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Larvicidal potential of certain plant extracts of the family Lamiaceae against *Anopheles subpictus* (Diptera: Culicidae)

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Abstract
The present study was undertaken to check and compare the virtue of plant extracts in relation to larvicidal activities against *Anopheles subpictus*. The lethality and toxicity of methanolic and aqueous extracts of *Ocimum sanctum*, *Ocimum basilicum*, *Leucas aspera* and *Coleus amboinicus* to early 4th instar larvae of *A. subpictus* were determined in the laboratory. All plant extracts of Lamiaceae showed moderate effect against the larvae of *A. subpictus* after 24 hours exposure as evidenced by low lethal concentration and lethal time and the lethality varied in larvae and the crude plant extracts. The mortality rate was recorded after 24 hours of exposure and LC_{50} was determined. Larval mortality between 80% and 92% was observed in methanolic extract and 60-85% in aqueous extracts of selected plant species. The highest larvicidal activity against *A. subpictus* was obtained with methanol extract of *O. sanctum*. The present findings have paramount implications in the practical control of mosquito larvae and adults in the aquatic ecosystem as the medicinal plants studied are routinely accessible in enormous quantities. These plant extracts are easy to prepare, inexpensive, and safe for mosquito control which might be used directly as larvicidal agents in small dimension aquatic habitats or breeding sites around human dwellings.

Keywords: *A. subpictus*, larvicidal, plant extract, lethal, mosquito control, lamiaceae

1. Introduction
The natural history of malaria involves cyclical infection of humans and female Anopheles mosquitoes. It is the only known carrier of malaria, also transmits filariasis, encephalitis and arbovirus infections. As vectors, Anopheles mosquitoes have affected the lives of more human than any other insect and engender a great impact on human genome evolution [1]. Vector control targeting the larval stages of mosquitoes was applied successfully against many species of Anopheles (Diptera: Culicidae) in several countries until the mid-20th Century [2].
The regulator of larval or pupal periods plays a major role in mosquito population management which includes environmental control, biotic control and insecticidal control [3]. However spraying of insecticides containing organophosphates, pyrethrum and bacterium byproducts can result in insecticide resistance, environmental pollution and pose a serious threat to ecosystem [4]. The concerns about insecticide resistance and environmental impacts have stimulated renewed interest in larval control involving temporary or permanent removal of anopheline larval habitats, as well as larviciding with biological agents or plant based products which are ecofriendly nature.
Solicitation of a mosquito fish, *Gambusia affinis* against this vector, has shown capable efficiency in decreasing the mosquito population and malaria cases in India [5]. Biological compounds tend to be more expensive than chemical controls but they affect fewer non-target organisms [6]. In recent years, plant derived products has been revived as they contain a rich source of bioactive phytochemicals that are safe and biodegradable into non-toxic by-products, which could be screened for insecticidal activities. Many plant extracts have been tested against various species of mosquitoes, focusing on larvicidal action [7]. For instance, leaf extracts of *Eclipta prostrata* and *Andrographis paniculata* were reported to be effective against fourth instar larvae of *A. subpictus* [8].

Essential oil from *Ocimum basilicum* (L.) was evaluated for larvicidal activity against *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus* [9]. Extract of *Ligusticum sinense*, essential oils of citronella, pine, *Dalbergia sissoo*, peppermint and *Rhizophora mucronata* are reported to exert high activity against different species of *Anopheles* [10]. In this context, a comparative study was undertaken to evaluate the larvicidal activity of *Ocimum sanctum* L., *Ocimum basilicum* L., *Leucas aspera* (Willd.) Link and *Coleus amboinicus* Lour of Lamiaceae family against *Anopheles subpictus.*

2. Materials and Methods

2.1 Biological material

Leaves of *Ocimum sactum*, *Ocimum basilicum*, *Leucas aspera* and *Coleus amboinicus* were collected in the month of February, 2021 at Thiruverumbur town of Tiruchirappalli city (Latitude 10.784770 N, Longitude 78.784050 E). The collected plants species were identified and authenticated at St. Joseph's College, Tiruchirappalli (Fig. 1). Leaves were shade dried at room temperature for 7 days and ground to fine powder using a mechanical blender. *Anopheles subpictus* eggs were collected from the sewage water at St. Joseph's College, Tiruchirappalli. The eggs were placed in distilled water to hatch. The emerging larvae were reared and tested at 28±2 °C temperature, ≥45±10% relative humidity, and a 12:12 (light: dark) photoperiod and were fed tropical fish flakes.

Fig 1: Image of **A.** *Ocimum sanctum* L. **B.** *Ocimum basilicum* L. **C.** *Leucas aspera* (Willd) link **D.** *Coleus amboinicus* Lour.

