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Research Article

Entomology for Better Antimalarial Activity

Larvicidal Effects Of Pongamiaglabra and Syzygiumaromaticum On Aedes Aegypti Mosquito Larvae

Ananya Bar[1*], J. Andrew[1], Akshay Botle[2] and Manojsingh[3]

[1]*Department of Zoology and School of Entomology, St. John's College, Agra, 282002, India.*

[2]*Department of Chemistry, Wilson College, Mumbai-400007, India*

[2]*Department of Zoology, Kalinga University Raipur, India*

Abstract: Mosquitoes are considered as the most nuisance causing vectors as they are responsible for a widespread of diseases. Malaria-borne mosquitoes are responsible of killing two to three million people and infect an estimated two hundred million or more annually. A host of other mosquito-borne diseases including filariasis, yellow fever, dengue, and encephalitis kill and debilitate hundreds of millions more. The present study investigates the bio efficacy of two commercially available plant oils from Pongamia glabra (Karanja) and Syzygium aromaticum (Clove). These two plant oils were tested against Aedes aegypti (Dengue fever mosquito) to determine their lethal concentration doses (LC50). A larval toxicity assay was performed where a stock solution of 10% was prepared for both the oils at a concentration range of 100 to 900 ppm and 90 to 1300 ppm was set for Pongamia glabra and Syzygium aromaticum respectively. A batch of each 10 IVth instar larvae was subjected to the assay with test, combination and a control set for a period of 24 and 48 hrs. LC50 of two oils were determined while the combination of two oils showed a synergistic effect. The effect of P. glabra and S. aromaticum on the anterior and posterior regions of the midgut region of A. aegypti larvae were found damage to epithelial cells, basement membrane, peritrophic membrane, fat body cells and nuclei were observed. The results of the study showed that the synergistic method of using common eco-safe combination of plant oils were effective to handle and monitor *A. aegypti* larva. Pongamia glabra and Syzygium aromaticum are among the successful mosquito control agents.

Keywords: Aedes aegypti, Pongamia glabra, Syzygium aromaticum, Phytochemicals, LC50, Larvicidal Activity, Mosquitocidal Activity

***Corresponding Author**

Ananya Bar , Department of Zoology and School of Entomology, St. John's College, Agra, 282002, India.

