Development of an eco-friendly mosquito larvicide for dengue/ chikungunya vector control

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Abstract

This study used hydro-distillation to evaluate the larvicidal potential of essential oils (EOs) extracted from seven plant species against *Aedes aegypti* larvae. The chemical profiles of the volatile oils were characterized through gas chromatography-mass spectrometry (GC-MS). The major constituents identified were thymol (74.93%) in *Plectranthus amboinicus* leaves, terpinen-4-ol (27.13%) in *Myristica fragrans* nuts, citral (27.55%) in *Citrus limon* leaves, caryophyllene oxide (36.11%) in *Hyptis suaveolens* leaves, and asarone (85.51%) in *Acorus calamus* rhizomes. Larvicidal bioassays revealed LC_{50} values below 50 ppm (23.40 to 47.34 ppm), with the order of efficacy being *A. calamus* > *M. fragrans* > *T. ammi* > *I. verum* > *C. limon* > *H. suaveolens* > *P. amboinicus*. Twenty-one binary and thirty-five ternary combinations were tested to determine synergistic, additive, and antagonistic interactions using Wadley's, Ting-Chao Chou, Co-Toxicity Factor, and Chi-square comparison methods. Binary combinations, such as *P. amboinicus* and *M. fragrans* and *T. ammi* and *M. fragrans* exhibited better synergism, this combination analysed by GC-MS, *P. amboinicus* and *M. fragrans* contains, carvacrol (36.23%), thymol (39.04%), and caryophyllene oxide (3.84%). Similarly, the combination of *T. ammi* and *M. fragrans* showed thymol (61.96%) and caryophyllene oxide (7.19%). Based on the essential oil yield and activity the *T. ammi* and *M. fragrans* binary combination formulated as soft-gel capsules, this formulation showed 100% larval mortality. Essential oil mixtures work very well, showing they could be good, eco-friendly options to replace chemical insecticides for controlling mosquito larvae in the future.

Keywords: *Aedes aegypti*; Dengue; Plant essentiol oil; GC-MS analysis; Larvicide; Synergism

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Introduction

Chikungunya, Dengue, Yellow, and Zika fever are spread by *Aedes* mosquitoes. Dengue was listed by the WHO as one of ten diseases that could be dangerous in 2019, and current outbreaks in a number of nations support this finding. In South East Asia, dengue cases rose by 46% in 2019 compared to 2015. Environmental circumstances have constantly changed as a result of global warming, and suitable locations have been created to boost insect populations. Unfortunately, filthy waste management practices and unhygienic conditions for family-use water storage can lead to an increase in mosquito populations in both urban and rural areas (Estrada et al. 2012). Controlling the *Aedes aegypti* (*Ae. aegypti*) population is the only practical means of preventing dengue. Therefore, using larvicides to stop mosquito breeding is the best way to manage mosquito infestations. In many parts of the world, *Ae. aegypti* has become resistant to pyrethroid, carbamate, organochlorine, and organophosphate pesticides (Rawlins and Wan 1995; Rawlins 1998; Poulton et al. 2024). Vector resistance has been brought about by the frequent use of synthetic insecticides to control mosquitoes. The use of synthetic pesticides has led to major environmental issues, including contamination of the air, soil, and groundwater as well as toxicity to humans and aquatic ecosystems (Liu et al. 2005; Peter et al. 2010; Pradeep and Sarala 2003; Severini et al. 1993).

Over the years, many synthetic insecticides have been employed. Since the 1960s, organophosphates like Temephos have been employed as larvicides in a number of foreign countries (Braga et al. 2004; Gubler and Duane 2006; Romi et al. 2003). The development of resistance, side effects on non-target populations (particularly humans), and adverse impacts on the environment (contaminating soil, water, and air) have all significantly increased in recent years (Chang et al. 2014). Scientists have developed alternative control methods due to the numerous drawbacks of chemical control approaches (Alavanja et al. 2004). Natural chemical substitutes for insect and pest management have been the focus of contemporary research globally (Dharmagadda et al. 2005; Cheng et al. 2003; McClements and David 2011). Essential oils have strong larvicidal effects and are bio-based pesticide phos is more poisonous than Trachyspermum ammi EOs against Daphnia magna water fleas, according to Seo et al. Numerous plant essential oils (EOs) have been shown to have a larvicidal action on mosquitoes, including leaves and sprig EOs from Clausena excavation, which exhibited larvicidal activity against *Ae. aegypti* and *Ae. albopictus* (Seo et al. 2012; Cheng et al. 2009).