2.2 Preparation of plant extracts

For methanolic extract, 250 g of finely powered leaves were extracted with methanol in a Soxhlet apparatus (boiling point range 50–80 ºC) for 8 hours. For aqueous extracts, 250 g of leaf powder was extracted with water in a boiling water bath (60 ºC) for 4 hours and filtered through muslin cloth. Both the extracts were concentrated under reduced pressure (22–26 mm Hg) at 45 ºC. The obtained residue was stored at room temperature.

2.3 Phytochemical screening

The phytochemical screening test was performed as described by Trease and Evans [11] and Sofowora [12].

2.4 Fourier-transform infrared spectroscopy (FTIR) Analysis

FTIR identifies the presence of organic and inorganic compounds in the sample. FTIR spectrum of aqueous and methanolic extracts of *O. sanctum, O. basilicum, L. aspera, C. amboinicus* was recorded in the range of 4000–400 cm−1 using FTIR spectrophotometer (Spectrum RX I, PERKIN ELMER).

2.5 Larvicidal bioassay

The larvicidal activity of the plant extracts was evaluated according to the World Health Organization guidelines for laboratory [13]. The biological activity of the extracts of collected plant leaves used in the laboratory was found in late L3 and/ or early L4 larval stages of populations of *Anopheles subpictus* to obtain the lethal concentrations (LC_{50}) value. The methanolic and aqueous crude extracts were evaluated at the concentrations of 200, 400, 600, 800 and 1000mg/L. Batches of 20 healthy third/fourth instars of larvae were transferred to petri plates containing 100 ml of distilled water. Then various concentrations of crude extracts were added and the experiments were replicated thrice along with control (water served as control). All the plates were left undisturbed for 24 hours including control. Effect of sub lethal (lethal concentrations of cumulative effect) plant extracts were established through LC_{50} value determined with the test larvicide after 24 hours of exposure to plant extracts. Daily observation was made with verification of larval stages, behavior changes, possible mortality of the larvae and the temperature. The experiment was conducted until the last pupa or adult died or until the last adult completely emerged.

The number of lifeless larvae (no appendage movement or leaping reaction in water) was counted after 24 hours of revelation and the percentage mortality was calculated from the average of three replicates. The mortality rate was calculated by this formula: Percentage of mortality=Total no. of dead larvae/Total no. of larvae introduced x 100 using the scale range

- Mortality in variation of 98 – 100% indicates defenselessness.
- Mortality in variation of 80 – 97% is indicative of the survival of resistance and further study is needed.
- Mortality fewer than 80% specifies resistance.

2.6 Statistics analysis

Larval mortality counts were in tune for the mortality in control, if any, by using Abbott's formula [14] to give an estimation of the plant extracts reasonable mortality. The rectified mortality data were subjected to regression analysis of % table of Probit mortality on log dosage [15]. The significant variance in LC_{50} is established on the non-overlapping of 95 % Fiducial limits and P-values < 0.05 were

considered to be statistically important [15].

3. Results and Discussion

The aim of this study was to establish the larvicidal activity of *Ocimum sanctum*, *Ocimum basilicum*, *Leucas aspera* and *Coleus amboinicus* against *Anopheles* and identify the compounds responsible for the observed activity. This work adds to the current efforts worldwide to discover new mosquito control agents. Plant bioactive chemicals are generally considered as nontoxic, easily available at affordable prices, biodegradable and show broad spectrum target-specific activities against different species of vector mosquitoes [16].

3.1 Phytochemical analysis

Phytochemical screening of selected plants showed the presence of alkaloids, flavanoids, terpenoids, saponins and phenolics (Table 1). Among the selected plants, methanolic extract of *Ocimum sanctum* showed higher amount of terpenoids while the aqueous extract showed saponins (Table 2). Tanins and phenolic compounds were absent in *Leucas aspera* of both methanol and aqueous extracts.

Table 1: Phytochemical analysis of Aqueous extracts of *O. sanctum, O. basilicum, L. aspera, C. amboinicus.*

S. No	Secondary metabolites	*O. sanctum*	*O. basilicum*	*L. aspera*	*C. amboinicus*
1.	Alkaloids	+	+	++	-
2.	Flavonoids	+	++	+	+
3.	Tannins	++	+	-	+
4.	Saponins	+++	++	+	++
5.	Terpenoids	+	++	+	-
6.	Phenols	+	+	-	+

Table 2: Phytochemical analysis of methanolic extracts of *O. sanctum, O. basilicum, L. aspera, C. amboinicus.*

S. No	Secondary metabolites	*O. sanctum*	*O. basilicum*	*L. aspera*	*C. amboinicus*
1.	Alkaloids	+	++	++	+
2.	Flavonoids	+	++	+	++
3.	Tannins	++	+	-	+
4.	Saponins	-	++	+	+
5.	Terpenoids	+++	+	+	+
6.	Phenols	++	+	-	+

3.2 FTIR analysis

Depending on the infrared absorption frequency range 600–4000 cm−1, the specific molecular groups prevailing in the sample were determined through spectrum data obtained for aqueous and methanolic extracts of selected plants. In general, crude methanolic extracts showed more number of asymmetric bands than aqueous extracts of selected plants of Lamiaceae. All the samples of both the extracts showed characteristic absorption peaks in the range of 640 cm–1 to 690 cm–1 which accounts for the presence of aromatic compounds and peaks in the range of 3390 cm–1 to 3452 cm–1 indicates the presence of amines (Fig. 2). Essential oils containing biological active constituents are known to possess insecticidal and nematicidal activities [17, 18]. Eugenol has been reported to be largely seen in *Ocimum* and responsible for the therapeutic potential [19].

