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Larvicidal efficacy of *Ocimum sanctum* oil against *Culex quinquefsciatus* by *in-silico* and *in vivo* methods

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Abstract
Culex quinquefsciatus is an insect vector responsible for transmission of lymphatic filariasis, a vector-borne disease that leads to swelling of lymph nodes and lymph vessels. The disease is more common in the tropics and primarily effects arms, legs and genitalia. The present study aims to determine the efficacy of *Ocimum sanctum* oil as a potential control agent of the mosquito larvae. *In-silico* methods are used to determine the effect of phytochemicals present in *Ocimum sanctum* against the target enzyme receptors of the mosquito larvae. Targeted inhibition of biologically important enzymes leads to disruption of key biological pathways ultimately leading to the death of the organism. Direct toxicity tests are performed using *Ocimum sanctum* oil and its effect on total protein content of the larvae are also noted. The results of the *in-silico* study corroborated with direct toxicity tests confirm the efficacy of *Ocimum sanctum* oil as a potential larvicide against *Culex quinquefsciatus*.

Keywords: *Culex quinquefsciatus*, *in-silico*, larvicide, *Ocimum sanctum*, vector

Significance Statement: Mosquito vectors transmit a number of diseases that cause harm to public health, one example of which is *Culex quinquefsciatus* responsible for transmission of lymphatic filariasis. The present study aims to find plant-based alternatives to chemical larvicides that the normally used for vector control.

1. Introduction
Among the 112 genera of mosquitos, Anopheles, Culex and Aedes are the vectors of serious health disorders such as malaria, filariasis, japanese encephalitis, dengue, chikungunya, yellow fever etc. that are responsible for the untimely death of millions of people worldwide (Kamaraj *et al*. 2011) [18]. *Culex quinquefsciatus* is responsible for the transmission of lymphatic filariasis caused by parasite *Wuchereria bancrofti*, it is a widely distributed tropical disease. The parasite damages the lymphatic system and cause severe swelling in the legs, arms and genitalia. It also increases the risk of frequent bacterial infections that harden and thicken the skin.
Lymphatic filariasis has infected around 120 million people worldwide, and 44 million people have common chronic manifestation (Bernhard *et al*. 2003) [5]. To control disease bearing mosquitos, insecticide and pesticides are predominantly used. However prolonged chemical control of vectors is inherently associated with the resistance to insecticides (Lima *et al*. 2003) [20]. Different mosquito vectors have been reported to be resistant against organophosphorus compounds like malathion. Besides this, residual contamination left in human food and water as well as environment leads to adverse effects in living organisms (Bisset *et al*. 1997) [7]. These circumstances have created the need for the development of safe, biodegradable and target specific agents for pest control. Plant oils present an alternative source for mosquito control as they are environmentally safe, less hazardous to non-target biota, simple, inexpensive and can be applied effectively. Plants have potent biochemicals and are components of phytomedicine, more than 2000 plant species are known to produce chemical factors effective in pest control (Ghosh *et al*. 2012) [14]. *Ocimum sanctum* L. (Tulsi), the whole

herb in its natural form shows nutritional and pharmacological properties and has been used for thousands of years in traditional Ayurveda (Pattanayak *et al*. 2010) [23]. Different oils of *Ocimum sanctum* have been used for the treatment of diseases like malarial fever, ringworms, and other cutaneous afflictions (Butani 1982) [9]. It is also used as a mosquito repellent and has toxic properties (Batta and Santhakumari 1972) [3]. *Ocimum sanctum* essential oil have also showed larvicidal activity against *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles stephensi* (Pathak *et al.* 2000) [22].

The present study elucidates the effect of *Ocimum sanctum* oil as a larvicide in the control of *Culex quinquefsciatus*. To understand the viability of the oil, an *in-silico* study was performed. The phytochemicals present in *Ocimum sanctum* leaves were taken as ligand molecules and their effect against specific target receptors were tabulated. Two enzymes of the target vector were selected. Glutathione S Transferase is an enzyme that catalyses the attachment of exogenous xenobiotic substances to glutathione for the purpose of detoxification (Enayati *et al*. 2005) [13]. Thus, inhibition of the enzyme will affect he natural ability of the insect to detoxify exogenous compounds such as larvicides. Thymidylate synthase on the other hand is the only enzyme in folate metabolism by which the 5,10-methylenetetrahydrofolate is oxidised in presence of deoxyuridine monophosphate (dUMP) to produce deoxythymidine monophosphate (dTMP) as by product (Costi *et al.* 2002) [12]. It plays a crucial role in production of DNA precursors and inhibition of the enzyme may result in DNA damage. Both the enzymes play crucial roles in the normal biological functions of the insects' body and their targeted inhibition will deter these functions. The interaction of the phytochemicals of *Ocimum sanctum* with these enzymes thus indicates their effectiveness as control agents for the target insect.