They possess a range of biological properties, such as larvicidal activity (Osanloo et al. 2019; Soleimani et al. 2017a; Soleimani et al. 2017b), leishmanicidal (Moemenbellah et al. 2020; Noorpisheh et al. 2020), antibacterial (Osanloo et al. 2020; Sedaghat et al. 2011), and repellent (Moemenbellah et al. 2021; Pirmohammadi et al. 2016). Due to their rapid environmental degradation, selective action on target species, and minimal side effects on non-target organisms, essential oil (EO)-based insecticides have recently been proposed as alternatives to synthetic products for mosquito control. Numerous studies on the use of EOs against mosquitoes have been published in the literature. (Mahnaz et al. 2013; Sedaghat et al. 2011). However, because some of its constituents are volatile, EOs have few uses as insecticides and repellents.

These days, it is anticipated that the worldwide pesticide market will favour more environmentally friendly and healthful products. In this sense, the EO sector is expanding at a rate of 5% annually (CBI 2009), and it can supply the raw materials needed to produce potent botanical insecticides, which are projected to capture 7% of the worldwide pesticide market by 2025 (Isman 2015). Additionally, it has been noted that plant essential oils and their components readily evaporate in water, thus their leftovers are not very problematic (Park et al. 2011, 2012; Seo et al. 2012).

They are less likely to develop resistance since they are user-friendly, environmentally friendly, and include a variety of bioactive substances. (Isman 2000). In order to improve insecticidal effects and lower the likelihood that the targeted pest population will develop resistance, focus is being concentrated on creating combinations of EO-based chemicals. Numerous essential oils extracted from plant species have been shown to have antibacterial and antifungal properties in addition to acting against a variety of insects. (Abbassy 1981; Curtis et al. 1991; Regnault 1997). EOs must be prepared to prevent the vaporisation of their volatile components in order to maintain their biological activity. (Bakkali et al. 2008; Buranasuksombat et al. 2011; Osanloo et al. 2017). EOs provide various pesticida Alavanja l effects (Benelli 2012; Burt 2004), including insecticidal effects. Because of these qualities, EOs are used in the culinary, cosmetic, and medicinal sectors as well as in the creation of novel botanical insecticides, such as larvicides against vectors. (Dias and Moraes 2014; Pavela 2009, 2011, 2014).

Natural molecules having potential insecticidal action, notably those obtained from plants, have gained attention because to mosquito resistance to synthetic insecticides. These items are seen to be viable substitutes for traditional synthetic pesticides in the management of various insect pests and vectors. In this regard, essential oils and their components have drawn a lot of interest as possibly beneficial bioactive substances against insects. (Chowdhury et al. 2008). Many research studies have shown that medicinal plant and their essential oils can kill the larvae of the *Aedes aegypti* mosquito and other insects from the *Culex* and *Anopheles* groups (Pitasawat et al. 2007; Wafa et al. 2014).

Recent studies show that natural substance from plants work well as larvicides for *Aedes aegypti* mosquitoes. Essential oil and extracts from the various medicinal plant have proven to be very good at killing mosquito larvae, even when the mosquitoes no longer respond to other kinds of treatment (Pereira Filho et al. 2021; Aljameeli 2023). Lab tests show that *Bocageopsis multiflora* and *Myrciaria floribunda* are harmful to both adult and young mosquito insects. This means that certain plants from tropical areas might help in keeping diseases-spreading insects under control (Silva et al. 2024; Santos et al. 2025). It has also been discovered that plant-based nanoparticles are better at killing larvae and are more friendly to the environment (de Assuncao et al. 2024). Nano-formulations and method using essential oils have made botanical larvicides more stable and effective, showing great potential for developing better insects control solutions (Duarte et al. 2020). The essential oil from the leaves of *piper purusanum* plant work well to kill the eggs and early larvae stage of *Aedes aegypti* mosquitoes, which spread dengue and malaria. This essential oil and its main components did not hurt many other water creatures that are not affected, but they slowed down the growth of the larvae and stopped the eggs from hatching. This shows that the essential oil might be the good and safe way to control disease-spreading insects in an environmentally friendly way (de Oliveira et al. 2022).