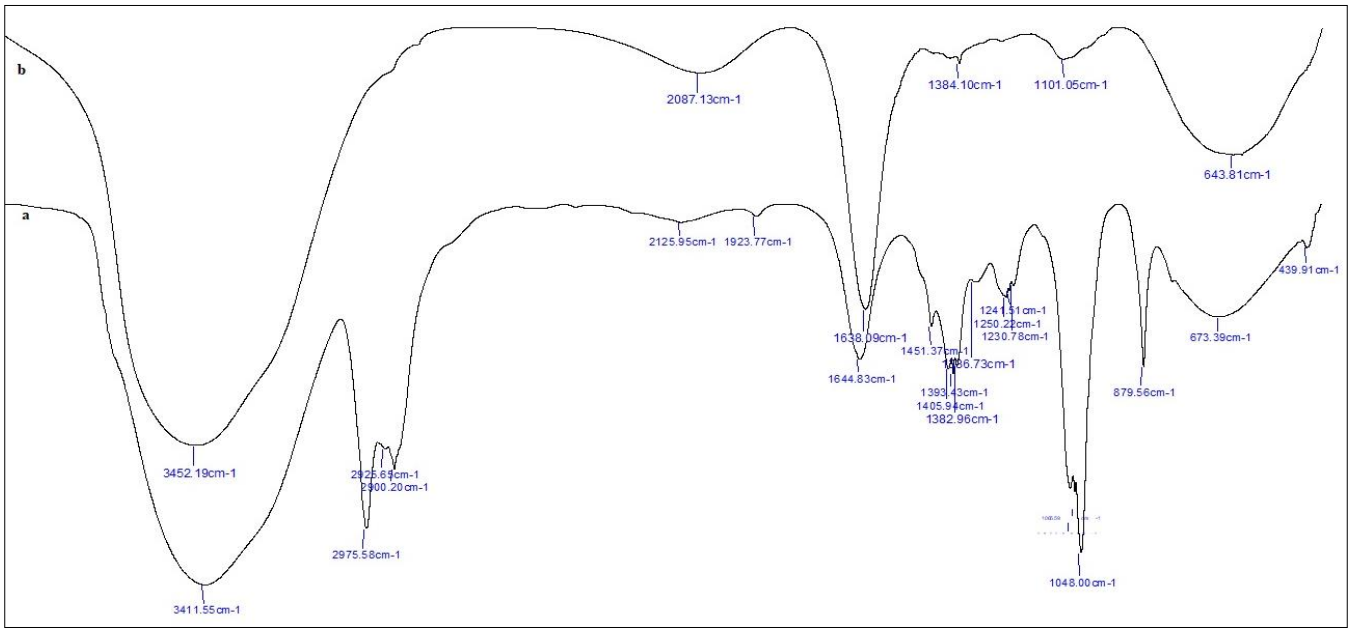

Fig 2: FTIR spectra of methanolic (a) and aqueous (b) extracts of *Ocimum sanctum*

Bands around 1638 cm–1 and 1384 in aqueous extracts of selected plants are believed to be arising from the N–O asymmetric and symmetric stretching bands, respectively, of the aromatic NO_2 group. Aqueous extracts of selected plants showed a characteristic peak at 1634 cm–1 attributing to amides which are not observed in methanolic extracts (Fig. 3).

