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RESEARCH ARTICLE

Toxicological Study of Essential Oils of *Mentha piperita* and *Citrus sinensis* against *Aedes aegypti* Mosquito Species

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Abstract

Background: Insects like mosquitoes cause various kinds of public-health diseases called vector-borne diseases. One of the prevention efforts is by using repellents and to control the population by using larvicidals. **Aim:** The present study aimed to investigate essential oils as larvicidals. **Methods:** EOs of *Mentha piperita, Citrus sinensis* used for the larvicidal study against Aedes aegypti. 500 ml water and 30 larvae of late 3rd or early 4th instar used to observe mortality rate. The control was set up with the same conditions only with dechlorinated water. Serial dilution of essential oils like 100µL, 500 µL, 1000µL, and 2000 µL used. **Results:** Peppermint oil at 100 µL observed with an inhibition of 100 ± 0% at 24 hours. 500 µL observed inhibition of 100 ± 0% at 20 hours. 1000µL observed inhibition of 100 ± 0% at 4 hours. 2000 µL observed inhibition of 92.67 ± 2.79% at 1 hour and 100 ± 0% within 4 hours. Orange oil at 100 µL observed with inhibition of 100 ± 0% at 20 hours. 500 µl observed inhibition of 100 ± 0% at 20 hours. 1000µL observed that the inhibition of 83.33 ± 2.360% at 4 hour and 100 ± 0% at 20 hour. 2000 µL observed inhibition of 76.67 ± 2.36% at 1 hour and 100 ± 0% at 4 hour. **Conclusions:** 100 % inhibition was observed for Peppermint oil at 100 µL at 24 hours, 500 µL at 20 hours, 1000µL at 20 hours and 2000 µL at 4 hours. The % inhibition will increase with the increased concentrations and 500 µL to 1000 µL concentrations show 100 % mortality within the same time duration. While increasing to 2000 µL within 4 hours, 100 % mortality was observed due to high concentrations. Similarly, 100 % inhibition was observed of Orange oil at 100 µL at 20 hours, 500 µL at 20 hours, 1000 µL at 20 hours and 2000 µL at 4 hours. The % inhibition will be increased with increased concentrations. Low concentrations of 100 µL showed a long time duration of up to 20 hours and high concentrations of 2000 µL within 4 hours for complete mortality. Results showed *M. piperita* and *C. sinensis* against *Aedes aegypti* have potential larvicidal. **Major Findings:** Essential oils of *M. piperita* and *C. sinensis* have significant larvicidals against *A. aegypti* mosquito species.

Keywords: *A. aegypti* Mosquito, *M. piperita, C. sinensis*, Toxicological

1. Introduction

Insects like mosquitoes will cause various kinds of public health diseases like vector-borne diseases; Malaria, Dengue, and Chikungunya are some of them. These vector-borne diseases are spread mainly by *Aedes*, and *Culex* genus of mosquitoes. Insects like mosquitoes cause diseases in around 600 million in all countries. Especially the newly born babies, children's are significantly the most affecting group of people[1].

Currently, control of mosquitoes are more challenging task due to some kind of reasons like environmental conditions, resistance towards controlling agents, more populations, and their dependent waste. Various cities of municipalities face big challenges in controlling mosquitoes and their related health issues mainly controlling aspects depend on how to use the larvicidal or mosquitocidal agents to control at larvae breeding stage itself[2]. Some of the vector controlled methods like spraying mosquito repellents, in this technique mosquito repellents that are sprayed

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on clothing or skin are the most frequently used. Some of the effective sprays have high DEET concentrations and are effective for several hours, even when strolling through dense woods with hundreds of mosquitoes. Mosquito coils work with concentrations of 0.3-0.4 % of coil mass, the main active element in mosquito coils are similar *Pyrethrums*. When a mosquito coil is burned, the insecticides in the coil evaporate with the smoke, preventing mosquitoes from entering the room and swarming all around the surroundings. Mosquito repellent liquidizers contain synthetic Pyrethroids and are used in mosquito repellent liquidizers. Some physical insect repellents, including mosquito nets, are believed to be superior to mosquito repellent than coils and other repellents that pose health risks. Sleeping beneath mosquito netting ensures mosquito defence and greater protection without posing any risks. Some mosquito nets are currently medicated with 25% Deltamethrin. Mechanical methods: Electric mosquito zappers, for example, use ultraviolet light to attract mosquitoes and afterward kill them when they come into touch with a lethal dosage of electrical current[3].