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1. INTRODUCTION

In the world, around 17% of the infectious diseases present are due to vector borne sources which include diseases like Malaria, Dengue and Yellow fever.[1] Diseases caused from mosquitoes can lead to a significant damage resulting in high mortality rates, increased morbidity and also add economic burden on mankind. From the present data available, approximately 3500 species of mosquitoes are known beyond the tropical and subtropical regions of the world from which they are classified accordingly.[2] Mosquitoes majorly breed in water, usually on moist surfaces, in tree holes and in containers but sometimes they even deposit their eggs directly in water.[3,4,5] Dengue is considered as one of the most ubiquitous disease present in the world transmitted by mosquitoes.[6,7,8] The disease has encroached around 2.5 billion people around the world with its virus and its occurrence has intensified 30 folds in these past years.[9,10]Human life is not only threatened but also widely affected due to these dreadful vectors. Among some species of mosquitoes the Aedes aegypti is the prominent one for transmitting viral diseases such as Yellow fever, Zika, Chikungunya, Dengue. Also the eggs of these mosquitoes are able to withstand dry conditions and are able to remain viable for months even in the absence of water. Thus with every passing decade, these diseases contribute in the increasing economic burden in the cosmopolitan areas.[11,12] In India dengue is severe and prominently found in the areas of Maharashtra, Kerala, Karnataka, Andra Pradesh and Tamil Nadu. [13] Dengue fever was first accounted in 1956 in Vellore district in Tamil Nadu whereas the first hemorrhagic outburst of fever was documented from the eastern coast in 1963 from a data obtained by National Vector Borne Disease Control Programme (NVBDCP) in India.[14] Thus, A. aegypti is considered as the prime vector for such diseases in India.[15] So, it is necessary to control Aedes aegypti. Therefore, some remedial measures has to be implemented to control the widespread of mosquito disease, which can be done by depending upon the applications of conventional insecticides.[16,17] Personal protection and public awareness are the most economic ways for protecting ones against mosquitoes followed by eradicating breeding grounds of these vectors through environmentally friendly larvicides.[18-23] In the recent years humans, domestic animals, wildlife and the environment has suffered a major problem due to the chemical synthetic insecticides used for controlling mosquitoes. [24] Moreover, from a prolonged usage of these insecticides most of the mosquito species have acquired resistance to these compounds making them ineffective.[25] Several studies reveal that Aedes aegypti has acquired resistance to almost all insecticides including the organochlorines, carbamates, organophosphates and pyrethroids. [26] As a result, there is a need to find a safe and eco-friendly alternative to minimize the peril from mosquito.[27]Phytochemicals are coming into interest due to their unique properties as they are environmentally safe, they have target specificity, development of resistance against them is almost negligible, they have high acceptability and moreover they are suitable for use in rural areas also.[28] Plants are considered equivalent to the natural industrial chemical factories as they consist similar phyto-chemical properties such as steroids, alkaloids, terpenoids, phenolics, essential oils etc. Also the oils extracted from these plants are of low costs and are widely used as alternatives for insecticides.[21-40] Essential oils have gained a lot of impetus where larvicidal activities of the extracts from Neem[41], Ipomoea carnea[4], Calophyllum inophylu[43], lemongrass[44], Syzygium aromaticum[45] and Ocimum basilicum[46] have been studied. The phytochemicals present in these extracts impart good killing actions on mosquitoes and larvae.[47]Various oils from plants have effects on mosquito's larvae. The LC50 and effects of plant oils like Syzygium aromaticum[48]; Pongamia glabra[27] were described by various authors. In this study, the mentioned plants were used to evaluate the effects on larvae of A. aegypti. So the present study paves its attention towards a few plant species of medicinal purpose which were screened for their essential oils to study the inhibitory effects of them on A. aegypti. The toxicological effects of the plant oils with respect to their synergistic effects were observed followed by the histological effects on the gut of the larvae.

2. MATERIALS AND METHODOLOGY

2.1 Details of Medicinal plant Oils

Pongamia glabra and Syzygium aromaticum were selected for the study where commercially available seed oil of Pongamia glabra and flower bud oil of Syzygium aromaticum were chosen for experimental purpose detecting their larval toxicity bioassay due to their inherent insecticidal properties. These oils were purchased from a standard local company. The studies were conducted in-situ under controlled laboratory conditions at St. John's College, Department of Zoology and School of Entomology, Agra, India.

2.2 Preparation of Stock Solutions

Prior the experimental setup for bioassay stock solutions for P. glabra and S. aromaticum were prepared by dissolving their oils in 10 ml of Dimethylsulphoxide (DMSO)[49] and a stock solution of 10% in a ratio of 1:1 stock solution was taken to study the larval bioassay.

2.3 Preparation of different concentrations of individual plant oils for larval bioassay

In order to determine the efficiency of the oils in larval bioassay, a series of different ranges of concentrations for individual oils were prepared, where a concentration series ranging from 100 ppm to 900 ppm at interval of 100 ppm was taken for P. glabra. Similarly for S. aromaticum a concentration series ranging from 90, 100, 300, 500, 700, 900, 1100 and 1300 ppm were selected. To determine the combination effect of two oils (P. glabra. Similarly + S. aromaticum) a series of 8, 10, 20, 30, 40, 50, 60, 70, 80 ppm were selected.

2.4 Collection of Larvae

Aedes aegypti was chosen for the present study. The Classification of the organism is given in Table 1.Various mosquito harboring sites in the city of Agra were selected viz. Bichpuri, Dayal Bagh, Kamlanagar, St. John's College Agra, Agra Cantonment, Sikandra, Shaheed Nagar and Tajganj area. The mosquito breeding sites were surveyed from January 2009 to December 2010. The larvae were collected from the breeding sites in a net with 6 cm of width.