In our study *P. amboinicus (Loureiro) Sprengel* –Leaves, *A. calamus Linn.* –rhizome, *T. ammi L. Sprague* – Seeds, *I. verum Hook. f. (Illiciaceae)* – fruits, *C. limon (L.) Burm. f.* – leaves, *H. suaveolens (L.) Poit.* – leaves, *M. fragrans (Houtt.)* – nuts used for essential oil extraction.

Materials and methods

Extraction of essential oil

In this work *A. calamus* rhizome, *M. fragrans* nuts, *ajwain* dry fruits, and star anise dry flower purchased from a local shop from Pondicherry and these other waste particles were removed and ground into powder in a Mixer. *P. amboinicus*, *C.limon,* and *H. suaveolens* fresh leaves were collected from Pondicherry, and the leaves were cleaned and washed with distilled water. Essential oil extracted by a Clevenger apparatus by hydro distillation method initially took 8 hours, based on the yield extraction time, which varied from 4 to 6 hours. 100 grams of the powdered plant material (for fresh leaves 100gm) material was soaked in 750mL of double distilled water in a 1000mL capacity round bottom (RB) flask, this RB flask was kept in a heating mantle and fixed with a Clevenger apparatus and condenser. In heating, the mantle temperature is maintained at 60 to 70°C, and water circulation is maintained to condense the boiled water and EOs from the plant materials. Essential oil was separated from water, and a trace quantity of water in the separated EOs was removed and filtered using anhydrous sodium sulfate. The obtained EOs quantity was measured and stored in an amber glass vial at 4 to 10°C for further usage (Figure-1).

GC-MS analysis

Column Rtx-5MS (5% Diphenyl/95% Dimethyl poly siloxane), 30m x 0.25mm ID x 0.25 mdf, GC Program 8890GC/5977B GC/MSD-Agilent equipment, Software Mass Hunter Workstation, Detector Single Quadrupole Mass Spectrometer, Split 10:1, Carrier gas 1 millilitre per minute, 2 microlitres of sample injection volume The oven temperature program is set at 110°C for 3.50 minutes. Up to 200°C at a rate of 10°C per minute—no hold Up to 280 °C at a rate of 5 °C per minute with a 12-minute hold, 280°C is the injector temperature. GC ran for 40.50 minutes in total. NIST Version 2020 was utilised by the MS Program Library. The source temperature is 250 °C, and the inlet line temperature is 290 °C. 70 eV of electron energy 50-550 amu mass scan (m/z), Solvent Delay: 0–3.5 minutes 40.50 minutes was the total MS running duration (Sridhar and Sunil 2021).

Bioassay

Essential oils do not form a homogeneous solution with water. Dimethyl sulphoxide (DMSO) makes a homogeneous solution for these organic solvents. Larvicide bioassays were performed using 200ml of juice paper cups with four replicates, with 99ml of tap water added to each cup and 25 late third instar *Ae. aegypti* larvae collected from our center's mosquito rearing and colonisation section in ICMR-VCRC. The stock solution was made by 1000mg of essential oil taken into a 10mL amber colour standard volumetric flask and made up volume with DMSO solvent 100000ppm. From these stock solutions, a serial dilution solution was made with DMSO solvent to obtain a concentration of 500 to 15000 ppm. For the test, 1 ml of serially diluted solution was added to 99 ml of water with a larvae cup to make the lowest concentration to the highest concentration between 5 and 150 ppm. For control, 1ml DMSO solvent alone was added, and after the addition of the test and control cup, it was slightly shaken for homogenous. For binary and tertiary combinations, the essential oil was selected based on their LC_{50} values, and these combination test solutions were also prepared using DMSO solvent. Test and control maintained 27 ± 2 °C, RH 76 ± 4%, and at 12 hours dark and 12 hours a night. After 24 hours, larval food was provided, and death of moribund larval was noted in 48hrs from replicate. The test was repeated three times on different days and in various larval batches, from larval mortality test narrow range concentration between 0% to 100% mortality four to six concentrations repeated with 4 replications and three experiments for LC_{50} and LC_{90} calculation. In the control group, 10% of pupae formed, or more than 20% of larvae mortality observed the test to be discarded. In cases where larvae mortality is below 20%, and pupation is less than 10% in control, the treated mortality should be corrected using Abbot's formula.

$$\text{Mortality (\%)} = \frac{X - Y}{X}\ 100$$

Where X = Larvae survival Percentage in the control units and Y = Larvae survival Percentage in the treatments (WHO 2005).

