Mosquitocidal activity of twenty-eight plant essential oils and their binary mixtures against Culex quinquefasciatus, (Diptera: Culicidae)

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ABSTRACT

Background: Mosquito control programme using synthetic insecticides has been facing the challenges of resistance development. However, synergistic combinations of plant essential oils (EOs) having different modes of actions and potent lethal toxicity may further negate the concern of resistance development.

Methods: In this study, the toxicity of 28 EOs and the two synthetic insecticides, Temephos and Malathion were evaluated individually and based on the performance, binary combinations of effective EOs were prepared and tested against the larval and adult stages of Culex quinquefasciatus. Mixtures were prepared by blending LC10 or LD10 concentration/doses of candidates at different volume ratios.

Results: Results demonstrated that among 155 numbers of combinations of different volume ratios, 1:1 ratio of A. sativum (bulbs) L. + C. paradisi (peels) Macfd. (AsB + CpP) was found to be the most potent against adults, whereas, 1:1 volume ratio of Allium sativum (bulbs)+ Citrus paradisi (leaves) (AsB + CpL) was found to possess highest activity against larvae after considering its dose and synergistic interaction. GC-MS analysis revealed the presence of diallyl trisulde, diallyl disulfide, beta-citronellol, ocimene as major constituents of AsB + CpP combination and diallyl disulfide, linalool, citronellal, caryophyllene oxide etc. in AsB + CpL combination.

Conclusions: Therefore, the said mixtures of the plant essential oils and or mixtures of the constituent compounds can be used as effective control agents for the control of the filarial vector, C. quinquefasciatus.

1. Introduction

Medical science and entomologists have been facing a diverse set of challenges to protect humans and domesticated animals from harmful mosquitoes. Considering the increasing populations of vector mosquitoes and cases of mosquito borne diseases, quick and proper counter measures are needed at the present time to improve the effectiveness of a vector control strategy. Prompting the adoption of effective mosquito control strategies has resulted in an urge to look for a highly potent, eco-friendly and cost-effective plant-based product. And the current study was aimed to establish low-dose plant essential oil-based synergistic combinations that would be effective against the larval and adult stages of the filarial vector, Culex quinquefasciatus.

In recent years, researchers are giving more emphasis on developing mixtures to contribute a new dimension to prevent resistance evolution in the vector control strategy. A mixture of compounds or essential oils exerts either synergistic effect, antagonistic effect or no effect. Plant essential oil mixtures employing synergistic actions are considered to have a higher and longer lasting effect than pure organic compounds alone and therefore they are gaining more attention. Synergistic combinations of two or more agents can overcome side effects associated with high doses of single one by decreasing the risk of resistance development, sparing doses on each compound, or accessing context-specific multi-target mechanisms (Lehar et al., 2009; Youssef et al., 2019). There have been fewer records of the use of essential oil mixtures as mosquitocidal, although few synergistic activity of mixtures of essential oils with synthetic insecticides have been reported against field crop pests and stored product pests (Suwannayod et al., 2019). Moreover, the study on the potentiality of combinations purely made up of plant essential oils against mosquitoes is rare. In the current investigation, 155 numbers of binary mixtures are prepared from the selected essential oils. All total, twenty-eight essential oils are extracted from twenty-five numbers of plants to study their mosquitocidal activities which are selected based on traditional knowledge and literature review (Table S1). Among these, some plants are already explored and some are investigated for the first time against C. quinquefasciatus. Based on their efficacy in terms of
sublethal concentration, effective plant essential oils are selected for combination study to find out synergistic combinations. To compare the toxicity of synthetic insecticides with the plant essential oils, the larval and adult stages of *C. quinquefasciatus* are also treated with two commercially available WHO recommended synthetic organophosphates namely Temephos and Malathion respectively.

Aiming to reduce the dose of synthetic insecticides to be released in the environment and to prevent resistance occurrence, combinations of synthetic organophosphates with the effective plant oils are also attempted to get synergistic interactions in any mixtures. The constituent profile of EOs of a plant species may vary in different regions. However, insight on constituents in combination may further help to develop efficient mixtures by adding its ingredient irrespective of geographical barrier etc. Therefore, to understand the constituent profile of the best combinations which are proved as the most effective against larval and adult stages of *C. quinquefasciatus* in the study, GC-MS analysis is carried out and analysed.

### 2. Results

- P. Diallyl disulfide, Q. Citronellal, R. Diallyl trisulfide, S. Carophyllene oxides.

The oil yield percentage of the selected essential oils is mentioned in Table S7. The highest oil yield was recorded for the peel part of the plant *C. grandis*. On the other hand, the EOs from *Homalomena aromatic* (leaves) and *Zingiber officinale* (leaves) yielded the lowest amount. While recording the larvicidal activity of the selected essential oils, fourteen numbers of oils were found to possess no larvicidal activity since they did not cause any mortality even at the highest concentration (1000 ppm) (Table S2). LC50 values were determined for the rest of the fourteen EOs. Among the effective EOs, the LC50 value was found lowest in case of *M. piperita* (leaves) oil with LC50 value of 2.31 ppm. The remaining candidates were found to possess LC50 values at a range of 18.23–275.66 ppm (Table S3). The highest larvicidal activity was exhibited by Temephos with LC50 concentration of 0.7 ppm. No mortality was recorded in the positive and negative control. In case of the bioassay against the adult stage, results demonstrated that some of the essential oils like *A. sativum* (bulbs), *A. marmelos* (leaves), *C. aurantifolia* (peels) and *E. maculata* (leaves) possessed strong adulticidal activity (Table S4). LD50 values could be calculated for seven numbers of plant essential oils. Among these, the lowest LD50 dose was recorded in case of *Citrus paradisi* (peels) oil (0.35 μg/cm²), which was followed by essential oil of *A. sativum* (bulbs). For Malathion, the recorded LD50 value was 0.3 μg/cm². In positive and negative control, no adult mortality was recorded.