2.4.1 Rearing of larvae

Samples of the larvae were brought to the laboratory and reared. Various instars were separated and reared in specific

containers where the larvae were fed on yeast.[50] Larvae were preserved and identified using themorphology of head, neck, thorax, abdomen, siphon and anal segment as specific key for identification.[51,52,53]

2.4.2 Larval toxicity assay

For Test, experimental containers having 100 ml. of tap water with the above mentioned concentrations (ppm) of two oils along with combination were added.For Control, similar setup was done with plastic containers containing 100ml of tap water without any oil added into it. In the mentioned sets, 10 IVth instar larvae were kept at 35±5°C room temperature which were supplemented with yeast as a nutrition source and the larval activity was noticed. Triplicate sets were maintained and observations recorded were tabulated. Mortality was recorded after 24 and 48 hrs.[54]

3. STATISTICAL ANALYSIS

In toxicity assay, data recorded was subjected to log probit analysis to obtain the lethal concentration value (LC) using Origin 8 software. These computer generated programs provided LC values with appropriate regression line and slope. LC50 was chosen as the value of the activity.[55,56] Safe concentration was recorded, below which larvae and adults remained alive. For making the combinations, the concentrations were made below safe concentration using the formula given below.[27]

$$Safe\ \ concentration(SC) = 48\ \ hrs.LC_{50} \times A \times S \quad where \quad A = 0.3$$

$$S = \frac{24\ \ hrs.\ \ LC_{50}}{48\ \ hrs.\ \ LC_{50}}$$

Synergistic factors (SF) were also calculated using the formula –

$$SF = \frac{LC_{50}\ \ value\ \ of\ \ the\ \ insecticide\ \ alone^{*}}{LC_{50}\ \ value\ \ of\ \ the\ \ insecticide\ \ with\ \ the\ \ assumed\ \ synergist}$$

(Values of SF > 1 indicate synergism and SF < 1 indicate antagonism) respectively.

* LC50 value of maximum effective plant product.

3.1 Histological studies

Histological studies were conducted with control and treated dead larvae. The dead larvae were immediately preserved in 10% formalin and dehydrated and cleared in xylol. Paraffin blocks were made and sectioned in the microtome. The sections were spread over the slides and processed by the standard staining method using Haematoxylin-Eosin. The stained slides were observed under a compound microscope for histological details. Mid gut regions (anterior and posterior) were photographed under an image documentation system under 10X magnification and were transferred on a computer to observe the histological differences between control and experiments, after toxicity bioassay.

4. RESULTS

4.1 Effects of different plants oils on A. aegypti larvae

The larvicidal activity observed due to treatment with oils of selected plant species was shown in Table 2 and Table 3.

4.2 Syzygium aromaticum

The LC50 value of Syzygiumaromaticum oil on A. aegypti larvae at 24 hr was found to be 445.8 ppm, while at 48 hr, it was observed to be 435.8 ppm. On the other hand 133.7 ppm concentration was found to be safe for larvae to survive (Table 2). Maximum 100% mortality in the larvae after 24 hr. at 1300 ppm and 97% mortality in 48 hr at 1100 ppm were further observed (Figure. 1 A and B). The larvae died in the presence of S. aromaticum oil and sank to the bottom of the container. No adults were formed within 48 hrs. However in control, a few pupae and adults were formed and no mortality was observed.

4.3 Pongamia glabra

The LC50 value of Pongamia glabra oil on A. aegypti larvae at 24 hr. was found to be 645.4 ppm, while at 48 hr it was observed to be 380.2 ppm. On the other hand 193.6 ppm concentration was found to be safe for larvae to survive (Table 2). Maximum 80% mortality in the larvae after 24 hr. at 900 ppm and 96% mortality in 48 hr. at 800 ppm were further observed (Figure 2 A and B). The larvae died in the presence of P. glabra oil and floated parallel to water. At lower concentration, a few numbers of pupae and adults were developed. However in control, a few pupae and adults were formed and no mortality was observed.