Potentiation/additive or no effect / antagonistic activity

For Chi-square comparison (χ^2) and Co-Toxicity Factor methods, Individual Essential oil LC_{25} and binary mixture 1:1 ratio (doses LC_{25}/LC_{25}) were taken for larvicidal activity against late 3rd instar larvae of *Ae. aegypti*. Each mixture was evaluated in four duplicates, with untreated controls in three trials conducted on different days. The mortality percentages were calculated after 48 hours.

Chi-square comparison method (CSC) (χ^2)

The following formula calculates expected mortality:

$$E = O_a + O_b\ (1 - \frac{O_a}{100})$$

Where E is the anticipated mortality and O_a, O_b are the activity of the individual essential oil samples at LC_{25} dosage. The activity of the EOs combination was determined as antagonistic, with no effect or potentiation calculated by χ^2 values using the formula below:

$$\chi^2 = \frac{(O_m - E)^2}{E}$$

O_m is a binary combination of actual mortality, E is the anticipated mortality, χ^2 with Degree of freedom = 1, and p = 0.05 is 3.84. If χ^2 values above 3.84 and more than theoretically calculated mortality were considered potentiation (or antagonistic), in case χ^2 values less than 3.84, that combination is called additive effects (Andrade et al. 2018; Hummelbrunner and Isman 2001; Pavela 2015).

Co-toxicity Factor (CTF) method

The following formula to distinguish between additive, antagonistic, and potentiation:

$$CTF = (O - E) \times 100/E$$

Where O represents observed mortality (%), and E represents anticipated mortality (%). If CTF values for any combination are more than or equal to 20, that combinations show synergistic interaction. If the CTF values are less or equal to -20, they show antagonism, and between the CTF values above -20 and below 20, they show an additive effect. The anticipated mortality was determined by the SPSS data of individual EO at LC_{25} values added and calculated for combination EO expected mortality. Binary mixture test solutions were prepared by adding individual EO values at LC_{25}. These solutions were tested against mosquito larvae to evaluate the observed death rate. (Dhinakaran et al. 2019; Pandiyan et al. 2019; Sameeh et al. 2010; Sun and Johnson 1960). Expected mortality was calculated based on about 25% larval mortality observed in the profit analysis of each essential oil concentration added to a binary combination. Observed mortality was determined experimentally by adding individual essential oil at an LC_{25} dose into the binary combination testing cup, and larval death was observed at 48 hours. The percentage mortality substituted into profit analysis.

For Wadley's & Ting-Chao Chou's methods: To study the interaction between essential oils in a mixture, each essential oil mixed in 1:1 ratio in their LC_{50}/LC_{50} for binary and 1:1:1 ratio in their $LC_{50}/LC_{50}/LC_{50}$ values for ternary combination and treated against *Ae. aegypti* late 3rd instar larvae. The mortality percentages were determined after 48 h for LC_{50} value calculation.

Wadley's (W) method

The Wadley method is used to compute the predicted response LC_{50} for a combination, which is then compared to the observed response LC_{50} as follows:

$$\text{Expected LC}_{50} = \frac{(a+b)}{(\frac{a}{LC_{50(a)}}) + (\frac{b}{LC_{50(b)}})}$$

Where a and b are the concentrations of essential oils A and B in the mixture, and $LC_{50(a)}$ & $LC_{50(b)}$ are the experimental LC_{50} values of the compound A and B individual essential oils, respectively. The combination EO interaction ratio (R) between the estimated LC_{50} value and experimental LC_{50} value is calculated as

$$\text{R} = \frac{\text{Expected LC}_{50}}{\text{Observed LC}_{50}}$$

Where the R-value above 1.5 is defined as potentiation, if the R-value is between 0.5 and 1.5 it is called additive effect, and the R-value below 0.5 is defined as antagonistic (Gisi 1985; Tak et al. 2017; Wadley 1945, 1966).

Ting-Chao Chou method (TCC)

In online free Compusyn version 1 software downloaded and used for combination index (CI) determination, individual larvicidal data, and combination mixture (with ratio) data entered in software after all data entry generated report. CI = combination index, CI < 1 indicates synergism(S); CI = 1 indicates additive effect(A); CI > 1 indicates antagonism(AG), @ 0.5 Fa = fraction affected at 50%, @ 0.9 Fa = fraction affected at 90% (Chou, 2006; Chou, 2018; Zhang et al. 2015).