Research at present, there is a need to concentrate on natural-based larvicidals and repellents to protect ourselves from vector-based diseases[4]. The herbal-based oils used in the ancient period and reported by the Indian system of medicines like *Ayurvedhum* and *Siddha* traditional systems[5]. The ancient literature knowledge helps to predict the plant-based larvicidal and repellent to use as household aspects and in current research there is a need for scientific validation to ensure the therapeutic benefits and larvicidal properties[6]. Some of the toxicological studies helped the herbal oils as potential larvicidals and repellents[7]. The residual portion of synthetic larvicidal will pollute the environmental conditions like spoiling the water and soil irrigation lands *etc*[8]. Similarly, synthetic repellents will cause respiratory issues while inhaling the synthetic mosquito coils. Some of adverse effects like skin reactions, neurological, and cardiovascular disorders are the most common adverse effects of overexposure of synthetic coils and vaporous[9-11]. Some findings of vapour phase of mosquito repellents leads to carbonyl compounds, mainly formaldehyde and acetaldehyde, which have a potent irritating effect on the upper respiratory tract[12,13].

2. Materials and Methods

2.1 *Mentha piperita* (Peppermint Oil)

Peppermint plants are used for extraction f the compounds for the purposes of larvicidal studies and repellent properties[14]. *M. balsamea*, water mint, and spearmint are some of the other synonyms for the plant materials. The oil derived from the *M. piperita* has many health benefits and healing properties for various illnesses as well as the aroma properties of peppermint oil used for fragrance and food industry[15]. The flavour property utilised mainly various food products and the fragrance behaviors used for the cosmetic industry, especially cosmetic beautifying products. This peppermint oil is used to investigate the larvicidal and repellent properties against mosquito bites and this is an alternative approach compared to synthetic larvicidal and repellent formulation currently used in the pharmaceutical market. This use of synthetic chemical resistant property can minimized by using this herbal-based extracted essential oils[16].

2.2 *Citrus sinensis* (Orange Peel Oil)

The essential oil derived from the peels of orange was used for the larvicidal study design. In addition, already this oil has potential usage in the soap industry[17,18]. The fragrance behavior is mainly the aromatic chemical composition and this aroma is used in perfumes, cosmetics products, *etc*[19,20].

2.3 Authentication of Herbals

The herbs for the study were authenticated by Dr Sunil Research Officer, Department of Pharmacognosy Siddha Central Research Institute, Arumbakkam, Chennai, Tamil Nadu, India. After identification, the plant material was processed for extraction. (Authentication Reference No: 532.24052301-13)[21,22].

2.4 Extraction of *M. piperita* (Peppermint Oil)

Mentha piperita leaves were used for the extraction of essential oils. 100g of raw herb was used for the study. Hexane is used as the oil extraction solvent. Soxhlet was used for the distillation process. The solvent heated to release vapor and the distillation process and further condensation helped the oil separation process. The

components from peppermint leaves are mixed with hexane in a hot condition and the vapor carries the components and condense process releases the desired oil portion. The cycle of a process performed for a few hours for better yield value. Finally, after the extraction solvent was removed typically using a rotatory evaporator. Then the yield of oil is calculated from the residue portion towards the input of raw herbs.

2.5 Extraction of *C. sinensis* (Orange Peel Oil)

The *C. sinensis* peel is used for the oil extraction process. Before the extraction process, the orange peels were washed with water and cleaned properly. Then preheated in a vessel at 40° for nearly 10-15 minutes of time duration. Then the steam distillation process is performed. The sample was taken in a round bottom flask and added sufficient quantity of water, the condensation was then tried at 100°C for 90 minutes. The loss of water maintained by the replacement of water and the distillate was simultaneously collected; further oil is separated using a chloroform solvent mixture. The separation process was performed using a separating funnel and the oil-mixed organic layer was collected by the chloroform evaporation process. This was done in a water bath and the residue oil was collected, yield % was observed.