4.4 Effect of combination of plant oils on A. aegypti larvae

For the synergistic effect on IV instar A. aegypti larvae, plant oils were combined in equal proportion (1:1) below the level of their safe concentration limits. They were shown in Table 3.

4.5 P. glabra and S. aromaticum

The combination of plant oils of P. glabra and S. aromaticum on A. aegypti larvae showed synergistic effect. The LC50 at 24 hr. was 77.6 ppm and at 48 hrs. 35.5 ppm. On the other

hand 23.3 ppm concentration was found to be safe for larvae to survive (Table 3). At higher concentration i.e. (80+80) ppm dosage cent % mortality at 24 hr. while 96 % mortality at (60+60) ppm at 48 hr were being noticed (Figure 3). The larvae died in the presence of P. glabra and S. aromaticum and settled at the bottom of the container. A few pupae were developed and no adults were formed within 48 hrs. However in control, a few pupae and adults were formed and no mortality was observed.

4.6 Histology of A. aegypti IV instar larvae

Control: In A. aegypti larva, the anterior midgut was round in shape. Peritrophic membrane was present in the inner border of the tract and it had food particles. Epithelial cells with prominent densely stained nuclei which were round in shape and basement membrane were noticed. Well-formed brush borders were present. Fat body cells were in compact form (Figure 4).Posterior midgut is oval shaped and it has epithelium cells with densely stained nuclei, well developed brush borders and basement membrane. Peritrophic membrane is present (Figure 4). Fat bodies are in compact form.

4.7 Histopathological effect of P. glabra and S. aromaticum on A. aegypti larvae

This combination had the highest effect on A. aegypti. In P. glabra and S. aromaticum treated mid gut region, the anterior midgut became elongated. Epithelial cells of the gut in some areas were swollen towards the medial border of the gut lumen. Peritrophic membrane got damaged (Figure 5).Posterior midgut became elongated. Peritrophic membrane was damaged. Nuclei were present in epithelial cells which were densely stained and became oval in shape in some cells. Whereas, some epithelial cells got damaged and were intensely stained (Figure 5). Fat bodies are found to be damaged and lose their compact form (Figure 6).

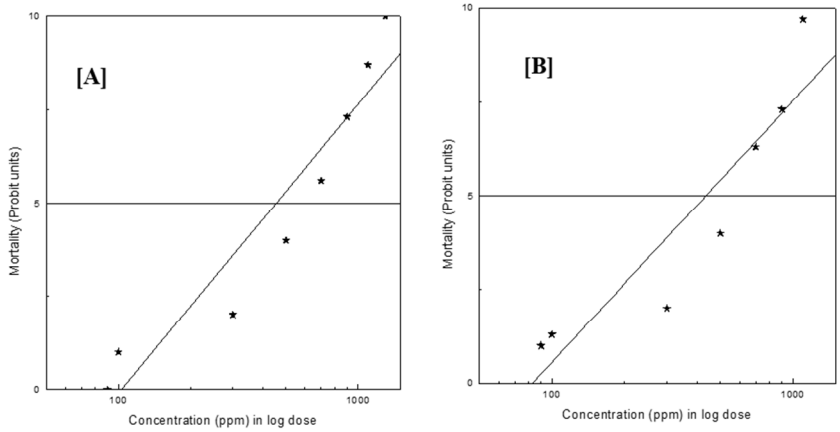


Fig1.LC_{50} value of *Syzygiumaromaticum* oil on *A. aegypti* larvae at 24 hrs. (A) and 48 hrs. (B)