After the initial *in-silico* analysis *in vivo* toxicity tests are performed. The results of both *in-silico* and *in vivo* tests are then corroborated to elucidate the effectiveness of *Ocimum sanctum* oil as a potential larvicide against *Culex quinquefsciatus.*

2. Materials and Methods

2.1 *In-silico* analysis

2.1.1 Receptor and ligand preparation

The receptors are selected based on their biological activity in the body of the organism. Two receptors Glutathione S Transferase (1R5A) and Thymidylate synthase (1F4B) were selected for the study (Fig 1). The molecular structures of both the receptors are downloaded from the RCSB PDB Database (https://www.rcsb.org/) (Berman *et al*. 2000) [4]. The list of ligands was compiled through literature review (Chaudhary *et al* 2020; Singh and Chaudhuri 2018) [10, 26]. Accordingly, six ligands were selected, Apigenin, Carvacrol, Cirsimaritin, Eugenol, Linalool and Ocimarin (Fig 2). The molecular structures of the selected ligands are downloaded from ZINC database (http://zinc.docking.org) (Irwin and Shoichet 2005) [17].

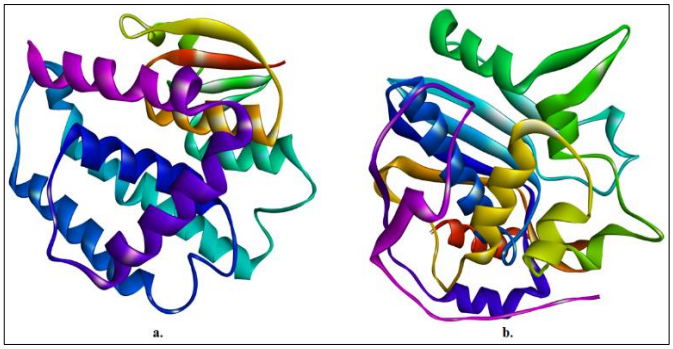

Fig 1: Structure of receptors, a. Glutathione S Transferase (1R5A) and b. Thymidylate Synthase (1F4B)

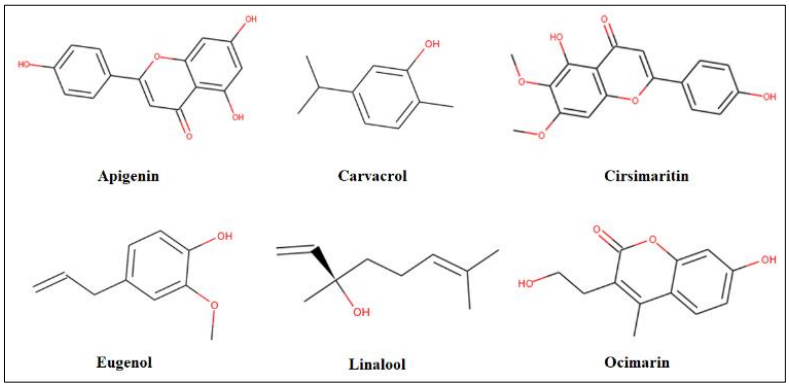

Fig 2: Structures of the six selected ligands

2.1.2 Molecular Docking

Docking stimulations are performed using a computer software Molegro Virtual Docker (MVD 2010.4.0) for Windows. The software uses integrated scoring functions to predict the different probable binding orientations of the ligands with the receptor molecules ad provides 3-dimensional image of the predominant receptor ligand interaction (Bitencourt and Azevedo 2019) [18]. The receptor and ligand molecules are uploaded on the software interface and the stimulations are run. The docking results thus obtained are tabulated for further analysis.