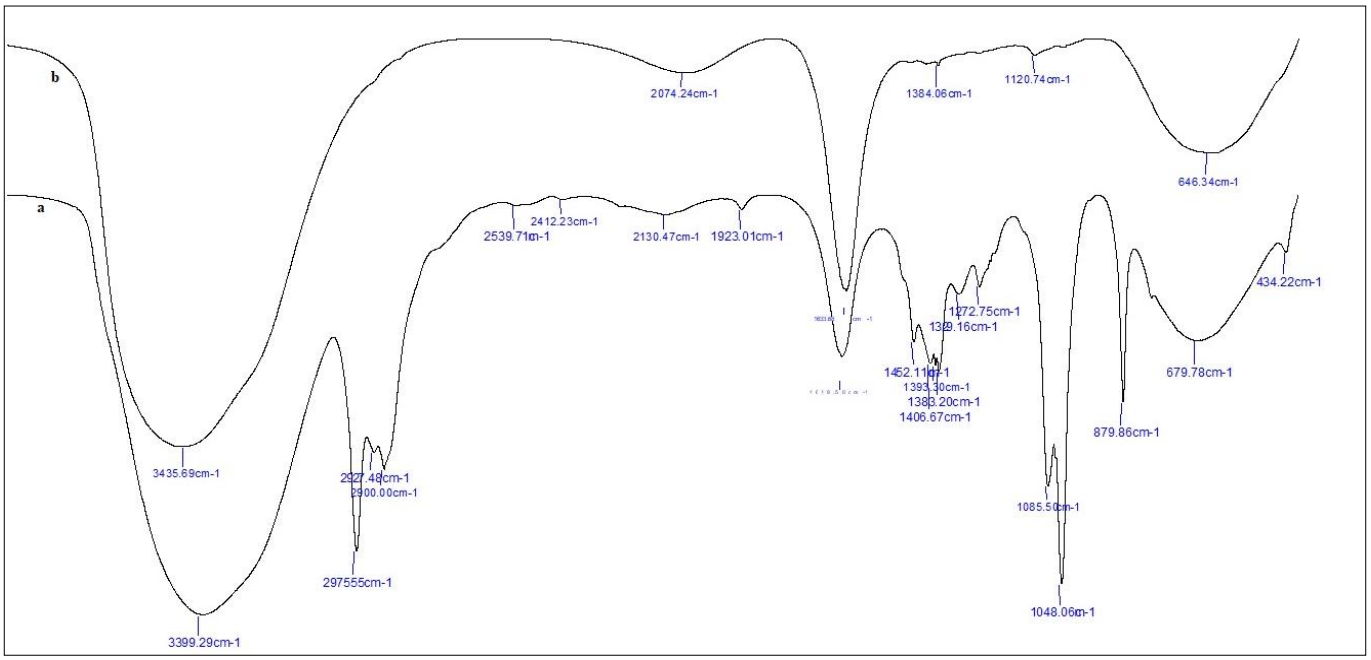

Fig 3: FTIR spectra of methanolic (a) and aqueous (b) extracts of *Ocimum basilicum*

Methanolic extracts of selected plants showed absorption band at 2975 cm–1 region due to asymmetric C–H stretching of CH3 of alkane group and at around 2927 cm–1 region due to asymmetric C–H stretching of CH2 of alkane group. The band at 2898 cm–1 is assigned due to the symmetric C–H stretching in CH2 of the alkane group (Fig. 4).

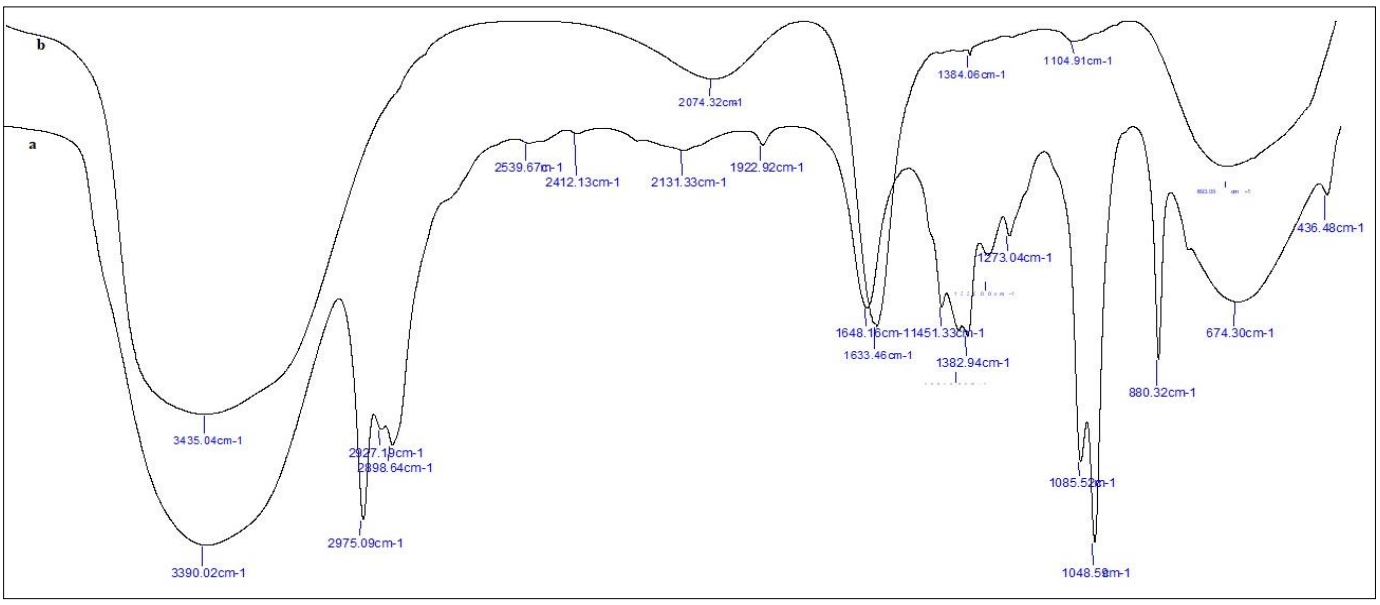

Fig 4: FTIR spectra of methanolic (a) and aqueous (b) extracts of *Leucas aspera*