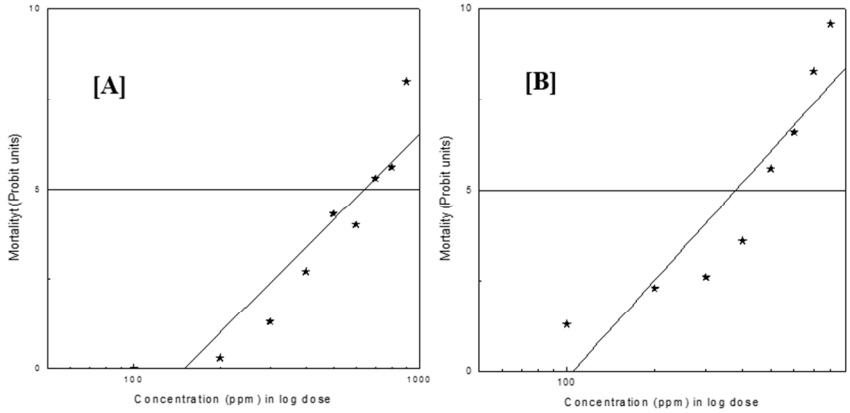


Fig 2. LC50 value of Pongamiaglabra oil on A. aegypti larvae at 24 hrs. (A) and 48 hrs. (B)

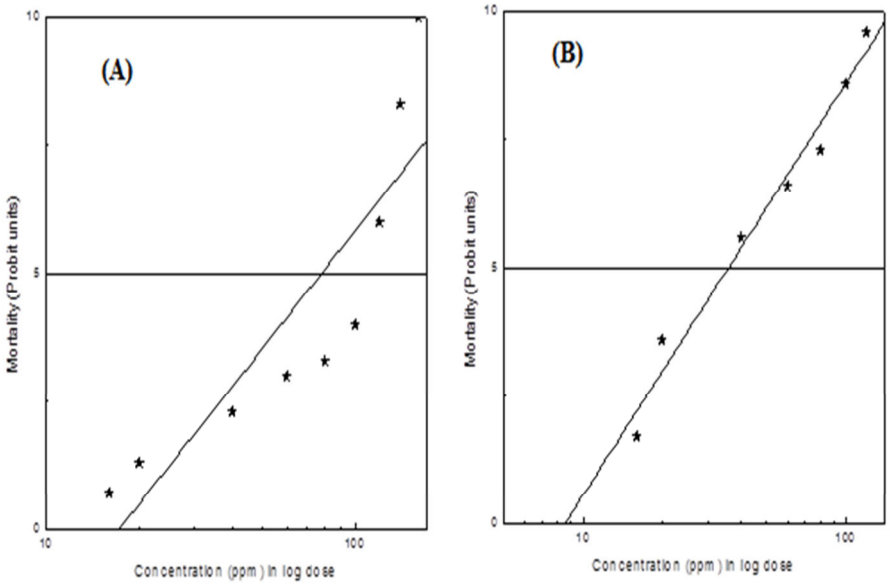
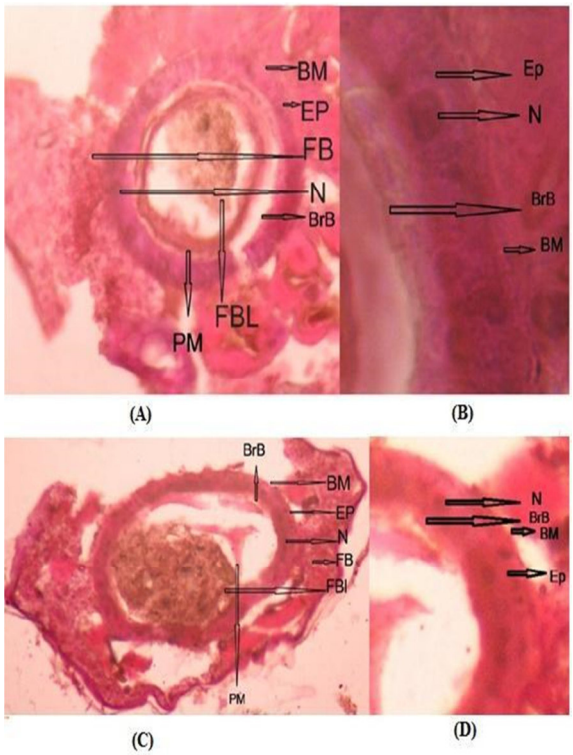
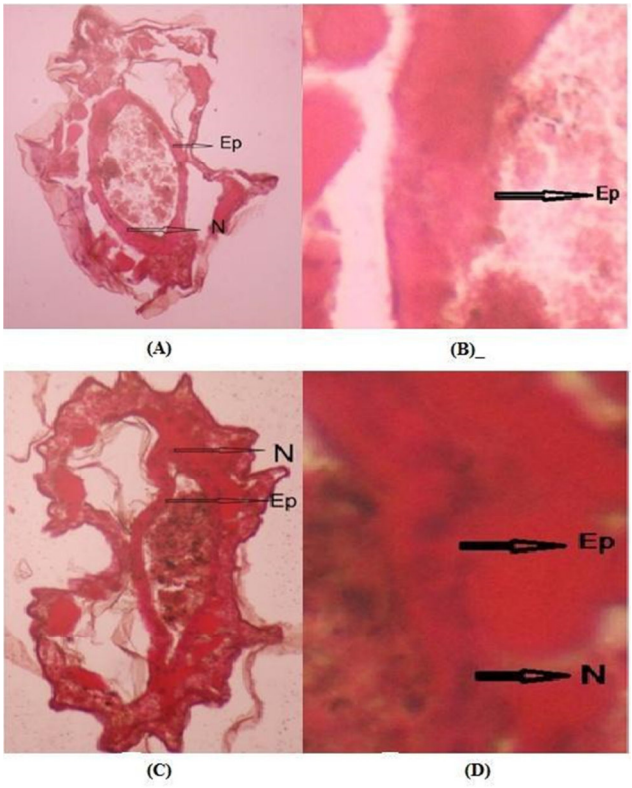