Formulation

The effective combination was further carried out for formulation studies for field conditions. Soft gel gelatin capsules (each capsule contains 500 mg of EO emulsion - oil in water; essential oil quantity LC_{99}*2) were formulated with the help of Sun Beam Lifesciences Pharmaceuticals Pvt. Ltd. (Figure-2). The obtained capsules were evaluated for their larvicidal efficacy against *Ae. aegypti, An. stephensi* and *C. quinquefasciatus* late 3rd instar larvae in simulated field conditions. Initially, 1 tablet/1L, 1 tablet/2L, 1 tablet/3L, 1 tablet/4L, 1 tablet/5L, and 2 tablet/5L at time intervals of 24hrs,48hrs,72hrs and 96 hrs. once 100% larval mortality was attained within 96hrs, a fresh batch of larvae of 50 numbers were introduced to the same bioassay unit.

Statistical analysis

LC_{50} and LC_{90} values were determined using SPSS -16.0 software. Compusyn version 1 software was used for the TCC method synergism calculation.

Results

Essential oil yield

The yields of extracted essential oils from *P. amboinicus* leaves*,* Rhizome of *A. calamus, M. fragrans* seeds*, Citrus limon* leaves, and *H. suaveolens* leaves are 0.8%, 3.2%, 1%, 4% and 0.48% respectively.

GC-MS/MS analysis.

The GC-MS analysis of EO, obtained from *P. amboinicus* leaves contains chemical components that include thymol-74.93%, caryophyllene- 7.07%, and caryophyllene oxide 5.94%. The GC-MS analysis of EO, obtained from Rhizome (root) of *A. calamus* contains chemical components: γ-asarone 2.85%, α-asarone 85.51%, β-asarone 3.55%. The GC-MS analysis of EO, obtained from *citrus* lemon leaves *contains* chemical components: Linalool 3.73%, cis-geraniol - 4.05%, neral 22.98%, geraniol 4.82%, caryophyllene oxide 4.76%. The GC-MS analysis of EO, obtained from the nuts of *M. fragrans contains:* Terpinen-4-ol 27.13%, alpha- terpineol 3.76%, safrole 6.43%, methyleugenol 4.32%, caryophyllene 2.16%, myristicin 18.12, elemicin - 14.41%. The GC-MS analysis of EO, obtained from *H. suaveolens* leaves *contains:* Caryophyllene oxide 36.11%, 14-hydroxycaryophyllene 4.49%, abitatriene - 9.64%, totara-8,11,13-triene-7-β,13-diol - 5.06%. The GC-MS analysis of EO, obtained from *P. amboinicus leaves + M. fragrans* contains compounds Terpinen-4-ol 8.60%, carvacrol- 36.23%, thymol 39.04%, caryophyllene oxide 3.84%, safrole 2.76%. The GC-MS analysis of EO, obtained from *T. ammi* + *M. fragrans* contains (-)-Terpinen-4-ol - 7.56%, Thymol 61.96%, Caryophyllene 3.75%, Caryophyllene oxide 7.19% shown in Table 1a-g and Figure-3 a-g.

Individual EOs Larvicidal activity

The outcomes of the preliminary screening of the five EOs from *P. amboinicus, A. calamus, M. fragrans, Citrus lemon*, and *H. suaveolens* tested from 5ppm to 150ppm range, all essential oil showed complete larval mortality between 100 to 150ppm. Each EO's LC_{50} and LC_{90} values are obtained by considering a narrow range of 5 to 6 concentrations with mortality rates ranging from

10 % to 90%. The LC_{25}, LC_{50} and LC_{90} values are determined using by probit analysis in SPSS software 16.0. The LC_{50} values for mosquito larvicidal activity of obtained EOs are as follows: *P. amboinicus* – 47.34 ppm*, A. calamus* - 23.34 ppm, *M. fragrans* - 38.15 ppm, *citrus lemon - 45.78ppm* and *H. suaveolens* - 43.25 ppm shown in Table 2. *T. ammi* and *I. verum* individual larvcidal activity data taken from our previous study (Pandiyan et al. 2019) *T. ammi* LC_{50} 39.48 and *I. verum* LC_{50} 41.30. These LC_{50} dose concentrations prepared binary combinations for potentiation/antagonistic/additive interaction activity. The LC_{25} dose was calculated by probit analysis, and these LC_{25} dosages were used to prepare binary combinations for potentiation /antagonistic/additive interaction between the EOs activity testing.