Methanolic extract of *Ocimum sanctum* have a very weak and extended band at 2125 cm–1 C of alkynes. The relatively stronger band around 1100 cm–1 and 1120 cm–1 is assigned as C–O stretching vibration of primary and secondary alcohols. A notable absorption peak at 878 cm–1 seen in methanoilc extract of *C. amboinicus* is probably due to the bending vibrational mode of O–N–O of the NO_2 group confirming the presence of nitro group (Fig. 5). FTIR and EDS spectra of *Eclipta alba* and *Eclipta prostrate* described the presence of characteristic functional groups of carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, nitrates, chlorates, and carbohydrate which are responsible for its biological properties [7].

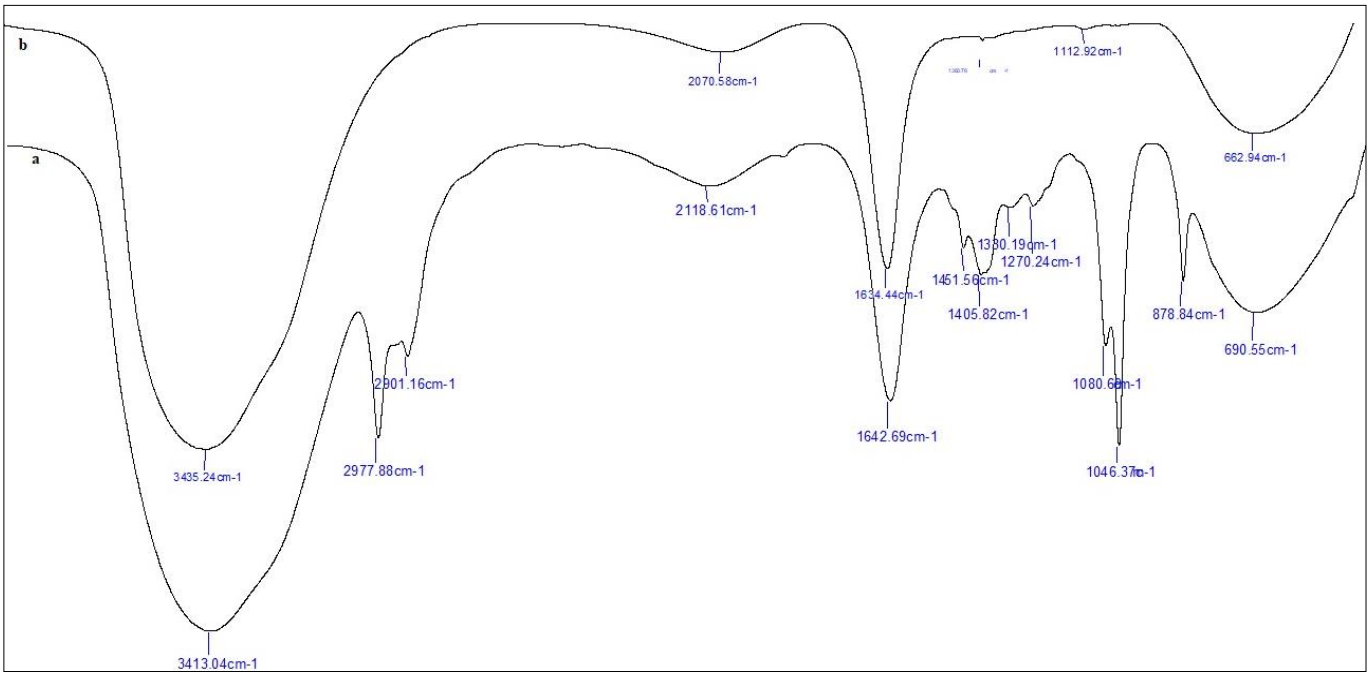

Fig 5: FTIR spectra of methanolic (a) and aqueous (b) extracts of *Coleus amboinicus*

3.3 Larvicidal activity

With respective to the larvicidal activity of the crude plant extracts analyzed, it was observed that after 24 and 48h of exposure, both aqueous and methanolic extracts demonstrated relevant results against L_3 *A. subpictus* larvae. Among the selected plants, aqueous extract of *Ocimum basilicum* (LC_{50}-0. 603 µg/ml) showed highest lethality at low concentration followed by *Leucas aspera* (LC_{50} 0.661 µg/ml), *Coleus amboinicus* (LC_{50} 724.43 µg/ml) and *Ocimum sanctum* (LC_{50} 891.25 µg/ml) (Table 3).