Based on the individual toxicity of the selected essential oils and the two organophosphates, the effective oils were selected for the preparation of binary mixtures to study their joint - activity against the larval and adult stages of the same target species. Six numbers of EOs and temephos were short-listed for the preparations of binary mixtures against the larval stage. Against the adult stage, four numbers of EOs and the organophosphate malathion were selected for the preparations of binary mixtures. Initially, blending of LC50 concentrations of the selected candidates were considered, but the results demonstrated 100% mortality in each case (Table S5). As a result, it was difficult to underscore the most potent binary mixtures as all the mixtures had caused 100% observed mortality. Finally, the concentration was reduced to LC10 (against larvae) or LD10 (against adults) and binary mixtures were prepared at different volume ratios by mixing LC10 or LD10 concentrations/doses of each candidate present in the mixtures. It is obvious that if such results would be obtained even after reducing the concentration then it would be a better mosquito control agent from the point of cost, dose and toxicity towards the non-target organisms. For larvicidal activity, 83 numbers possessed synergistic effect in case of larvicidal activity. Four binary mixtures with antagonistic effect and eighteen numbers with no effect were also recorded (Table 1). Most of the temephos-based combinations were found to have synergistic interaction. Maximum numbers of *C. paradisi* (leaves) essential oil-based combinations were found to cause...
100% larval mortality except C. paradisi (leaves) + C. grandis (leaves) mixture at 2:1 volume ratio. The lowest larval mortality (3.3%) was observed in C. grandis (leaves) + Temephos (1:1) and O. sanctum (leaves) + M. piperita (leaves) (1:1) mixtures. Fifty numbers of binary mixtures were tested against the adult stage of C. quinquefasciatus, out of which 14 combinations were found to exhibit synergistic interaction with observed mortality at the range of 53–100% (Table 2). Malathion-based combinations were found to show antagonistic or no effect except in Citrus paradisi (peels) + Malathion (All volume ratios) and A. sativum (bulbs) + Malathion (1:1) mixtures. However, fourteen numbers of mixtures were found to act antagonistically against the target individuals.

Based on the dose of the essential oils present in the mixtures and its effect in terms of observed mortality, the most effective binary combinations were selected which were A. sativum (bulbs) + Citrus paradisi (peels) against adults and A. sativum (bulbs) + C. paradisi (leaves) against larvae respectively. Besides this, the availability of the selected essential oils, their high yielding nature and environmental and health safety attributes were also taken into account for selection of combination. It is worthwhile to mention that both the plants considered for combinations are edible plants and locally available as well as cultivated commercially for their edible parts.

A number of compounds are found to be present in it and based on its area percentages, probable major constituents are reported. From GC-MS analysis, the constituent profile of A. sativum (bulbs) + Citrus paradisi (peels) combination showed the presence of diallyl trisulfide, diallyldisulfide, beta-citronellol, ocimene as probable major constituents of the combination (Figures 1 and 2). However, in the binary mixture of A. sativum (bulbs) + C. paradisi (leaves), the major constituents identified were diallyldisulfide, citronellal, diallyltrisulfide, caryophyllene oxide etc. The MS, molecular weight, retention index, area percentage and retention time are mentioned below (Figures 3 and 4, Tables 3 and 4).

The constituent profile of individual essential oils selected in the present study was mentioned in Table S6.

3. Discussion

In the present investigation, as larvicides essential oil of M. piperita (leaves), A. sativum (bulb), C. paradisi (leaves), O. sanctum (leaves), C. grandis (peels) and C. grandis (leaves) are found to be highly effective against the target species. However, treated larvae are found to be moderately responsive to the oil of C. aurantifolia (leaves), C. linearis (leaves), A. marmelos (leaves), E. maculata (leaves), C. sinensis (leaves) and L. alba (leaves). Treated adults are found as highly responsive toward the oils of A. sativum (bulbs), C. paradisi (peels), A. sativum (bulbs), Citrus paradisi (peels), E. maculata (leaves) respectively but with higher LD50 dose in comparison to larvae. The result of the current study revealed that in some cases, the same plant essential oil acted differently against the different developmental stages of the same target species, C. quinquefasciatus. These differential toxic effects might be due to their difference in the levels of penetration and persistence in different life stages of C. quinquefasciatus (Sarma et al., 2019). However, in some other cases, the same plant oil is found effective against both the larval and adult stages and therefore such oils can be applied to control this mosquito species irrespective of their developmental stages. In the present investigation, the larval and adult stages of the target species are found to be vulnerable to A. sativum (bulbs) oil. The larvicidal activity of A. sativum (bulbs) oil is in conformity with the findings of Muturi et al. (2018) where they observed biocidal activity of the garlic oil against Culex pipiens and mentioned the constituent compound, allyl disulfide as the responsible factor. It seems that garlic oil is comparatively less volatile because it contains constituent molecules having higher weight with thiol groups than commonly occurring terpene compounds of other essential oils and thereby strongly affecting every developmental stage of

Figure 2. MS of four major constituent compounds of the binary mixtures (1:1 volume ratio) of A. sativum (bulbs) + Citrus paradisi (peels) oil mixture. A- Diallyl-trisulfide, B- Diallyl disulfide, C- Beta citronellol, D- Ocimene.
Table 1. Larvicidal activity of binary mixtures of different volume ratios against *C. quinquefasciatus* after 24 Hours.