2.6 Mosquito Culture

Larvae (*A. aegypti)* were collected from Chennai metropolitan city stagnant water areas and confirmed in CMRC (Central Malarial Research Centre), DMS CAMPUS, Thenampet, Chennai. The collected larvae were impregnated with the medium of DE-chlorinated water for sustainability. The larvicidal study was performed at 27± 2°C and 60-70% RH under 14:10 h light and dark cycles. Brewer's yeast, dog biscuits, and algae collected from ponds are the diet for the larvae[23]. Guidelines for laboratory and field testing of mosquito larvicidal as per WHO protocol were used for the study reference[24]. In addition, instructions for determining the susceptibility or resistance of mosquito larvae to insecticides are also considered for the study[25].

2.7 Larvicidal Bioassay

In the preliminary screening with the laboratory trial, the stock solution with different serial dilutions was prepared. The larvicidal activity was assessed by the procedure of WHO (Guidelines for testing of larvicides) with some modifications and as per the method of Rahuman *et al*[26]. The larva study was carried out the method of WHO protocol. 5 times the study was repeated for each oil concentration for accuracy and reproducible data. The mortality was studied after 0, 1 hour, 4 hours, 20 hours, 24 hours, and 48 hours. The count of dead larvae was done by needles that were pricked in the neck or siphon. The control was set up with the same conditions only with dechlorinated water (without adding any essential oil) and reagents. Solubilizer: polysorbate 802 ml (tween 80 used for the study). Each contained 0.5 L of de-chlorinated water and 30 numbers of larvae. Stock concentrations were prepared by diluting the plant oil extracts with distilled water and drops of Tween 80 (Polyoxyethylene Sorbitan Monooleate) to obtain volumes v/v) respectively. Serial dilution of essential oils like 100 µL, 500 µL, 1000 µL, and 2000 µL used for the study. For each concentration 5 replicates with a total of 150 larvae are utilized for the experiment. The numbers of dead larvae were counted after 0, 1 hour, 4 hours, 20 hours, 24 hours, and 48 hours of exposure[27,28].

2.8 GC Fingerprint Studies

Method: Agilent GC instruments used for the study. Initial temperature 60°C for 2 minutes and 10°C per minute, gradually increased and 280°C hold. Internal temperature 260°C, 1µl injection volume flow 1.5 ml per minute and helium gas used with column HP 560 mm dimension.

3. Results

3.1 Larvidal Results of *M. piperita* against *Aedesaegypti* Mosquito Species

3.1.1 Larvicidal Potential of Peppermint Oil (100 µL)

The results of the larvicidal potential of 100 µL peppermint oil added to the culture and observed that inhibition of 6.67 ± 2.36 %, 10 ± 2.36%, 20 ± 2.36%, 100 ± 0%, 100 ± 0% at 1 hour, 4 hours, 20 hours, 24 hours,

48 hours respectively. 100 % mortality was observed at the 24-hour time point.

3.1.2 Larvicidal Potential of Peppermint Oil (500 µL)

The results of the Larvicidal potential of 500 µL peppermint oil added to the culture and observed that inhibition of 27.33 ± 2.79 %, 80 ± 2.36%, 100 ± 0 %, 100 ± 0%, 100 ± 0% at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. Within 4 hours 80 % and in 20 hours 100 % mortality was observed.

3.1.3 Larvicidal Potential of Peppermint Oil (1000 µL)

The results of the larvicidal potential of 1000 µL peppermint oil added to the culture and observed that inhibition of 39.33 ± 2.79 %, 100 ± 0%, 100 ± 0 %, 100 ± 0%, 100 ± 0%at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. Within 1 hour 39.33 % and in 4 hours 100 % mortality was observed.

3.1.4 Larvicidal Potential of Peppermint Oil (2000 µL)

The results of the larvicidal potential of 2000 µL peppermint oil added to the culture and observed that inhibition of 92.67 ± 2.79 %, 100 ± 0%, 100 ± 0 %, 100 ± 0%, 100 ± 0% at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. Within 1 hour 92.67and 4 hours 100 % mortality was observed.

3.2 Larvidal Results of *C. sinensis* against *Aedesaegypti* Mosquito Species

3.2.1 Larvicidal Potential of Orange Oil (100 µL)

The results of the larvicidal potential of 100 µL Orange oil added to the culture and observed that inhibition of 19.33 ± 2.79 %, 34.66 ± 3.80%, 100 ± 0, 100 ± 0%, 100 ± 0% at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. 100 % mortality was observed at the 20-hour time point.