2.1.3 Visualization

The receptor ligand binding orientations obtained from the docking stimulation are visualized using a software Discovery Studio Visualizer 2021 Client. The software provides both 3-dimentional and 2-dimentional image outputs which can be used to visualize the interaction of ligand with the active locations in the target receptor.

2.2 Toxicity tests (Wet lab analysis)

Fresh leaves of *Ocimum sanctum* were collected and washed properly. Leaves were then crushed and placed in Clevenger

apparatus at 60 ºC which was also half filled with water. After 4 hours of continuous distillation, oiled oil was collected in a vial and tightly corked (Clevenger 1928) [11]. A culture of *Culex quinquefasciatus* larvae was maintained in clear polystyrene cups containing 30 larvae each at room temperature. Around 14 culture sets were maintained and the larvae were allowed to grow up to 3rd instar (Kauffman *et al* 2017) [19]. The bioassay was carried out in accordance with the WHO protocol (World Health Organization 1996 and 1981) [29, 30], with minor changes as formulated by Pavela (Pavela 2015) [24]. For the bioassay initially 10 and 1000ppm concentration of *Ocimum sanctum* oil was prepared using DMSO as an emulsifying agent, further based on the result of the two concentration a series of four different concentration were prepared. The variants evaluated were as follows: Positive control- larvae in distil water, Negative control- larvae in dimethyl sulfoxide (DMSO), Treated-essential oil diluted in dimethyl sulfoxide (DMSO) at concentrations of 10, 45, 100, 1000 ppm, (distilled water mixed with the same amount of DMSO as that of essential oil variants) each treated set had three replicates and larval mortality was recorded after 24 h. It has been observed that essential oils bring about a number of biochemical changes in the insect body. So, protein estimation was performed by Lowry's' method (Lowry 1951) to determine the biochemical changes in the larvae due to the inclusion of oil in the body of insects. The protein concentration can be determined accurately by measuring the absorbance using the Beer-Lambert Law, this method do not require any incubation period so measurements can be performed rapidly and with high reproducibility, the protein solution is also used directly without any modification or inactivation (Grimsley and Pace 2003) [16]. UV absorbance spectroscopy is one of the oldest methods for determining protein concentration (Warburg and Christian 1942) [28].

3. Results and Discussion

Essential oils are known to have negative impact on mosquito larvae. They effect the growth and development of larvae and also cause death in appropriate concentrations. These properties are established through a number of studies such as the one by Thomas *et al*. (Thomas *et al.* 2004) [27]. *Ocimum sanctum* is an herb commonly used in traditional medicine for treatment of a number of health disorders. The active phytochemical constituents of the plant possess a number of properties that make it effective as a medicine. The plant is traditionally known to have insecticidal properties. The present investigation was performed to study the efficacy of *Ocimum sanctum* oil against larvae of *Culex quinquefsciatus.* The study was completed by using both *in-silico* methods and direct toxicity tests. The preliminary *in-silico* analysis was performed by conducting computerized stimulations of receptor ligand interactions in a docking software. The receptors, Glutathione S Transferase and Thymidylate synthase were selected based on their function in the body of the target organism. The ligand molecules are active plant phytochemicals present in oils of *Ocimum sanctum.* The result of the docking stimulations obtained from the docking software are indicted by docking scores as shown in graphs (Fig 3). All the selected phytochemicals show negative docking scores which indicate that the ligand molecules used for the study can effectively bind to the active site of the target receptors and as a result can effectively inhibit the natural functions of the receptor molecules. Among the tested ligands, the best receptor-ligand interactions are shown by Cirsimaritin. The ligand showed the best results with both the selected target receptors. The interactions displayed as 3D and 2D images shows the interaction of ligand with the active sites of the target receptors and their interaction with specific amino acids of the target receptor respectively (Fig 4 and Fig 5).

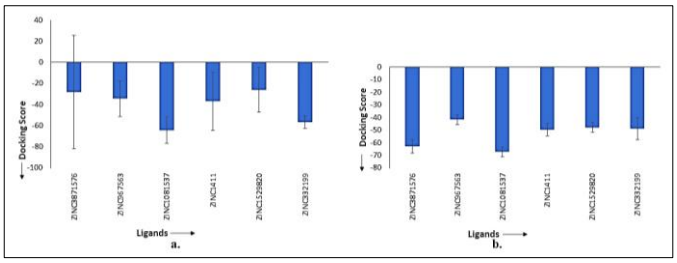

Fig 3: Graphs indicating molecular docking scores of selected ligands with the target receptors, a. Glutathione S Transferase (1R5A) and b. Thymidylate Synthase (1F4B).