| Volume ratios | Oil A                | Oil B                | LC10 (A) (Percent mortality) | LC10 (B) (Percent mortality) | Expected mortality | Observed mortality | Chi-square | Effect   |
|---------------|----------------------|----------------------|-------------------------------|-------------------------------|--------------------|--------------------|------------|----------|
| 1:1           | *Allium sativum* (bulbs) | *Citrus paradisi* (leaves) | 18.3                          | 11.6                          | 27.8               | 100                | 187.51     | Synergistic |
|               | *Ocimum sanctum* (leaves) | *Citrus grandis* (peels) | 18.3                          | 8.3                           | 25.1               | 26.6               | 0.089      | No Effect  |
|               | *C. grandis* (leaves) | *Mentha piperita* (leaves) | 18.3                          | 8.3                           | 25.1               | 6.6                | 13.63      | Synergistic |
|               | Temephos             | Temephos             | 18.3                          | 3.3                           | 20.75              | 96.6               | 277.26     | Synergistic |
|               | *Ocimum sanctum* (leaves) | *Citrus grandis* (leaves) | 11.6                          | 5                             | 16.02              | 100                | 440.24     | Synergistic |
|               | *M. piperita* (leaves) | *C. grandis* (leaves) | 11.6                          | 3.3                           | 13.67              | 100                | 545.20     | Synergistic |
|               | Temephos             | Temephos             | 11.6                          | 3.3                           | 13.67              | 100                | 545.20     | Synergistic |
|               | *Citrus grandis* (peels) | *Citrus grandis* (leaves) | 8.3                           | 3.3                           | 11.3               | 86.6               | 501.77     | Synergistic |
|               | *M. piperita* (leaves) | Temephos             | 8.3                           | 8.3                           | 15.9               | 38.3               | 31.56      | Synergistic |
|               | *Ocimum sanctum* (leaves) | *C. grandis* (leaves) | 8.3                           | 3.3                           | 10.76              | 12.67              | 0.33       | No effect  |
|               | *M. piperita* (leaves) | *O. sanctum* (leaves) | 3.3                           | 8.3                           | 11.3               | 28.3               | 25.57      | Synergistic |
|               | Temephos             | Temephos             | 3.3                           | 3.3                           | 5.91               | 3.3                | 1.28       | No effect  |
|               | *C. grandis* (leaves) | *M. piperita* (leaves) | 5                             | 8.3                           | 12.9               | 3.3                | 7.14       | Antagonistic |
|               | Temephos             | Temephos             | 5                             | 3.3                           | 7.85               | 9.5                | 0.34       | No effect  |
| 1:2           | *Allium sativum* (bulbs) | *Citrus paradisi* (leaves) | 18.3                          | 5                             | 22.1               | 100                | 273.53     | Synergistic |
|               | *Ocimum sanctum* (leaves) | *Citrus grandis* (peels) | 18.3                          | 1.67                          | 20.7               | 25.1               | 46.33      | Synergistic |
|               | *C. grandis* (peels) | *M. piperita* (leaves) | 18.3                          | 0                             | 18.3               | 26.6               | 102.78     | Synergistic |
|               | Temephos             | Temephos             | 18.3                          | 0                             | 18.3               | 25                 | 2.45       | No effect  |
|               | *Ocimum sanctum* (leaves) | *M. piperita* (leaves) | 11.6                          | 3.3                           | 13.6               | 100                | 548.89     | Synergistic |
|               | Temephos             | Temephos             | 11.6                          | 0                             | 11.6               | 100                | 673.67     | Synergistic |
|               | Temephos             | Temephos             | 11.6                          | 0                             | 11.6               | 100                | 673.67     | Synergistic |
|               | *C. grandis* (leaves) | *C. grandis* (leaves) | 8.3                           | 0                             | 8.3                | 101.33             | 1805      | Synergistic |
|               | *M. piperita* (leaves) | *O. sanctum* (leaves) | 8.3                           | 0                             | 8.3                | 100                | 548.89     | Synergistic |
|               | Temephos             | Temephos             | 8.3                           | 0                             | 8.3                | 100                | 548.89     | Synergistic |
|               | *C. grandis* (leaves) | *C. grandis* (leaves) | 8.3                           | 0                             | 8.3                | 16.67              | 5.53       | Synergistic |
|               | *M. piperita* (leaves) | *M. piperita* (leaves) | 3.3                           | 3.3                           | 5.91               | 3.3                | 1.85       | No effect  |
|               | Temephos             | Temephos             | 3.3                           | 3.3                           | 7.85               | 11.67              | 1.85       | No effect  |
|               | *M. piperita* (leaves) | *Temephos*           | 3.3                           | 0                             | 3.3                | 100                | 2833.60    | Synergistic |
|               | Temephos             | Temephos             | 3.3                           | 0                             | 3.3                | 100                | 2833.60    | Synergistic |
| 2:1           | *Allium sativum* (bulbs) | *Citrus paradisi* (leaves) | 0                             | 11.6                          | 11.6               | 100                | 673.67     | Synergistic |
|               | *Ocimum sanctum* (leaves) | *C. grandis* (peels) | 0                             | 8.3                           | 8.3                | 11.6               | 1.31       | No effect  |
|               | *C. grandis* (peels) | *M. piperita* (leaves) | 0                             | 3.3                           | 3.3                | 60                 | 974.20     | Synergistic |
|               | Temephos             | Temephos             | 0                             | 3.3                           | 3.3                | 88.4              | 64.85      | Synergistic |
|               | *O. sanctum* (leaves) | *Temephos*           | 0                             | 3.3                           | 18.33              | 33.60              | 835.39     | Synergistic |
|               | Temephos             | Temephos             | 0                             | 3.3                           | 9.75               | 100                | 835.39     | Synergistic |
|               | *C. grandis* (peels) | *M. piperita* (leaves) | 5                             | 8.3                           | 12.6               | 100                | 606.25     | Synergistic |
|               | Temephos             | Temephos             | 5                             | 8.3                           | 12.6               | 100                | 606.25     | Synergistic |
|               | *C. grandis* (leaves) | *M. piperita* (leaves) | 5                             | 3.3                           | 7.85               | 11.67              | 1.85       | No effect  |
|               | Temephos             | Temephos             | 5                             | 3.3                           | 11.67              | 100                | 462.70     | Synergistic |
|               | *C. grandis* (leaves) | *M. piperita* (leaves) | 1.67                          | 3.3                           | 18.96              | 36.67              | 16.54      | Antagonistic |
|               | Temephos             | Temephos             | 1.67                          | 8.3                           | 12.67              | 88.33              | 711.82     | Synergistic |
|               | *M. piperita* (leaves) | *O. sanctum* (leaves) | 1.67                          | 5                             | 5.95               | 36.67              | 158.60     | Synergistic |
|               | Temephos             | Temephos             | 1.67                          | 11.67                         | 11.89              | 100                | 652.38     | Synergistic |
|               | *C. grandis* (leaves) | *M. piperita* (leaves) | 0                             | 8.3                           | 8.3                | 25                 | 33.60      | Synergistic |

(continued on next page)
the target species due to its high persistence. Like *A. sativum* (bulbs) oil, *E. maculata* (leaves) oil is also found toxic against larvae and adults of the target species. The reported major constituents of the *E. maculata* (leaves) oil are eucalyptol and eudesmol which are individually found to be effective against *Aedes aegypti* in earlier studies (Sarma et al., 2019) and the same compounds may be responsible for the biocidal activity of the oil against the life stages of *C. quinquefasciatus* in the present case. In our previous study, we have reported *C. grandis* (peels) oil as potent larvicide for the first time against *C. quinquefasciatus*, where eudesmol, nootkatone etc. are found as the major constituent compounds which may be the possible factors for its toxicity against larvae (Mahanta et al., 2017). Besides these, *C. grandis* (leaves) and *C. aurantifolia* (leaves) oils are also found as effective larvicides in the current study. Two of the selected plant oils namely *M. piperita* (leaves) and *O. sanctum* (leaves) oils are found efficient only against the larval stage of the target species.  