3.2.2 Larvicidal Potential of Orange Oil (500 µL)

The results of larvicidal potential of 500 µL Orange oil added to the culture and observed that inhibition of 27.33 ± 2.79 %, 58 ± 2.9%, 100 ± 0 %, 100 ± 0%, 100 ± 0% at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. Within 4 hours 58 % and within 20 hours 100 % mortality was observed.

3.2.3 Larvicidal Potential of Orange Oil (1000 µL)

The results of the larvicidal potential of 1000 µL Orange oil added to the culture and observed that inhibition of 39.33 ± 2.79 %, 83.33 ± 2.360%, 100 ± 0 %, 100 ± 0%, 100 ± 0%at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. Within 4 hours 83.33 ± 2.36 and within 20 hours 100 % mortality was observed.

3.2.4 Larvicidal Potential of Orange Oil (2000 µL)

The results of the larvicidal potential of 2000 µL Orange oil added to the culture and observed that inhibition of76.67 ± 2.36 %, 100 ± 0%,100 ± 0 %, 100 ± 0%, 100 ± 0% at 1 hour, 4 hours, 20 hours, 24 hours, 48 hours respectively. Within 1 hour 76.67 ± 2.36 and 4 hours 100 % mortality was observed.

4. Discussions

4.1 Extractions of Oil

The percentage yield of peppermint oil from *M. piperita* 1.3ml/100g was obtained by the Soxhelet process and preliminary phytochemical and GC fingerprints were performed to confirm the peppermint oil and this was further utilized for the toxicological studies. Similarly, the percentage yield of orange oil obtained by the Steam distillation apparatus method was 2.95%.

4.2 GC Fingerprints of Peppermint Oil

It is characterised by the presence of oxygenated monoterpene such as menthol, menthone, menthofuran, menthyl acetate, 1,8-cineole, and pulegone[29]. Some of the compounds reported like 3-octanol, 1,8-cineole, limonene, trans-sabinene hydrate, menthone, isomenthone, menthofuran, neomenthol, menthol, pulegone, menthyl acetate, and β-caryophyllene *etc*[30].

4.3 GC Fingerprints of Orange Oil

The GC showed chemical composition of Orange oil. Due to complexity of essential oils, it is important to know the chemical composition and establish a fingerprint elution[31]. Monoterpene, sesquiterpenes, and oxygenated compounds (alcohols, esters, oxides, and epoxides) were identified and compound like α-pinene,

sabinene, β-myrcene, limonene, linalool, citronella, α-terpineol, decanal, Z-carveol, E-carveol, carvone, citral, methylbenzoate, α-copaene, β-cubenene, caryophyllene, germacrene D, valencene, δ-cadinene, β-sinensal, α-sinensal reported in the GC studies[32].

4.4 Dose-response Bio-assay

Previous studies of different kinds of plant extract and essential oils revealed the potential larvae mortality rate and inhibit the bits of mosquitoes, especially the *A. genesis*[33-36]. Some previous studies like Cheng *et al.*, compared the essential oils from the leaves of eight provenances of indigenous *Cinnamon* (*Cinnamomum osmophloeum* Kaneh.) and reported an excellent inhibitory effect against the *A. aegypti*[37,38]. Similarly the larvicidal activity of *Cinnamon* and other oils were recorded by Zhu *et al*[39,40]., against *A. albopictus*, *A. aegypti,* and *C. pipiens* pallens. Amer and Mehlhorn reported the larvicidal properties of *Lippia citriodora* against *A. aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus*[41,42].

The dose-response bioassay performed, the results were revealed in Figure 1 and 2 that 100 µL concentration 100 % mortality was observed within 24 hours, similarly 500 µL at 20 hours, 1000 µL at 4 hours, and 2000 µL within 4 hours. The level of toxicity increased with exposes to the oil at higher concentrations (Figure 5 and 6). Several reports are available on other oils that reveal their efficacy against mosquito larvae.