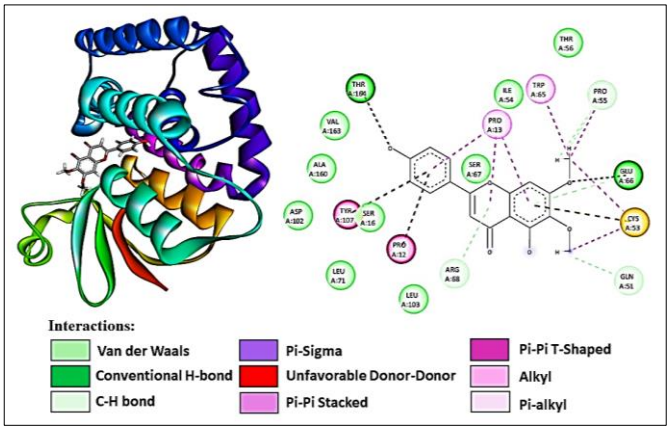

Fig 4: Interaction of Cirsimaritin with Glutathione S Transferase, a. 3D interaction and b. 2D interaction

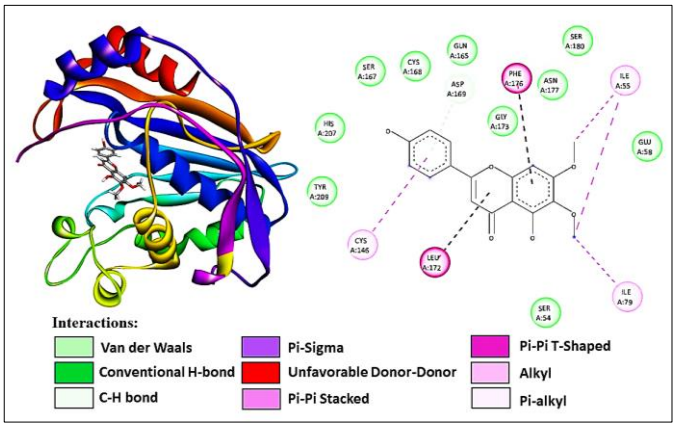

Fig 5: Interaction of Cirsimaritin with Thymidylate Synthase, a. 3D interaction and b. 2D interaction

The *in-silico* analysis showed the potential of active phytochemicals of *Ocimum sanctum* against two enzymes of mosquito larvae. The enzymes were selected as such that their inactivity would cause major biochemical disturbances in the body of the insect. The *in-silico* analysis shows that all the selected phytochemicals of *Ocimum sanctum* shows inhibitory effect on the enzymes of the target pest. Similar *in-silico*

studies by Andrade-Ochoa *et al.* (Andrade-Ochoa *et al* 2018) [1] used terpenes, terpenoids and related compounds as potential larvicides of *Culex quinquefasciatus* and obtained promising results**.** *In-silico* studies using phytochemicals of different plant-based compounds has also shown potential effect against Culex species. To corroborate with the *in-silico* analysis, direct toxicity tests were conducted using oil of *Ocimum sanctum.*

Direct toxicity tests using crude oil of *Ocimum sanctum* was performed to analyse its effect on larvae of *Culex quinquefaciarus*. On treatment with different concentrations of the prepared oil, dose dependent variations are observed after 24 hours of exposure (Table 1 and Fig 6). 100% mortality is observed on treatment with 1000 ppm and least number of deaths were recorded on treatment with 10ppm of the solution, with a LC50 value of 52.34 ppm. The effect of the oil is also evident on the total protein content of the larvae (Table 2 and Fig 7). These effects also show dose dependant changes and the highest effect was observed on treatment with 1000ppm of solution.