### Table 1 (continued)

| Volume ratios | Oil A | Oil B | LC10 (A) (Percent mortality) | LC10 (B) (Percent mortality) | Expected mortality | Observed mortality | Chi-square Effect |
|---------------|-------|-------|-----------------------------|-----------------------------|-------------------|-------------------|------------------|
| 1:3 A. sativum (bulbs) | C. paradisi (leaves) | 18.3 | 21.7 | 35.22 | 100 | 119.21 | Synergistic |
| O. sanctum (leaves) | 18.3 | 13.3 | 28.67 | 46.67 | 11.41 | Synergistic |
| C. grandis (peels) | 18.3 | 18.3 | 32.76 | 18.3 | 638.25 | Synergistic |
| C. grandis (leaves) | 18.3 | 0 | 18.3 | 19.1 | 0.40 | No effect |
| M. piperita (leaves) | 18.3 | 5 | 22.1 | 19.1 | 0.40 | No effect |
| Temephos | 18.3 | 3.3 | 20.46 | 31.67 | 6.14 | Synergistic |
| C. paradisi (leaves) | O. sanctum (leaves) | 11.6 | 13.3 | 22.57 | 100 | 265.63 | Synergistic |
| M. piperita (leaves) | 11.6 | 5 | 15.45 | 100 | 462.69 | Synergistic |
| C. grandis (peels) | 11.6 | 18.3 | 27.02 | 100 | 197.12 | Synergistic |
| C. grandis (leaves) | 11.6 | 0 | 11.6 | 100 | 673.67 | Synergistic |
| Temephos | 11.6 | 3.3 | 13.67 | 100 | 546.80 | Synergistic |
| 3:1 A. sativum (bulbs) | C. paradisi (leaves) | 0 | 11.6 | 11.6 | 100 | 673.67 | Synergistic |
| O. sanctum (leaves) | 0 | 5 | 5 | 76.67 | 1027.31 | Synergistic |
| C. grandis (peels) | 0 | 8.3 | 8.3 | 6.67 | 0.32 | No effect |
| C. grandis (leaves) | 0 | 3.3 | 3.3 | 53.3 | 757.57 | Synergistic |
| M. piperita (leaves) | 0 | 8.3 | 8.3 | 14.2 | 4.1 | Synergistic |
| Temephos | 0 | 3.3 | 3.3 | 25 | 142.69 | Synergistic |
| C. paradisi (leaves) | O. sanctum (leaves) | 21.67 | 5 | 24.95 | 100 | 225.75 | Synergistic |
| M. piperita (leaves) | 21.67 | 8.3 | 27.32 | 100 | 193.35 | Synergistic |
| C. grandis (peels) | 21.67 | 8.3 | 27.32 | 100 | 193.35 | Synergistic |
| C. grandis (leaves) | 21.67 | 3.3 | 23.73 | 100 | 251.27 | Synergistic |
| Temephos | 21.67 | 3.3 | 23.73 | 100 | 251.27 | Synergistic |
| C. grandis (peels) | C. grandis (leaves) | 18.3 | 3.3 | 21.67 | 100 | 251.27 | Synergistic |
| O. sanctum (leaves) | 18.3 | 8.3 | 24.56 | 100 | 251.27 | Synergistic |
| Temephos | 18.3 | 3.3 | 21.67 | 100 | 251.27 | Synergistic |
| C. grandis (leaves) | M. piperita (leaves) | 18.3 | 8.3 | 24.56 | 100 | 251.27 | Synergistic |
| O. sanctum (leaves) | 18.3 | 5 | 22.1 | 15 | 2.28 | No effect |
| Temephos | 18.3 | 3.3 | 21.67 | 100 | 251.27 | Synergistic |
| O. sanctum (leaves) | M. piperita (leaves) | 21.67 | 3.3 | 23.73 | 100 | 251.27 | Synergistic |
| Temephos | 21.67 | 3.3 | 23.73 | 100 | 251.27 | Synergistic |

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bonds in its constituent profiles [limonene and carvone in *M. piperita* (leaves) and eugenol and methyl eugenol in *O. sanctum* (leaves)] may play the pivotal role for the larvicidal effect (Sarma et al., 2019; Pandey et al., 2013). Moreover, the *C. paradisi* (leaves) oil is found as a strong larvicidal agent with LC50 value of 22.18 ppm against *C. quinquefasciatus* for the first time in our investigation. Earlier it was reported as potent ovicide and larvicide against *Aedes* and *Anopheles* mosquitoes respectively (Dosoky and Setzer, 2018). Sabinene, beta-ocimene, gamma-terpinene, beta pinene etc. are some of the reported major constituents of this oil which may be responsible for the strong larvicidal activity of the plant oil (Paoli et al., 2016). Moreover, *Citrus paradisi* (peels) oil is also found as an adulticide for the first time in our investigation which may be due to the activity of its constituent compounds like limonene, linalool, pinene etc (Ivoke et al., 2013). In addition to *A. sativum* (bulbs) and *Citrus paradisi* (peels), *A. marmelos* (leaves) and *O. sanctum* (leaves) were used in the study.