Fig 3. LC50 value of Pongamiaglabra and Syzygiumaromaticum oil on A. aegypti larvae at 24 hrs. (A) and 48 hrs. (B)



Basement membrane (BM); Epithelial cell (EP); Fat body (FB); Nucleus (N); Brush border (BrB); Food bolus (FBL); Peritrophic membrane (PM)

Fig 4.Histology of anterior (A, B) and posterior (C,D) midgut of A. aegypti larvae in Control



Epithelial cell (EP); Fat body (FB); Nucleus (N)

Fig 5.Histopathological effect of P. glabra and S. aromaticum on A. aegypti larvae in the anterior midgut (A and B) and posterior midgut (C and D)

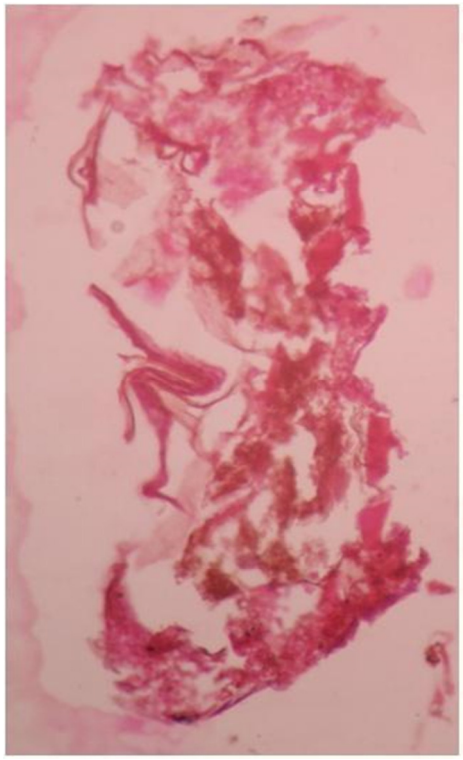


Fig 6.Histopathological effect of P. glabra and S. aromaticum on A. aegypti larvae on fat bodies

Table 1.The Classification of the organism				
Class	Insecta			
Order	Diptera			
	Sub-order	Nematocera		
	Family	Culicidae		
		Sub-family	Culicinae	
		Tribe	Culicini	
			Genus	Aedes
			Subgenus	Stegomyia
				Species aegypti