Binary combination larvicidal activity

The obtained LC_{50} values of individual EOs are used for evaluating potentiation/antagonistic/additive interaction between the EOs in binary combinations while screening for their insecticidal activity. A binary combination of the seven essential oils produces around total of 21 combinations (BC_1-IT, BC_2-IP, BC_3-IA, BC_4-IM, BC_5-IC, BC_6-IH, BC_7-TP, BC_8-TA, BC_9-TM, BC_{10}-TC, BC_{11}-TH, BC_{12}-PA, BC_{13}-PM, BC_{14}-PC, BC_{15}-PH, BC_{16}-AM, BC_{17}-AC, BC_{18}-AH, BC_{19}-MC, BC_{20}-MH, BC_{21}-CH). All these combinations were assayed against *Ae. aegypti* larvae for establishing synergism between EOs by CTF, CSC (χ^2).

Co-toxicity Factor method (CTF) and Chi-square comparison method (χ^2) (CSC) for binary combination

Based on the individual essential oil of LC_{25} values of bioassay carried out against late 3rd instar larvae of *Ae. aegypti* and mortality observed at 48hrs. *I. verum* 26.67%: *T. ammi* 27.33 %: *P. amboinicus* 19.67 %: *A. calamus* 13 %: *M. fragrans* 41 %: *C. limon* 13.33 %: *H. suaveolens* 11.67%. Addition of LC_{25} dose for binary combination observed percentage mortality at 48hrs against late 3rd instar larvae of *Ae. aegypti*, IT-100; IP-100; IA-1.67; IM-100; IC-100; IH-90.67; TP-100; TA-52.67; TM-100; TC-100; TH-89; PA-58; PM-100; PC-74; PH-100; AM-84; AC-19; AH-16; MC-100; MH-100; CH-26, Table-3.

Wadley's method for binary combination

Each essential oil combination was made in a 1:1 ratio (LC_{50}/LC_{50}) and tested against *Ae. aegypti* larvae at various concentrations to ascertain the binary combinations' LC_{50} and LC_{90} values. The results are shown in Table 4. The LC_{50} values by SPSS -16.0 software were IT-34.274; IP-51.704;

IA-78.406; IM-24.573; IC-32.018; IH-69.208; TP-34.813; TA-41.500; TM-19.979; TC-28.739; TH-77.795; PA-44.191; PM-21.709; PC-36.023; PH-85.696; AM-27.922; AC-46.121; AH-64.147; MC-23.652; MH-32.018; CH-56.719; and LC_{90} values were IT-51.183; IP-63.650; IA-92.530; IM-32.492; IC-39.656; IH-95.061; TP-45.009; TA-52.592; TM-38.529; TC-44.138; TH-87.991; PA-56.370; PM-30.633; PC-54.734; PH-95.410; AM-44.054; AC-62.770; AH-70.903; MC-29.660; MH-39.656; CH-71.972.

Ting-Chao Chou method for binary combination:

Each essential oil combination was made in a 1:1 ratio (LC_{50}/LC_{50}) and tested against Ae. aegypti larvae at various concentrations to ascertain the binary combinations' LC_{50} and LC_{90} values. The results are shown in Table 5. The LC_{50} values by Compusyn version 1 were LC_{50}: IT 34.276; IP 51.476; IA 77.69; IM 24.49; IC 29.73; IH 69.10; *TP* 34.76; TA 41.51; TM 19.90; TC 28.79; TH 77.52; PA 44.43; PM 21.68; PC 35.87; PH 85.54; AM 27.67; AC 45.96; AH 63.97; MC 23.56; MH 31.93; CH 56.86. The comparison of Wadley's and Ting-Chao Chou's methods for binary calculation of synergism/additive/antagonism is displayed in Table 6.

Ternary combination larvicidal activity

Wadley's, and TCC methods ternary combination of seven EOs (TC_1-ITP, TC_2-ITA, TC_3-ITM, TC_4-ITC, TC_5-ITH, TC_6-IPA, TC_7-IPM, TC_8-IPC, TC_9-IPH, TC_{10}-IAM, TC_{11}-IAC, TC_{12}-IAH, TC_{13}-IMC, TC_{14}-IMH, TC_{15}-ICH, TC_{16}-TPA, TC_{17}-TPM, TC_{18}-TPC, TC_{19}-TPH, TC_{20}-TAM, TC_{21}-TAC, TC_{22}-TAH, TC_{23}-TMC, TC_{24}-TMH, TC_{25}-TCH, TC_{26}-PAM, TC_{27}-PAC, TC_{28}-PAH, TC_{29}-PMC, TC_{30}-PMH, TC_{31}-PCH, TC_{32}-AMC, TC_{33}-AMH, TC_{34}-ACH, TC_{35}-MCH) 35 ternary combinations, all these were analyzed according to Wadley's and Ting-Chao Chou method for establishing potentiation /antagonistic/additive interaction among the EOs.