Oil like essential oil extracted from *C. sinensis* possesses excellent larvicidal efficiency. The dose-response bio-assay revealed in Figure 3 and 4 that 100 µL mortality at 20 hours with 100 % inhibition. Similarly 500 µL at 20 hours, 1000 µL at 20 hours, and 2000 µL at 4 hours. This higher concentration like 2000 µL has an immediate mortality effect within 4 hours (Figures 7 and 8) against the larvae of *A. aegypti* with the above experimental conditions.

4.5 Comparative Analysis of Larvicidal of Peppermint Oil *vs* Orange Oil

100 % inhibition was observed of Peppermint Oil at 100 µL at 24 hours, 500 µL at 20 hours, 1000 µL at 20 hours and 2000 µL at 4 hours. The larvicidal potential of % inhibition will be increased with the increase of oil concentrations and 500 µL to 1000 µL concentrations show 100 % mortality within the same time duration. While increased to 2000 µL within 4 hours 100 % mortality was observed due to high concentrations. Similarly, 100 % inhibition was observed of Orange oil at 100 µL at 20 hours, 500 µL at 20 hours, 1000 µL at 20 hours and 2000 µL at 4 hours. The larvicidal potential of % inhibition will be increased with increase of oil concentrations. Low concentrations of 100 µL showed a long time duration of up to 20 hours and high concentrations of 2000 µL within 4 hours for complete mortality.

4.6 Advantages of Natural-based-larvicidals

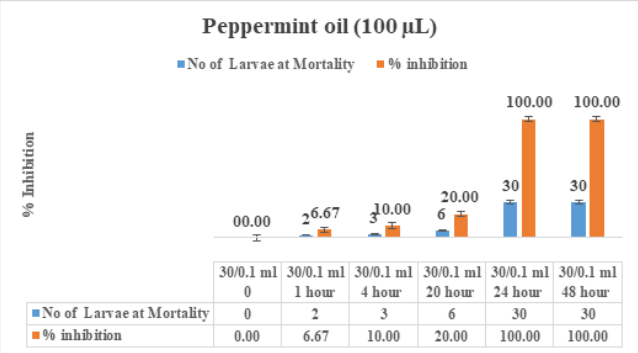
Using the level of synthetic larvicides leads to pollution of soil and surface water, soil, and living organisms.[43-47]. Naturally occurring larvicidals are safe[48,49]. Essential oils derived from natural plants are most important alternative approach to control vector borne disease for their ease of obtaining and minimal cost compared to synthetic chemical products which were available in the market[50].

5. Conclusions

This study concluded the essential oils of *M. piperita* and *C. sinensis* against *A. aegypti* mosquito species have potential inhibition and this helps to use this essential oil as a preventive aspect of vector-borne disease which will be spread by *A. aegypti* mosquito species. This study knowledge in future planned to incorporate this larvicidal potential herbal oils into mosquito inhibitory sprays and pharmaceutical topical preparations like creams and gels for the repellent products. In addition, this larvicidal knowledge will help the public health department and municipal corporations as an alternate approach for the herbal-based environmental eco-friendly pesticides when compared to currently using synthetic and chemical-based pesticides to control the mosquito species against the various kinds of vector-borne diseases.

6. Acknowledgement

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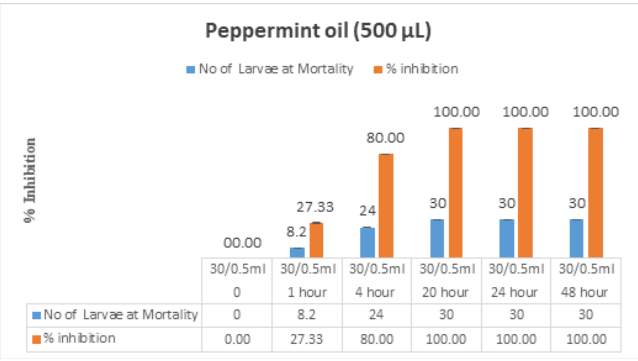
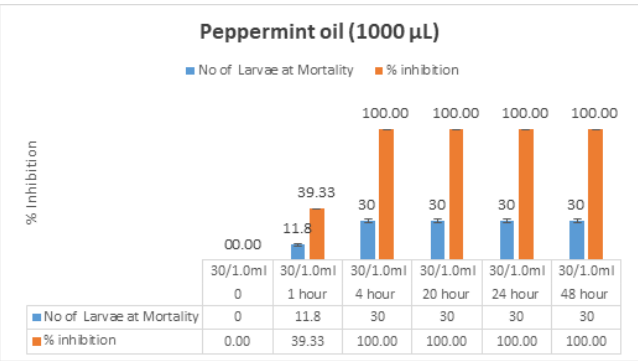


Figure 1. Larvicidal potential of peppermint oil (100 µL and 500 µL).