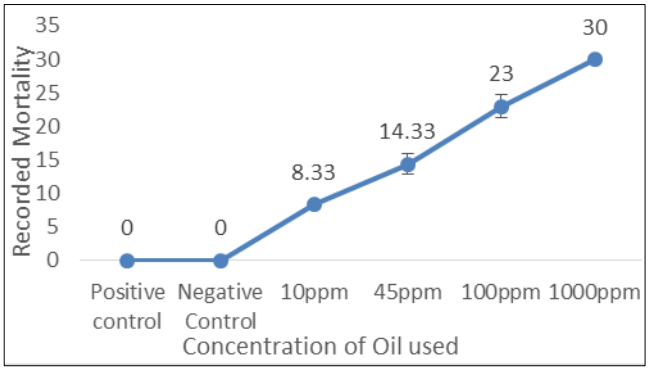

Fig 6: Graph showing mortality of *Culex quinquefsciatus* larvae when treated with *Ocimum sanctum* oil.

Table 1: Table showing weight and mortality of larva when treated with different concentrations of *Ocimum sanctum* oil N=30

Concentrations	Weight in gram	Death larvae
Positive control	0.054±0.001	0±0
Negative Control	0.056±0.001	0±0
10ppm	0.050±0.001	8.33±0.57
45ppm	0.049±0.001	14.33±1.52
100ppm	0.046±0.001	23±1.73
1000ppm	0.040±0.001	30±0

Table 2: Change in protein concentration of *Culex quinquefsciatus* larvae after treatment with *Ocimum sanctum* oil.

Concentration (ppm)	Absorbance	Concentration of protein (mg/g)
Positive control	0.054±0.001	7.50
Negative control	0.05±0.001	7.21
10	0.05±0.002	7.20
45	0.031±0.001	4.89
100	0.031±0.002	4.78
1000	0.023±0.001	4.50

The results obtained from the laboratory study also showed that the oil is effective as a larvicide. The treatment showed dose dependent changes and the highest mortality was achieved by using a solution of 1000ppm. This indicates that *Ocimum sanctum* oil is effective as a potential larvicide against *Culex quinquefsciatus*. The essential oil also effects the total protein content of the insect in a dose dependent manner. The results obtained in the study are similar to the results obtained by Anees (Anees 2008) [2] who worked on the larvicidal efficacy of *Ocimum sanctum* extracts and found that the highest efficacy is shown by acetone extracts. *Ocimum sanctum* extract is also found to be effective against *Culex quinquefsciatus* in studies of Ramar *et al.* (Ramar *et al*. 2017) [25] and Ghosh *et al.* (Ghosh *et al*. 2016) [15] amongst others. Thus, from the results obtained from the *in-silico* analysis and the laboratory tests, it can be ascertained that *Ocimum sanctum* oil would be very effective as potential larvicide in natural control of *Culex quinquefsciatus.*

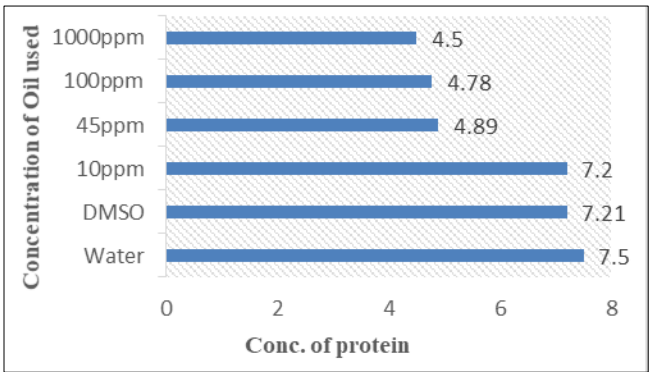

Fig 7: Bar diagram indicating change in protein concentration of *Culex quinquefsciatus* larvae after treatment with *Ocimum sanctum* oil.

4. Conclusion

The present study was conducted with an aim to analyze the efficacy of *Ocimum sanctum* oil in the natural control of *Culex quinquefsciatus* larvae. The mosquito as a vector of tropical diseases pose serious threat to human health so it was taken as a target for the study. The experiment was conducted by both *in-silico* and *in vivo* methods to obtain precise results. After careful experimentation it was clearly observed that *Ocimum sanctum* oil was effective in controlling mosquito larvae and will be very effective as a potential larvicide for control of *Culex quinquefsciatus.*

5. Acknowledgement

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6. Conflict of Interest: None Declared.

7. Author Contributions

N.I. and L.B.: Data collection and writing the original draft, B.K.: Planning the experimental procedure. review and editing, R.A. and S.S: Review and editing.

8. Data Availability Statement

The data used for the article are incorporated within the article. Any additional data will be available from the authors on reasonable request.

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