Wadley's method for ternary combination:

Essential oil combinations in a 1:1:1 ratio ($LC_{50}/LC_{50}/LC_{50}$) were created and tested against *Ae. aegypti* larvae at various concentrations to determine the LC_{50} and LC_{90} values in ppm for the ternary mixes. The results are shown in Table 7. The LC_{50} values by SPSS -16.0 software were ITP-51.63; ITA-51.20; ITM-35.028; ITC-33.992; ITH-43.937; IPA-52.328; IPM-36.212; IPC-49.654; IPH-51.349, IAM-38.60, IAC-47.427, IAH-54.52, IMC-30.612, IMH-43.653, ICH-43.872, TPA-48.158, TPM-34.899, TPC-53.142, TPH-42.610, TAM-30.609, TAC-34.592, TAH-

45.542, TMC-39.073, TMH-42.690, TCH-42.785, PAM-37.978, PAC-50.872, PAH-39.321, PMC-38.847, PMH-39.514, PCH-47.665, AMC-48.37, AMH-42.73, ACH-47.549, MCH-44.603; and LC_{90} values were ITP-69.88; ITA-59.78; ITM-44.273; ITC-40.616; ITH-66.772; IPA-76.55; IPM-47.798; IPC-73.053; IPH-80.419; IAM-48.46; IAC-61.949; IAH-75.95; IMC-43.897; IMH-51.335; ICH-65.522; TPA-75.60; TPM-43.530; TPC-75.115; TPH-63.408; TAM-49.815; TAC-47.532; TAH- 73.153; TMC-58.719; TMH-52.146; TCH-54.914; PAM-64.321; PAC-109.371; PAH-46.131; PMC-53.869; PMH- 51.528; PCH-62.955; AMC-81.63; AMH-80.23; ACH- 73.973; MCH-55.853.

Ting-Chao Chou method for ternary combination:

A 1:1:1 essential oil combination (LC_{50}/LC_{50}/LC_{50}) was created and tested against Aedes aegypti larvae at various concentrations to determine the LC_{50} and LC_{90} values for the ternary combinations. The LC_{50} values by Compusyn version 1 were LC_{50}: ITP-51.39; ITA-51.08; ITM-35.0; ITC-33.83; ITH-44.06; IPA-51.76; IPM-36.26; IPC-49.69; IPH-51.49; IAM-38.795; IAC-47.25; IAH-54.56; IMC-30.56; IMH-43.60; ICH-43.56; TPA-41.97; TPM-34.86; TPC-52.71; TPH-42.17; TAM-30.41; TAC-34.60; TAH-45.67; TMC-39.00; TMH-42.48; TCH-42.77; PAM-37.97; PAC-51.60; PAH-39.15; PMC-38.8; PMH-39.72; PCH-47.73; AMC-48.75; AMH-42.90; ACH-47.75; MCH-44.46, results are given in Table 8. The comparison of Wadley's and Ting-Chao Chou's methods for ternary calculation of synergism/additive/antagonism is displayed in Table 9.

Larvicidal toxicity of formulated material (soft gel capsules)

The effective binary combination TM (*T. ammi +M. fragrans*) selected for the further formulation, in these PM (*P. ambonicus+M. fragrans*) combination also very effective but consideration of for *P. ambonicus* essential oil yield is very low based on this we selected TM combination for formulation. One tablet in 1 Liters water - showed 100% mortality at 24hrs. After introducing 50 larvae in the same solution, they again showed a percentage mortality at 24hrs, again, larvae were introduced in the same set of solutions, but activity decreased at 24 and 48 hrs. 1tablet in 2liters water - 100 % mortality shown in 24 hrs and the same solution after 48hrs activity decreased; 1tablet in 3liters water - at 48hrs only showed 100% mortality, after introducing larvae in this same solution, less larval mortality displayed; 1 tablet in 4 Liters water at 48hrs only showed 100% mortality, same solution showed very less mortality after 72hrs. 1 tablet in 5Liters water- 100% mortality at 72hrs showed, 2 tablets in 5liters water – in 48hrs 100% mortality shown.