### Table 2. Adulticidal activity of binary mixtures of different volume ratios against *C. quinquefasciatus* after 24 Hours.

| Volume ratios | Oil A                     | LC10(A) (Percent mortality) | Oil B                     | LC10(B) (Percent mortality) | Expected mortality | Observed mortality | Chi square | Effect     |
|---------------|---------------------------|----------------------------|---------------------------|----------------------------|---------------------|---------------------|------------|------------|
| 1:1 A. sativum (bulbs) | C. paradisi (peels)       | 6.6                        | 6.6                       | 12.7                       | 100                 | 600.1               | Synergistic |
|               | A. marmelos (leaves)      | 6.6                        | 15                       | 20.6                       | 16.6                | 0.78                | No effect   |
|               | Eucalyptus maculata (leaves) | 6.6                       | 15                       | 20.6                       | 53.3                | 51.90               | Synergistic |
|               | Malathion                 | 6.6                        | 6.6                      | 11.64                      | 100                 | 670.75              | Synergistic |
|               | C. paradisi (peels)       | 15                         | 6.6                      | 20.6                       | 56.6                | 62.91               | Synergistic |
|               | Malathion                 | 15                         | 6.6                      | 20.1                       | 22.3                | 0.24                | No effect   |
|               | E. maculata (leaves)      | 6.6                        | 15                       | 20.6                       | 13.3                | 2.58                | No effect   |
|               | Malathion                 | 6.6                        | 6.6                      | 11.64                      | 100                 | 670.75              | Synergistic |
| 1:2 A. sativum (bulbs) | C. paradisi (peels)       | 6.6                        | 20                       | 24.8                       | 100                 | 228.02              | Synergistic |
|               | A. marmelos (leaves)      | 6.6                        | 53.3                      | 58.22                      | 93.33               | 25.17               | Synergistic |
|               | E. maculata (leaves)      | 6.6                        | 50                       | 53                         | 63.33               | 2.001               | No effect   |
|               | Malathion                 | 6.6                        | 13.3                      | 18.22                      | 21.2                | 0.48                | No effect   |
|               | A. marmelos (leaves)      | 15                         | 20                       | 32                         | 10                  | 16.1                | No effect   |
|               | E. maculata (leaves)      | 15                         | 50                       | 57.5                       | 23.33               | 20.30               | Antagonistic |
|               | Malathion                 | 15                         | 13.3                      | 26.05                      | 28.2                | 0.17                | No effect   |
|               | C. paradisi (peels)       | 6.6                        | 50                       | 53                         | 63.33               | 2.001               | No effect   |
|               | Malathion                 | 6.6                        | 13.3                      | 18.22                      | 100                 | 367.06              | Synergistic |
| 2:1 A. sativum (bulbs) | C. paradisi (peels)       | 96.67                      | 6.6                       | 96.24                      | 100                 | 206.27              | Synergistic |
|               | A. marmelos (leaves)      | 96.67                      | 15                       | 96.67                      | 96.67               | 0                   | No effect   |
|               | E. maculata (leaves)      | 96.67                      | 15                       | 96.67                      | 56.67               | 16.56               | Antagonistic |
|               | Malathion                 | 96.67                      | 6.6                       | 96.24                      | 73.33               | 5.45                | Antagonistic |
|               | A. marmelos (leaves)      | 53.33                      | 6.6                       | 98.82                      | 33.33               | 34.44               | Antagonistic |
|               | C. paradisi (peels)       | 53.33                      | 15                       | 60.05                      | 56.67               | 0.19                | No effect   |
|               | Malathion                 | 53.33                      | 6.6                       | 98.82                      | 100                 | 19.03               | No effect   |
|               | E. maculata (leaves)      | 20                         | 15                       | 32                         | 26.67               | 28.40               | Antagonistic |
|               | Malathion                 | 20                         | 6.6                       | 24.8                       | 96.67               | 206.27              | Synergistic |
| 1:3 A. sativum (bulbs) | C. paradisi (peels)       | 6.6                        | 66.67                     | 68.04                      | 60                  | 0.07                | No effect   |
|               | A. marmelos (leaves)      | 6.6                        | 80                       | 81.2                       | 100                 | 4.35                | Synergistic |
|               | E. maculata (leaves)      | 6.6                        | 63.33                     | 65.22                      | 63.33               | 0.06                | No effect   |
|               | Malathion                 | 6.6                        | 30                       | 34.2                       | 41.3                | 1.47                | No effect   |
|               | A. marmelos (leaves)      | 15                         | 66.67                     | 80.01                      | 26.67               | 35.50               | Antagonistic |
|               | C. paradisi (peels)       | 15                         | 63.33                     | 68.55                      | 46.67               | 6.98                | Antagonistic |
|               | Malathion                 | 15                         | 30                       | 40.5                       | 28.67               | 3.45                | Antagonistic |
|               | E. maculata (leaves)      | 6.6                        | 63.33                     | 65.22                      | 96.67               | 15.16               | Synergistic |
|               | Malathion                 | 6.6                        | 30                       | 34.2                       | 96.67               | 114.10              | Synergistic |
| 3:1 A. sativum (bulbs) | C. paradisi (peels)       | 93.33                      | 6.6                       | 93.42                      | 83.33               | 1.09                | No effect   |
|               | A. marmelos (leaves)      | 93.33                      | 15                       | 94.05                      | 40                  | 31.06               | Antagonistic |
|               | E. maculata (leaves)      | 93.33                      | 15                       | 94.05                      | 46.67               | 23.87               | Antagonistic |
|               | Malathion                 | 93.33                      | 6.6                       | 93.42                      | 93.33               | 0.0001              | No effect   |
|               | A. marmelos (leaves)      | 80                         | 6.6                       | 81.2                       | 63.33               | 3.94                | Antagonistic |
|               | C. paradisi (peels)       | 80                         | 15                       | 83                         | 46.67               | 15.90               | Antagonistic |
|               | Malathion                 | 80                         | 6.6                       | 81.2                       | 85.3                | 0.20                | No effect   |
|               | E. maculata (leaves)      | 66.67                      | 15                       | 71.1                       | 100                 | 11.74               | Synergistic |
|               | Malathion                 | 66.67                      | 6.6                       | 68.04                      | 96.67               | 12.05               | Synergistic |
|               | E. maculata (leaves)      | 63.33                      | 6.6                       | 65.52                      | 41.2                | 9.02                | Antagonistic |
Figure 3. MS of the constituent compounds of the binary mixtures of *A. sativum* (bulbs) + *Citrus paradisi* (leaves) oil mixture.