Table 3: Lethal concentration of aqueous extracts of *O. sanctum, O. basilicum, L. aspera* and *C. amboinicus* against the larvae in exposure of 24 hours

Species	LC_{50}	Regression equation	R^2	P value (P<0.05)
Ocimum sanctum	891.25	y=2.74x-3.10	0.93	0.007
Ocimumbasilicum	602.56	y=2.83x-2.87	0.89	0.016
Leucasaspera	660.69	y=3.12x-3.81	0.95	0.044
Coleus amboinicus	724.43	y=2.90x-3.32	0.95	0.005

Similarly methanolic extract of *Ocimum basilicum* was proven to be the most promising control of mosquito larvae which was confirmed by the LC_{50} 346.73 µg/ml. Comparably methanolic extracts of selected plants showed lethality at low concentrations than aqueous extracts (*Ocimum sanctum* – LC_{50} 524.81 µg/ml, *Leucas aspera* – LC_{50} 575.44 µg/ml, *Coleus amboinicus* – LC_{50} 575.43 µg/ml) values obtained after 24h of exposure (Table 4). The toxic effect of *Ocimum Sanctum* extract against the early fourth instar larvae of *Aedes aegypti* was least and this is in par with the work done by Senthilnathan *et al*. [20] where combination of all plant extract were tested against fourth instar larvae of *culex quinquefasciatus***.** Sakthivadivel and Daniel [21] observed that petroleum ether extract of *Leucas aspera* with LC_{50} value between 100 to 200 ppm against the larvae of *C. quinquefasciatus*, *A. stephensi* and *A. aegypti*. However use of plant extracts for developing mosquito larvicides and their mode of action was clearly explained by Pavela *et al*. [22] and Piplani *et al*. [23]

Table 4: Lethal concentration of methanolic extracts of *O. sanctum, O. basilicum, L. aspera* and *C.amboinicus* against the larvae in exposure of 24 hours

Species	LC_{50}	Regression equation	R^2	P value (P<0.05)
Ocimum sanctum	524.81	y=2.64x-2.17	0.96	0.003
Ocimumbasilicum	346.73	y=2.58x-1.56	0.95	0.004
Leucasaspera	575.44	y=2.54x-2.01	0.85	0.025
Coleus amboinicus	575.43	y=2.30x-1.35	0.90	0.0013

Aqueous extracts of selected plants showed varying levels of mortality (Table 5) whereas no dead larva was observed in control even after 24 hours of exposure. Among the species tested, *Ocimum basilicum* recorded highest mortality rate (85%) following *Leucas aspera* (80%), *Coleus amboinicus* (72%) and *Ocimum sanctum* (60%) respectively (Fig. 6). Larvicidal activity of *Ocimum sanctum* L. was reported very earlier by Keirn and Nair [24]. Essential oil of *Ocimum sanctum* L. revealed larvicidal efficacy against larvae of *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* [25].

Table 5: Mean Mortality Rate of aqueous extracts of selected plants against *A. subpictus* after 24 hours exposure

Conc (µg/ml)	Aqueous extract of *O. sanctum*			Aqueous extract of *O. basilicum*			Aqueous extract of *L. aspera*			Aqueous extract of *C. amboinicus*		
	% Dead larvae ± SE	Probit mortality	% Alive larvae	% Dead larvae ± SE	Probit mortality	% Alive larvae	% Dead larvae ± SE	Probit mortality	% Alive larvae	% Dead larvae ± SE	Probit mortality	% Alive larvae
0.2	5±0.04	3.36	95	12±0.04	3.82	88	7±0.02	3.52	93	7±0.02	3.52	93
0.4	13±0.05	3.87	87	28±0.10	4.42	72	20±0.07	4.16	80	17±0.06	4.05	83
0.6	22±0.08	4.23	78	40±0.14	4.75	60	42±0.15	4.80	58	32±0.11	4.53	68
0.8	50±0.18	5	50	56±0.20	5.15	44	53±0.19	5.08	47	55±0.21	5.13	45
1.0	60±0.22	5.25	40	85±0.31	6.04	15	80±0.29	5.84	20	72±0.26	5.58	28

