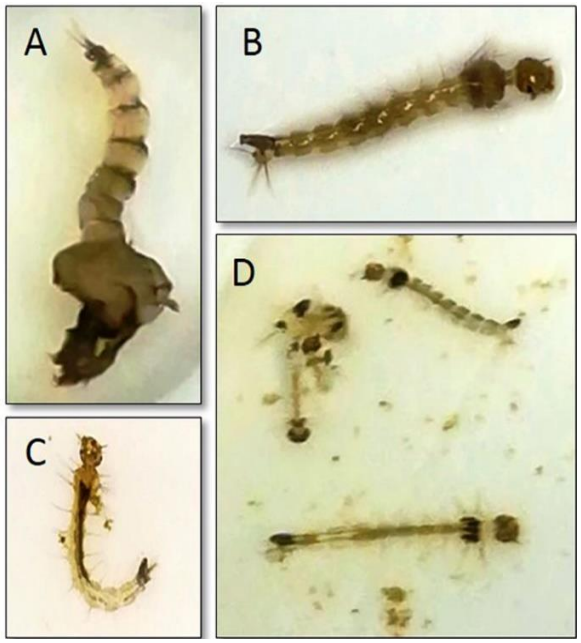

Fig. 6: The morphological deformations of *Aedes aegypti* mosquito larvae after 24 hrs post- treatment with the *Nigella sativa*. A) Abnormal Pupa, B) Larva with prolongation (Neck), C) Semi Albino larva, D) Albino larva.

Fig. 7: The morphological deformations on *Culex pipiens* mosquito larvae after 24 hrs post- treatment with the Methanolic extract of *Nigella sativa.* A). Larva with Prolongation (Neck), B). Albino pupa.

DISCUSSION

The methanolic extract of *N. sativa* was found significantly effective against the identified mosquito's species. This Larvicidal activity may lead to the presence of different biochemical compounds like phenols, flavonoids, terpenoids and alkaloids. These compounds may act individually or collectively by producing toxic effect against the exposed mosquitoes which was identified molecularly through DNA barcoding. Earlier Khater *et al* uses different types of commercially available oil against *Culex pipiens*. It shows that the fenugreek oil has potent ability to kill *Culex*. Recently Baz *et al* uses oil-resins of *Boswellia sacra*, *Eucalyptus camaldulensis, Pistacia lentiscus*, *Commiphora molmol* and *Araucaria heterophylla* against *Culex pipiens.* There results show that *Araucaria heterophylla* and *Commiphora molmol* has the ability to kill larvae of *Culex pipiens* (Baz *et al*., 2021). It is worthy to ensure environment safety during application of insecticides against pests and vectors. In order to be acceptable, insecticides need not to cause high mortality against the target organisms (Kabaru and Gichia 2001). More likely since in 1946 when DDT was introduced first for mosquito control and quickly after one-year *Aedes tritaeniorhynchus* and *Aedes sollicitans* developed resistance to DDT (Hemingway and Ranson 2000). Statistically, approximately 500 species of insects were found to be resistant against various kinds of insecticides (Shelton *et al*., 2007).

Hence, the importance of a suitable alternative to synthetic insecticides like phytochemicals which are safe, readily available, cost-effective and environment friendly may not be under estimated in the future. According to report of Bowers *et al*. (1995), the application of medicinal plants against mosquito will be better due to its cost-effectiveness and will be better alternate for expensive products that will stimulate local efforts to enhance public health. Therefore, this study will provide better understanding about the efficacy of available indigenous medicinal plants.

Molecular identification through DNA barcoding is a useful method generally for insects and especially for mosquitoes (Cywinska *et al*., 2006; Kumar *et al*., 2007). This research study also provides a reliable background for mosquito species identification in spite the identifications remain ambiguous regarding closely associated species. Identification of species is important in vector monitoring and control.

The effects of treatment of *Nigella sativa* extract against *Aedes aegypti* and *Culex pipiens* larvae showed the black patches and spots as pigmented areas on all body. Similarly the These observed and reported deformations on the larvae treated with *Nigella sativa* extract may be due to the fact that it contains many valuable valid active chemical constituents such as saponins and terpenes which might be viewed as playing the role of synthetic analogue of the juvenile hormones or other effective hormone mimics and their involvement and intrusion with the physiological processes during the insect metamorphosis and it might be attributed to the stoppage or inhibition by the hormones that regulate important vital processes that could lead to the imbalance in stimulation or inhibition of the secretion of the hormones of those chemical constituents. This situation may contradict other hormones or enzymes that are produced from the endocrine glands which spontaneously lead to an imbalance of the growth processes and larval fatalities (Silva and Mendes, 2007; Mehdi *et al*., 2012 and Grzybowski *et al*., 2013). Recently researchers are also working on the nanofabrication of plant extract. This will lead to find an ecofriendly way to treat the mosquitoes. For this purpose, some researcher has done work that show that green synthesized silver nanoparticles have the ability to kill mosquitoes (Murugan *et al*., 2015; Roni *et al*., 2015)

Conclusions: Our study shows that the Methanolic extract of black seeds has a potential Larvicidal activity as well as inhibition of Adult emergence. At 40 ppm of Nigella sativa shows a potent Larvicidal activity leads to kill almost 97 % of larvae. So it is recommended that 40 ppm of nigella sativa can be used for the biocontrol of *Culex pipiens* and *Aedes aegypti*. This activity may be attribute to the phenolic and chiral compounds present black seeds. Further studies are needed to isolate the active compounds responsible for this activity. Thus black seeds extract can be used to control the mosquito that is ecofriendly compare

to the chemicals one. Furthermore, the Eco-toxicological profile of Nigella sativa is needed to be done in future.

Authors contribution: NA, YA and KMA designed the project. The sampling, data collection, processing and interpretation of results were made by NA, MS and HMA. The data analysis was made by NA, MMA and YA. The manuscript was written by YA, KMA, NA and MS. All the authors read the manuscript and approved the contents.

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