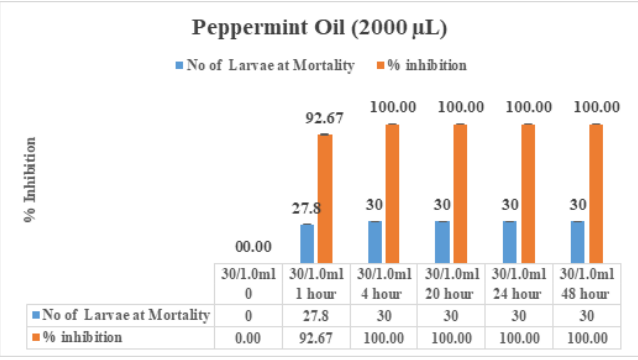
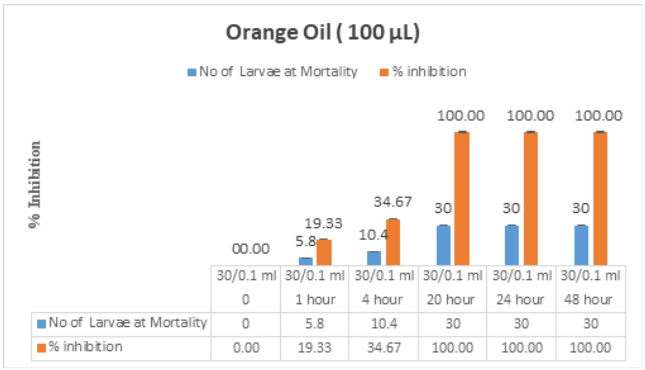


Figure 2. Larvicidal potential of peppermint oil (1000 µL and 2000 µL).



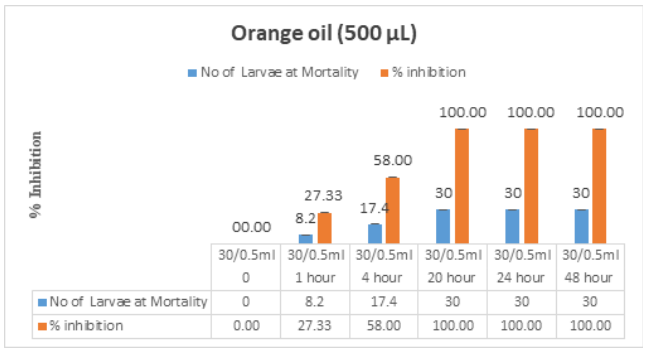
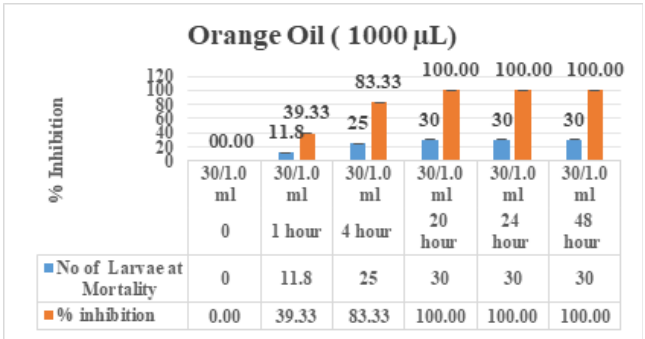


Figure 3. Larvicidal potential of orange oil (100 µL and 500 µL).



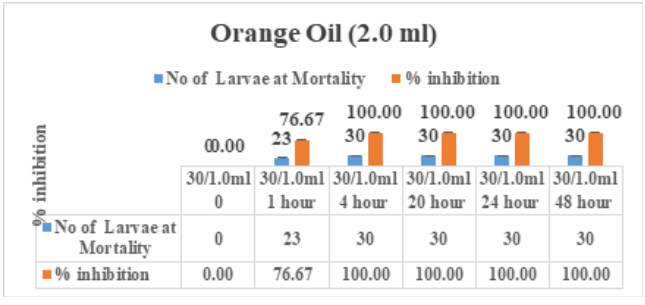


Figure 4. Larvicidal potential of orange oil (1000 µL and 2000 µL).