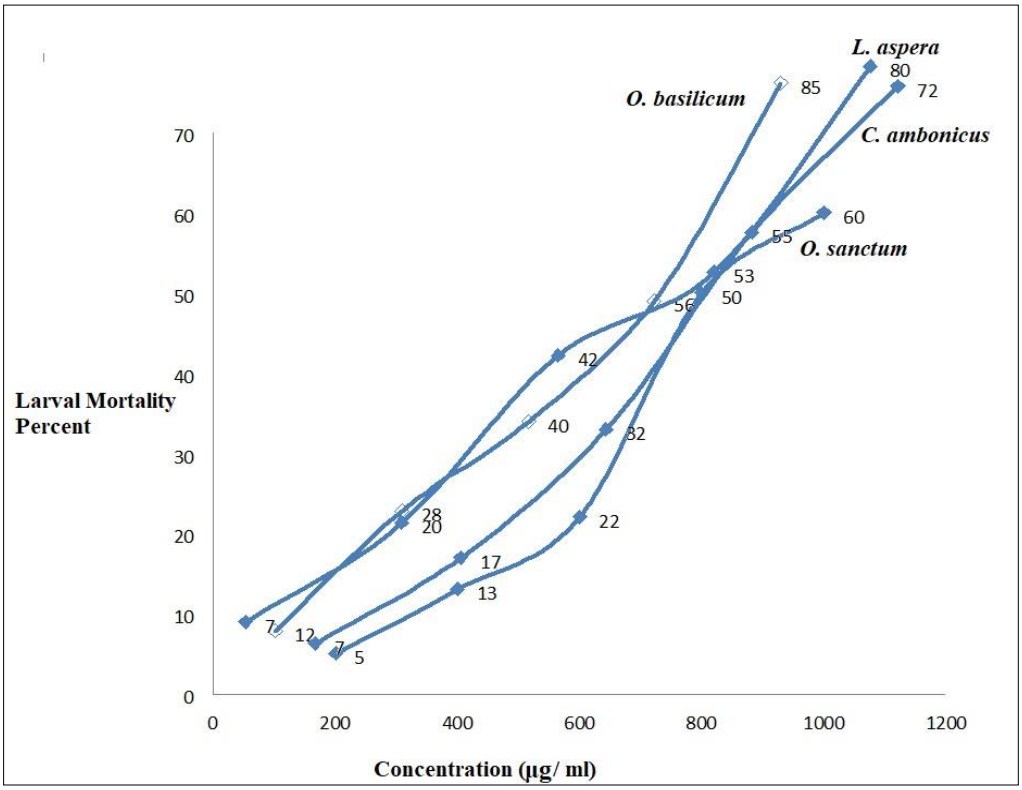

Fig 6: Mortality rate of aqueous extracts of *O. sanctum, O. basilicum, L. aspera* and *C. amboinicus* after 24 hours exposure

As well methanolic extracts showed varying mortality rates at different concentrations of the extracts tested (Table 6). No dead larva was observed in control even after 24 hours of exposure. After 24 hours of exposure, mortality rates tend to increase in response to increasing concentrations. Among the selected plants, *Ocimum basilicum* recorded highest mortality rate (92%) following *Leucas aspera* (83%), *Ocimum sanctum* (82%) and *Coleus amboinicus* (80%) respectively (Fig. 7). It is obvious that methanolic extract of *L. aspera* and *O. sanctum* exhibited approximately same mortality rates against *A. subpictus*. In general mortality rate of methanolic extract was higher when compared to the mortality rates exhibited by aqueous extracts. However, there was significant difference ($P < 0.05$) in the mortality rate of *Anopheles* species larvae between extracts of all for each concentration degree of freedom-1 for each extracts. Larvidal activity of *O. basilicum*, *Thymus vulgaris*, *Cymbopogan citatus*, *Mentha arvensis* and *P. graveolens* essential oils were reported against the late third instar of *C. quinquefasciatus*. The LC_{50} values of *O. basilicum*, *T. vulgaris*, *C. citatus*, *M. arvensis* and *P. graveolens* were 29.98, 30.31, 165.70, 178.04 and 226.52 ppm respectively [26]. Recently Dris *et al.* [27] tested *O. basilicum* leaf extracts against fourth instar *C. pipiens* L. larvae and informed LC_{50} value of 73.45 ppm. Additionally larvicidal activity of medicinal plant extracts (*Annona squamosa*, *Chrysanthemum indicum* and *Tridax procumbens*) against fourth instar larvae of *Anopheles subpictus* and *Culex tritaeniorhynchus* was reported by Kamaraj *et al.* [28]

Table 6: Mean Mortality Rate of methanolic extracts of selected plants against *A. subpictus* after 24 hours exposure

Conc (µg/ml)	Methanolic extract of *O. sanctum*			Methanolic extract of *O. basilicum*			Methanolic extract of *L. aspera*			Methanolic extract of *C. amboinicus*		
	% Dead larvae ± SE	Probit mortality	% Alive larvae	% Dead larvae ± SE	Probit mortality	% Alive larvae	% Dead larvae ± SE	Probit mortality	% Alive larvae	% Dead larvae ± SE	Probit mortality	% Alive larvae
0.2	17±0.06	4.05	83	32±0.11	4.53	68	18±0.07	4.08	82	18±0.06	4.08	82
0.4	33±0.12	4.56	67	52±0.19	5.05	48	27±0.09	4.39	73	32±0.11	4.53	68
0.6	52±0.19	5.05	48	68±0.25	5.47	32	38±0.14	4.69	62	43±0.15	4.82	57
0.8	70±0.25	5.52	30	82±0.29	5.92	18	65±0.23	5.39	35	58±0.21	5.20	42
1.0	82±0.30	5.92	18	92±0.33	6.14	8	83±0.30	5.95	17	80±0.29	5.84	20