Figure 4. MS of four major constituents of the binary mixture (1:1 volume ratio) of *A. sativum* (bulbs) + *Citrus paradisi* (leaves) oil mixture. P- Diallyl disulfide, Q- Citronellal, R- Diallyl trisulfide, S- Caryophyllene oxides.
Table 3. Constituent compounds of the binary mixture (1:1 volume ratio) of A. sativum (bulbs) + Citrus paradisi (leaves) oil mixture.

| Component                  | Molecular weight | Retention index | Chemical formula | Area (percent) | Retention time (minute) |
|----------------------------|------------------|-----------------|------------------|----------------|-------------------------|
| Diallyl trisulfide          | 178              | 1350            | C₆H₁₀S₃          | 34.98          | 11.93                   |
| Diallyl disulfide           | 146              | 1099            | C₆H₁₀S₂          | 32.15          | 6.27                    |
| Beta citronellol            | 156              | 1179            | C₆H₁₂O₂          | 12.21          | 4.15                    |
| Oxime                      | 136              | 976             | C₆H₁₀O₂          | 3.24           | 37.4                    |
| Beta-terpinyl acetate      | 196              | 1348            | C₆H₁₂O₂          | 3.07           | 39.26                   |
| Linalool oxide             | 170              | 1164            | C₆H₁₂O₂          | 2.40           | 34.75                   |
| Alpha terpinol             | 154              | 1137            | C₆H₁₂O₂          | 1.67           | 11.93                   |
| Ocimene                    | 256              | 1738            | C₆H₁₂O₄          | 1.51           | 20.94                   |
| Cycloheptatriene           | 92               | 786             | C₆H₈           | 0.70           | 7.78                    |

C. auranitifolia (peels) oils are also found to act as adulticides but with relatively higher LD50 dose in comparison to their early life stage.

In the present study, some of the plant essential oils like L. camara, F. benghalensis, Z. officinale, M. koenigii, C. fistula etc. are found to be ineffective against the target species, though these candidate oils were reported to possess insecticidal activities in previous studies (Rahuman et al., 2008; Govindarajan, 2010, 2013; Rajan and Varghese, 2017). Differences in the constituent profile of plant essential oils may affect its toxicity. Climatic condition, geographic location, method of extraction, time of harvesting, soil properties are some of the factors which determine the constituent compounds of an essential oil (Mahanta et al., 2017). Differences in susceptibility of the treated individuals to the different oils are due to differential rates of uptake, penetration through the chorion which is dependent on the exposure period, detoxification and failure of the toxicant to reach the target (Ramar et al., 2013). The constituent profiles of each of the selected essential oils are mentioned here (Table S6). Some of the reported compounds (e.g. limonene from P. alba, alpha pinene from E. odoratum, Eugenol from C. fistula, 1,8-cineol from C. lineartis etc.) of these plants are found to possess insecticidal activities against mosquitoes (Pavela, 2015; Sarma et al., 2019) though they are not able to cause mortality in the present study whenever tested as crude oils against C. quinquefasciatus. This may be due to the interference of some other minor compounds present in the crude essential oils. Again, the role of the environmental temperature in differential performance of EOs and their constituents cannot be overlooked. It has already been established that the same plant oil or constituent show different biocidal activities in different environmental temperatures. Pavela and Sedlak (2018) experimentally proved higher efficacy of Thymus vulgaris EO and its constituents carvacrol and thymol at the lower temperature (15 °C) in comparison to the higher temperature (30 °C) against larvae of C. quinquefasciatus. However, in the present study almost uniform laboratory temperature range is maintained during the experimental period. It is also reported in literature that some plant EOs may not show prominent acute toxicity at sublethal concentrations but the treatment may impact on fertility and vitality in the next generation (Pavela, 2012). In a recent study Pavela et al. (2020) showed noticeable reduction of fertility, lower hatching abilities as well as lower vitalities in houseflies in response to the treatment of the sublethal concentration of EO of Carlina acaulis and its constituent carlina oxide. Though, in the current study the effect of plant EO on fertility and vitality in the next generation was not studied, further work in this line may enrich information on efficacy of plant EO on this aspect.

Table 4. Constituent compounds of the binary mixture (1:1 volume ratio) of A. sativum (bulbs) + Citrus paradisi (leaves) oil mixture.

| Component                  | Molecular weight | Retention index | Chemical formula | Area (percent) | Retention time (minute) |
|----------------------------|------------------|-----------------|------------------|----------------|-------------------------|
| Diallyl disulfide           | 146              | 1099            | C₆H₁₀S₂          | 32.75          | 4.51                    |
| Citronellal                | 154              | 1125            | C₆H₁₀O₂          | 23.64          | 10.23                   |
| Diallyl trisulfide          | 178              | 1350            | C₆H₁₀S₃          | 16.45          | 13.83                   |
| Caryophyllene oxide        | 220              | 1507            | C₆H₁₂O₂          | 8.24           | 4.16                    |
| Linalool                   | 154              | 1082            | C₆H₁₀O₂          | 3.62           | 8.63                    |
disagreement with the results reported in their investigations as the mixtures of eugenol [one of the constituents of O. sanctum (leaves) oil] with camvone as well as limonene have manifested synergism against the larval stages of C. quinquefasciatus. The antagonistic interaction may be due to the involvement of some other minor constituent compounds present in O. sanctum (leaves) and M. pipertia (leaves) plant essential oils. In other volume ratios of these two combinations whenever the amount of A. sativum and O. sanctum oils are increased, the mixtures have shown synergistic activities. From this an assertion could be made that the Mentha oil is not so effective in combination though it is found to be the most effective one when tested alone. Finally, the most effective binary combination proposed in the current study against the larval stage is 1:1 volume ratio of A. sativum (bulbs) + C. paradisi (leaves). The co-activity of the monoterpenes [major constituents of C. paradisi (leaves) oil and organosulfur compounds major constituents of A. sativum (bulbs) oil] may lead to the synergy in the larval stages of C. quinquefasciatus.