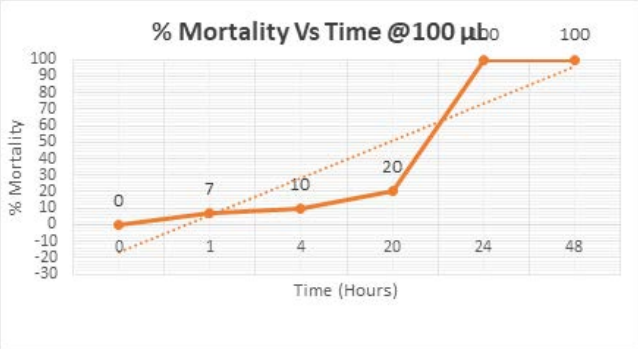


Figure 5. Peppermint oil: T max determinations of % mortality *vs* time at 100 µL.

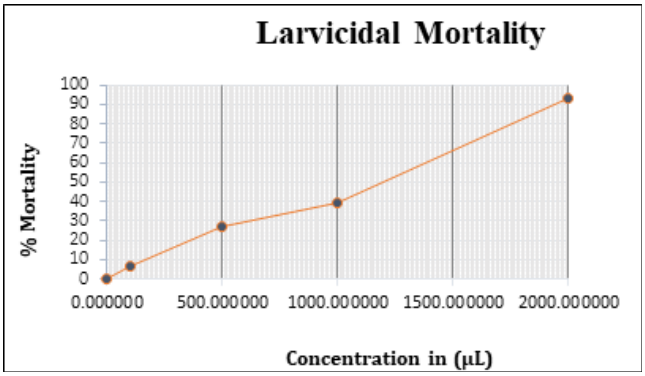


Figure 6. Peppermint oil: Larvicidal mortality *vs* different drug concentrations at 1 hour.

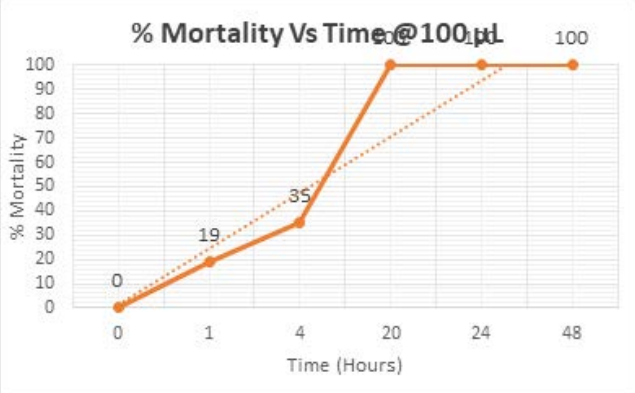


Figure 7. Orange oil: T max determinations of % mortality *vs* time at 100 µL.

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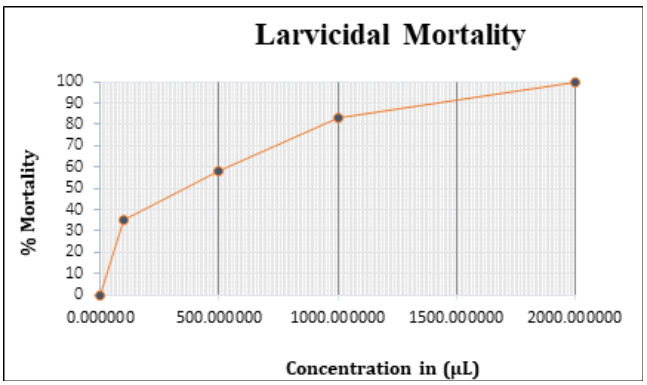


Figure 8. Orange oil: Larvicidal mortality *vs* different drug concentrations at 4 hour.

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7. Author Contribution

Kirubakaran N.: collected the data, extraction, larvicidal experiments for writing the research under various headings. Umashankar M. S.: Corresponding author: Design, preparation of study protocol, technical guidance, review of results, and review of manuscript. All the authors have read and approved the final manuscript.

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