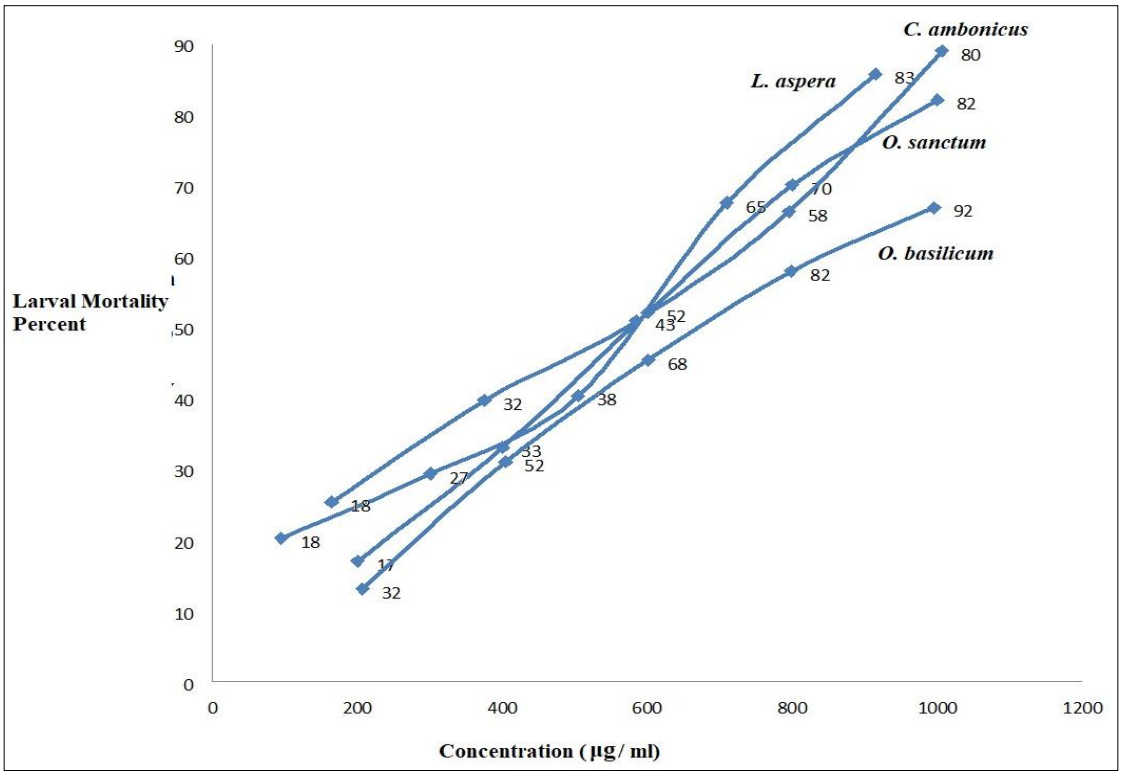

Fig 7: Mortality rate of methanolic extracts of *O. sanctum, O. basilicum, L. aspera* and *C. amboinicus* after 24 hours exposure

In the present study first and second instar larvae were extremely profound when compared with third and fourth instar larvae of both the test species. The present findings support the results of Muthukrishan *et al*. [29] as they observed the LC_{50} values of ethyl acetate extract of *L. aspera* were 75.40, 93.09, 132.20 and 138.60 against the first, second, third and fourth instar larvae of *C. quinquefasciatus*, respectively. Mwangi and Rembold [30] reported that the leaf extract of *L. aspera* exhibited high mortality, especially during the molting process or the successive processes of melanization and tanning. Murugam and Jayabalan [31] observed 90% larval mortality at 4% concentration of leaf extracts of *L. aspera* against fourth instar larvae (*Anopheles stephensi)*.

Carvacrol was also found to be the main essential of *Thymus herbabarona* essential oil and showed to be toxic to the Lepidopteran larvae *Limantria dispar* [32]. The *in vitro* antimalarial action of *C. dactylon* and other therapeutic plant extracts was detected to be important against *Plasmodium falciparum* [33]. In my observation *O.sanctum, O.basilicum, L,aspera, C. amboinicus* showed various phytochemicals and functional groups and the larvicidal activity of *O. basilicum and L. aspera were* exposed highest larvicidal activity against to *Anopheles* species rather than, *O. sanctum* and *C. amboinicus*

These tests indicated that lower concentrations are needed for the plant extracts to obtain a larval mortality of 90% of the population that can be used as larvicides to control mosquito. Over the past three decades plant products have drawn attention as potent insect control agents aiming only larval stages in the mosquito control programme.

4. Conclusion

Results obtained from this study have initiated on-going investigations into the incorporation of these crude plant extracts into the control of mosquito populations, with a view for developing an environmentally acceptable product of value in integrated vector control. The use of a plant extract that reduce mosquito populations at the larval stage can provide many associated benefits to vector control.

5. Acknowledgement

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