The susceptibility status of the adult stage of C. quinquefasciatus towards the prepared binary mixtures is also studied in the present investigation. Most of the combinations of 1:1 volume ratio has shown synergistic effect except the mixture of essential oil of A. sativum (bulbs) + A. marmelos (leaves); A. marmelos (leaves) + E. maculata (leaves) and Citrus paradisi (peels) + E. maculata (leaves). Similarly, at 1:3 volume ratio, when the volume of E. maculata (leaves) oil is increased up to three times than its original LC10 concentration, C. paradisi (peels) + E. maculata (leaves) mixture is found to possess synergism. However, A. marmelos (leaves) + E. maculata (leaves) mixture is not able to act synergistically despite the testing of its different volume ratios against the adult individuals. The synergistic interaction with 100% observed mortality is recorded in case of the mixture of A. sativum (bulbs) + C. paradisi (peels) oil mixture. However, 1:3 and 3:1 volume ratios of these mixtures are found to show no effect. From such findings, a conclusion can be drawn that the mixtures may act synergistically at an optimum volume ratio of the particular essential oil present in the mixture. Below or above the volume range, they may lose their synergistic interaction. Moreover, the mixture of A. sativum (bulbs) + E. maculata (leaves) and A. marmelos (leaves) + Citrus paradisi (peels) oil mixtures are also found to act synergistically only at 1:1 volume ratio. These oils may act at different target sites for which synergism occurs. The minor compounds may also interact due to which synergistic effect is observed even at the LC10/LD10 concentration/dose. Some of the Citrus paradisi (peels) oil-based combinations like 1:1 volume ratio of A. marmelos (leaves) + Citrus paradisi (peels) and A. sativum (bulbs) + Citrus paradisi (peels) oil mixtures are found to possess synergism. The co-toxicity of different major constituents viz. sulfur compounds from A. sativum (bulbs), limonene from Citrus paradisi (peels) oil, beta-terpinyl acetate from A. marmelos (leaves) oil with different mode of actions may be the possible reason for their synergistic interaction. Against the adult stage, 1:1 volume ratio of A. sativum (bulbs) + Citrus paradisi (peels) oil mixture is considered as the most effective combination.

In case of larvae and adults, some of the binary combinations consisting of temephos or malathion are not found to bear synergistic interaction though they are individually effective as larvicides and adulticides. The possible explanation for such effect is that the synthetic insecticides possess high hydrophobicity and affinity with the essential oils due to which the molecules of the synthetic insecticides get dispersed in the essential oil and as a result bioavailability is decreased (Sovan-nayod et al., 2019). However, in some cases the synergistic interaction is also reported (2019). In some cases at low concentration against different life stages of the filarial vector, C. quinquefasciatus. In the present study, the profound effect of prepared combinations on the target species even at LC10/LD10 is the major finding. Bio availability of active compounds at these optimum concentrations in prepared mixtures might be the probable cause for such outcomes (Mahanta et al., 2020).
promising alternative measures for reducing the use of synthetic insecticides, which might be effective in terms of cost, toxicity and resistance development. Plant essential oils generally are nontoxic to mammals and other vertebrates. Botanical insecticides developed on the basis of EO are gaining importance because of their environmental and health safety attributes. They are considered to be safe, and eco-friendly alternatives to the synthetic chemicals, although a few studies raise questions about the safety of certain plant essential oils and their constituent compounds. In fact, the most important issue to be considered for the application of essential oils is their rapid degradation under the impact of air and light. Their direct and indirect effects on the other non-target organisms and the economic aspects must be considered before commercialization.

In the present investigation, after testing of 28 EOs and its selected binary mixtures against larval and adult stages of C. quinquefasciatus, finally it gives some potential mixtures consisting of garlic oil and Citrus oil. To the best of our knowledge, this is for the first time we have tested 155 numbers of binary mixtures comprising of only plant crude oils or the mixtures of plant oils plus synthetic insecticides and proposed some profoundly effective combinations of a very low concentration of LC10/LD10 [earlier some authors have reported the joint-toxicity of binary mixtures by using LC50 or LC25 concentrations basically taking the constituent compounds of plant oils (Pavela, 2015; Youssefi et al., 2019)] and their constituent profiles against larvae and adults of the filarial vector, C. quinquefasciatus.

4. Materials and methods

4.1. Rearing of C. quinquefasciatus

Egg rafts of C. quinquefasciatus were collected from Regional Medical Research Centre (RMRC-ICMR), Dibrugarh, Assam, India. The collected eggs were reared in an insect culture room, Dept. of Zoology, Gauhati University and the colonies were maintained between 25 – 29 °C temperature and 80–90% relative humidity following the method described by Arivoli and Tennyson (2011). The egg rafts were kept in plastic trays containing 1000 ml of tap water and they were fed on finely powdered dog biscuits and yeast powder (3:1). After the fourth instar larval stage, pupae emerged. The pupal stage was transferred to a disposable cup half filled with water and kept in wooden netted cage of size measuring (1 × 1 m), where they finally metamorphosed in to the adult stage within 1–3 days. No food was provided to the pupal stage. The adult colony was provided with 10% glucose solution soaked in cotton kept in the glass petri-dishes. After 3–4 days of adult emergence, an albino rat was provided for blood-feeding. Gravid females after 1–2 days of blood feeding laid eggs on the plastic cups containing water.

The Institutional Animal Ethical Committee, Gauhati University gave the permission for using mosquitoes as experimental animals via Reference Number IAEC/Per/2019/RF/2019-021.

4.2. Collection of plant material

All of the selected plant species (Table 1) were collected from Guwahati city (Kamrup District, Assam) and the species were identified by the curator, Department of Botany, Gauhati University.

4.3. Essential oil extraction

After washing, collected parts of the plants (500 g) were cut into small pieces and the essential oil was extracted through hydro distillation method using Clevenger’s apparatus following the method described by Kumar et al. (2010). The chopped plant materials were placed in 5 L round bottom flask containing 2–3 L of water and allowed to heat over heating mantle for about 5–6 h which softens the plant materials and allows the release of essential oil in volatile form. The thermostat of the heating mantle was set at 50 °C for extraction of essential oil. The essential oil vapours were condensed and formed a two layered mixture having essential oil in the top layer at the recuperating container. Essential oils were collected and stored in clean glass vials after removing water traces with the help of micropipette and stored at 4 °C.