Table 2.The Classification of the organism		
Plant	***P. glabra***	***S. aromaticum***
24 hr. LC50 (ppm)	645.4	445.8
Regression equation	$Y=7.9x-17.2$	$Y=7.7x-15.6$
48 hr. LC50 (ppm)	380.2	435.8
Regression equation	$Y=8.9x-18.1$	$Y=6.9x-13.3$
S. C. (ppm)	193.6	133.7

Table 3.LC50, Synergistic factor (S. F.) and safe concentration (S. C.) of the combination of plant extracts on A.aegypti larvae							
Combination	**24 hr. LC50 (ppm)**	**Regression equation**	**Synergistic factor**	**48 hr. LC50 (ppm)**	**Regression Equation**	**Synergistic factor**	**S.C. (ppm)**
P. glabra + *S. aromaticum*	77.6	Y=7.6x-9.4	5.7	35.5	Y=8.0x-7.5	12.3*	23.3

(*) = synergism present

5. DISCUSSION

Mosquitoes are nuisance pests and vectors fro causing many diseases both in urban and rural areas.[57] They cause human death, suffering and impediment to economic development not only in India but all over the world.[58] Repellent which are used to prevent the mosquito bite could help in controlling the diseases caused by mosquito.[59] Controlling of mosquitoes is a vital strategy to check the transmission of diseases and disruption of epidemic which can be brought through chemical control. Thus there is a need for vector control using some other alternative method. It has been reported that before the discovery of synthetic organic insecticides there were ample herbal products available used as natural insecticides. Possible insect repellent are the toxicants extracted from the plants.[60] It is noted that many herbs and shrubs have promising medicinal properties, larvicidal mosquitoes and repellent properties of mosquitoes.[61] Several researchers have proposed different species of larvicidal plants in mosquito control.[27] The phytochemicals present in plants can inhibit the function of neuro-secretory cells or can interact with the epidermal cells that cause the tanning or cuticular oxidation cycle.[62] Research have shown that the behavior of plant compounds on target species varies with regard to the plant parts from which they are derived.[63] Hence, the plants which contain phytochemicals are being used as larvicides, insect growth regulators, repellents and oviposition attractants.[64]

5.1 Syzygium aromaticum:

Maurya *et al.*[65] reported that the flower bud hexane extracts of Syzygiumaromaticum, showed LC50 to be 85.90 ug/ml. against A. vagus and 149.56 µg/ml against Cx. vishnui. Ranan *et al.*[48] described that S. aromaticum leaf oil extracted by Clevenger apparatus, showed 93.33% mortality in 60 min. and 100 % mortality in 120 min at 250 ppm against early fourth instar larvae of Culex quinquefasciatus. Bhat *et al.*[66] described that S. aromaticum leaf and flower bud oil were used against Aedes albopictus fourth instar larvae. The leaf oil showed LC50 in 5.3 mg/L while bud oil showed 17.84 mg/L at 24 hrs. Although scant literature is available regarding the larvicidal effects of S. aromaticum flower bud oil against A. aegypti, and during the present investigations it was noticed that LC50 of S. aromaticum was obtained at 445.8 ppm in 24 hr. and 435.8 ppm in 48 hrs. The methanol and ether extracts of S. aromaticum were less toxic to the larvae in comparison to their inhibitory effects on the developmental stage of larvae which was found to be remarkable, showing complete inhibition of adult emergence at 200 and 600 ppm respectively against Culex pipiens.[67] The phyto-constituents present in flower bud oil are found to be eugenol (49.71%), caryophyllene (18.94%), benzene, 1-ethyl-3- nitro (11.12%), benzoic acid, 3-(1-methylethyl) (8.95%), elixene (3.87%), caryophyllene oxide (1.53%) and α- farnesene (1.11%) etc.[68] The larvicidal effects observed in the present study against A. aegypti is correlated to these constituents.