4.4. Larvicidal assay

To investigate the larvicidal activity, the standard World Health Organization procedure was followed with little modification. 20 numbers of third instar larvae were transferred to the disposable cups (depth 5–10 cm) having 100 ml of water where 100 and 1000 ppm concentrations were applied as mentioned above. Based on the results of the pilot test of the essential oil at 100 and 1000 ppm of concentrations, a wide range of concentrations (1 ppm, 5 ppm, 10 ppm, 25 ppm, 50 ppm, 100 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm) of essential oils were prepared using equal amount of dimethyl sulphoxide (DMSO) as emulsifying agent. For Temephos, three more concentrations (0.1ppm, 0.5 ppm and 1 ppm) were applied. Twenty numbers of 3rd instar larvae were transferred to each replica of each concentration. The mortality was recorded after 24 h by observing larval death. The LC50 and LC10 concentrations were calculated after 24 h of treatment. Each concentration was assayed in triplicate along with one negative control group in water without any treatment and one positive control group treated with equal concentration of DMSO. Larvae which did not respond to any mechanical stimulus were considered as dead, while those which did not show any kind of swimming movement were considered moribund. The moribund larvae which were unable to revive for next 24 h were considered as dead (Kumar et al., 2014). The mortality in the control groups if occurred between 5-10% then, the mortalities of treated group was corrected by using Abbott’s correction formula as follows

\[
\text{Mortality} \% = \frac{A - B}{A} \times 100
\]

(1)

Where

\( A = \) percentage survival in control group

\( B = \) percentage survival in treated group

4.5. Adulticidal assay

Impregnated filter paper method was followed by Ramar et al. (2013) to study the adulticidal activity of the selected essential oils against the target species. Initially two concentrations (10 μg/cm² and 1 μg/cm²) of each selected essential oil were prepared in 2 ml of acetone and applied on Whatman no.1 filter papers (size 12 × 15 cm²). 10 numbers of 4–5 days old non blood fed adult female mosquitoes were selected for each replication. Three replications were set for each concentration. Based on the results of pilot study of the essential oil, a wide range of concentrations (0.01–10 μg/cm²) of essential oils was prepared. For positive control, filter papers were treated with 2 ml of acetone alone and placed in exposure tubes (depth 10 cm). After the evaporation time of acetone (5 min), ten numbers of 3–4 days old sugar-fed female mosquitoes were released into the tubes. One control group with acetone was placed for each replication. Based on the results of the pilot study of the essential oil, a wide range of concentrations (0.01–10 μg/cm²) of essential oils was prepared. For positive control, filter papers were treated with 2 ml of acetone alone and placed in exposure tubes (depth 10 cm). After the evaporation time of acetone (5 min), ten numbers of 3–4 days old sugar-fed female mosquitoes were released into the tubes. One control group with acetone was placed for each of the oils tested. Adults were considered dead if they did not move when disturbed repeatedly with a soft brush. The LC50 and LC10 concentrations were calculated after 24 h of exposure by probit analysis and finally expressed in microgram/cm². If the recorded mortality percentage was found to exceed 20% in the control batch, the experiment was repeated. However, if mortality in the controls was found more than 5%, the recorded data were corrected using Abbott’s correction formula

\[
\text{Mortality} \% = \frac{A - B}{100 - B} \times 100
\]

(2)

Where
A = the percentage mortality in the treated group
B = the percentage mortality in the control.

No mortality was recorded at the control groups of larvae and adults.

Bioassays were performed maintaining a constant temperature (25–29 °C) relative humidity (80–90%) and photoperiod (12 h L: 12 h D).

4.6. Preparation of binary combinations

Considering the LC50 (Or LD50) concentration/dose of each of the selected plant oils (below 100 ppm for larvae; below 6 μg/cm² for adults) effective oils were short listed and they were further studied to explore the joint toxicity in mixtures at different volume ratios. Initially, the pilot study was performed taking 1:1 volume of LC50/LD50 concentration/dose of each of the candidate oil (Table S5) following the same method described above. However, each of the prepared binary mixtures comprising of LC50 concentration (Or LD50) of each candidate oil is found to cause cent percent mortality among the treated mosquitoes and hence the concentration was reduced to LC10 or LD10 and the rest of the combinations of oils were prepared based on LC10 concentration against the larval stage and LD10 dose against the adult stage of C. quinquefasciatus to find out the best effective combinations.

Mixtures of different volume ratios like 1:1, 1:2, 2:1, 1:3, and 3:1 of LC10 concentrations against larvae and LD10 dose against adults were prepared and applied against the respective developmental stages. DMSO was used as positive control in larvicidal activity and acetone was used as positive control in adulticidal activity of the prepared mixtures. In each bioassay, three replications were set for each combination and three replications for each of the ingredients present in the respective combination. The method of application and condition were kept similar to the methods used for bioassay of essential oil described above.

4.7. Toxicity of the binary mixtures

The activity of the binary mixtures of essential oils was observed by following the method described by Pavela (2015) with little modifications. Three replications with each replication comprising 20 larvae and adults were tested. Actual mortalities were compared to expected mortalities after 24 h of exposure based on the formula:

\[ E = O_a + (1 - O_a) O_b \]

(3)

\( E \) was the expected mortality and \( O_a \) and \( O_b \) were the observed mortalities of individual treatment at the given concentration or dose. The effects of mixtures were designated as either antagonistic or synergistic by analysis using \( \chi^2 \) comparisons. If the chi-square value was greater than the table value at definite degrees of freedom and at the same time, the observed mortality was higher than the expected mortality, then the combination was considered to possess synergism. Again, if the observed mortality was lower than the expected mortality, the mixture was considered to possess antagonistic effect. The combination with chi-square value lower than the table value and at definite degrees of freedom, it signifies neither synergistic nor antagonistic effect.

\[ \chi^2 = (O_a - E)^2 / E \]

(4)

4.8. GC-MS analysis

GC-MS analysis was carried out to identify the constituents of the most effective essential oil mixtures. Sample of the essential oils was analysed using Gas Chromatography- Mass Spectrometry. GC analysis was carried out on an Agilent GC 7890 A and Mass spectrophotometry in Accu TOF GC from Jeol instrument. Gas chromatograph equipped with an FID detector and a capillary column (HP5-MS). The carrier gas was helium at a flow rate of 1 ml/min. The splitting program was set as 1:10; 80–1M-5-200-3M-8-275-3M-5-280-E.

4.9. Statistical analysis

LC50/LD50 and LC10/LD10 concentrations/doses were calculated from the recorded data of average mortality of larvae and adults by probit analysis using SPSS software 21 (Statistical Package of Social Sciences) and Minitab software. 95% confidence limits of upper confidence limit and lower confidence limit, and chi-square values were also recorded. Standard error was calculated using MS- EXCEL.

Declarations

Author contribution statement

Sudarshana Mahanta: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Bulbuli Khanikor: Conceived and designed the experiments.

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Data availability statement

The authors do not have permission to share data.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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