5.2 Pongamia glabra

Geaorge *et al.*[27] noticed that the seed extracts of Pongamia glabra was prepared with petroleum ether through soxhlet extraction. The stock solution was made with acetone and tested against fourth instar larvae of Cx. Quinque fasciatus. The larvae were collected from the field and colonized. They were subjected to various concentrations of the extract of P. glabra under lab condition. The LC50 of P. glabra in 24 hr. was 282.57 ppm for the field collected larvae and 138.030

ppm for the laboratory colonized larvae. Shanmuga sundaram et al.[69] described that P. glabra oil cake when tested on Cx. Quinquefasciatus, A. Aegypti and A. Stephensi, at 24 hr., the LC50 was 0.26% for Cx. quinquefasciatus, 0.15% for A. aegypti and 0.21 % for A. stephensi respectively. In the present study, the LC50 of P. glabra seed oil was 645.4ppm in 24 hr. and 380.2 ppm in 48 hr. and in 900 ppm when tested against A. aegypti.

5.3 Synergistic effects of P. glabra and S. aromaticum and histology

The Pongamia spp. extract has been considered as a good synergist and hence has been combined with several pest control agents in the control of various pests.[27] In the present study, the combination of plant extracts of P. glabra and S. aromaticum, showed the LC50 value in 24 hr. at 77.6 ppm and in 48 hr. at 35.5 ppm and histological changes were being observed such as, elongation of anterior midgut region and damage of peritrophic membrane, swelling of epithelial cells of the gut in some areas, towards the medial border of the gut lumen; in the posterior midgut region, epithelial cells and basement membrane in some areas were damaged. Some of the damaged epithelial cells were intensely stained. Fat bodies were also found damaged and they lost their compact form. According to the chemical constituents present in P. glabra seed oil, included fatty acids like Palmitic acid 6.8%, Stearic acid 1.3%, Arachidic acid 2.56%, Oleic acid 46.43% and Linoleic acid 18.2% etc. which are responsible for the larvicidal nature and also was observed in the present study against both IV instar larvae of A. aegypti.[70]From the time immemorial, India has long heritage of use of herbal products, not only as medicine but also for insecticides etc.[71] The remarkable contribution of plants to the industry was possible because of the large number of phytochemical and biological studies all over the world.[72] Essential oils have received considerable attention as potentially useful bioactive compounds against insects.[73] The current studies therefore suggested the use of essential oil from seeds of A. indica and P. glabra and flower bud of S. aromaticum, as a mosquito larvicidal agents. The essential oils of these plants could be used in small water sources known as breeding sites such as pits, holes, coolers, water containers, etc. by the public to manage mosquito larvae. This will also benefit from the point of view of emissions control. The future aim of this research will be to establish an appropriate formulation with appropriate synergistic agents and field evaluation of the drug to evaluate the toxicological impact and bioefficacy.[74]The present study has identified plants with larvicidal activities, thus indicating their potential for application in the control of the dengue vector, A. aegypti. The results of the present investigations revealed the broad-spectrum of toxic properties of the tested botanical oils against the larval stages of A. aegypti. Combined effect or synergistic effect of the plant oils used had proved to be very advantageous in the control of this mosquito. Thus the present synergistic approach can be used as an eco-safe popular combination of plant oils for management and control of A. aegypti larva. Pongamia glabra, Syzygium aromaticum are some of the good mosquito control agents due to their effective insecticidal properties against A. aegypti larval stage when used as synergistic mixtures hence, are recommended for their application in control of A. aegypti mosquito larvae.

6. CONCLUSION

As the chemical insecticides are harmful for ecosystem, insecticides made by medicinal plant extracts and their combinations are the best choice. They are completely eco-friendly and cheap. The combinations tested were found effective on *A. aegypti*. They are useful for control of *A. aegypti,* Dengue vector. Moreover over, all control measure of anti *A. aegypti* campaigns have been extensively carried out in Agra city and the education of the inhabitants of the city regarding use of certain simple precautions is still desirable.

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8. AUTHORS CONTRIBUTION STATEMENT

Ananya Bar designed and performed experiments and analyzed the data. Akshay Botle and Manoj Singh; co-wrote the paper. Dr.J. Andrew.; Supervised the research.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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