Discussions

GC-MS Analysis

It is well recognized that a variety of factors, including plant age, seasonal variations, geographic origin, development stage, and extraction technique, can significantly alter the chemical makeup of essential oils. The main components of *P. amboinicus* leaves essential oil in the current investigation were thymol (74.93%), caryophyllene (7.07%), and caryophyllene oxide (5.94%). These findings are consistent with those of Oliveira et al. (2011), who found that thymol (75.39%) and caryophyllene (10.52%) were the most common compounds in the spring. De Costa et al. 2010) 64.3 % of Thymol, in the other hand, Senthilkumar et al., reported Thymol (21.66%), it has less content than that of our study (Senthilkumar and Venkatesalu 2010). It is well recognized that the age of the plant, seasonal and geographic variations, and extraction technique all affect the chemical makeup of the essential oils. These influences change the quantity of the particular molecule like Carvacrol, thymol, beta-caryphellene and caryophellene oxides (Maldaner et al. 2021; Blacio et al. 2025).

According to Baser et al., the percentage of carvacrol in the essential oil of *Origanum* species varies depending on the geographic location, ranging from 23.4% to 80.4%. (Baser et al. 1993). In our study *H. suaveolens* leaves Eos contain Caryophyllene oxide 36.11%, 14-hydroxycaryophyllene 4.49%, abitatriene - 9.64%, podocarpa-8,11,13-triene-7 beta.,13-diol, 14-isopropyl- 5.06%, the result matched with Pino et al., caryophyllene oxide (35.1%) (Pino et al. 2003). *H. suaveolens* EO chemical composition beta-caryophyllene, caryophyllene oxide, eucalyptol and other sesquiterpenes found that varies according growth and environment (Sonigra and Meena 2005).

M. fragrans nuts EO chemical contents in our study Terpinen-4-ol 27.13%, alpha- Terpineol 3.76%, Safrole 6.43%, Methyleugenol 4.32%, Caryophyllene 2.16%, Myristicin 18.12%, Elemicin - 14.41%. This chemical component matched with SP Piaru et al., Terpinen-4-ol 27.13%, Myristicin 6.8% (Piaru et al. 2012). It is widely known that *M. fragrans* essential oil can alter, and the levels of terpinene-4-ol, safrole, methyleugenol, myristicin, and elemicin can be influenced by factors such as the environment and how the plant is handled after collection (Ashokkumar et al. 2022; Khairan et al. 2024).

Acorus calamus rhizome chemical content in our EO, gamma-Asarone 2.85%, Asarone 85.51%, beta-Asarone 3.55%, Liu et al. reported alpha-asarone (50.09%), (E)-Methyl eugenol (14.01%), and Eugenol methyl ether (8.59%), followed by beta-asarone (3.51%), aplha-cedrene (3.09%) and camphor (2.42%) (Liu et al. 2013). The *A. calamus* essential oil chemical composition alpha and beta-asarone quantity significantly varies. Asarone quantity increase in some place 90%, whilst in others the forms of swsquiterpenoids varies greatly (Wijerathna et al. 2023; Sytykiewicz et al. 2025).

Citrus Limon leaves EO chemical content in our study, Linalool 3.73%, cis-Geraniol - 4.05%, Neral 22.98%, Geraniol 4.82%, Citral 27.55%, Limonen-1,2-diol - 2.76%, 2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)- 2.09%, Caryophyllene 2.30%, Caryophyllene oxide 4.76%. Wen et al., According to a Chinese article, the principal ingredient (21.3%) of the leaf oil of *C. limon* geranial is cis-Citral (14.0%), Cajeputene (9.9%), Neryl alcohol (4.5%), cis-Geranylacetone (2.8%), β-Citronellal (2.6%). Kamiyama reported β-Pinene & Sabinene 12.4%, Limonene 23.1%, neral 16.4%, and Geranial 24.3% and Yahaya et al., reported Carvene (22.82%), Neral (25.20%), and cis-2-Pinen-4-ol (20.43%) for *C. limon* leaf EOs (Wen et al.1989; Kamiyama 1967;Yahaya et al. 2019). Kacaniova et al. observed the essential oil of *C. limon* variability within the expected range of thymol, caryophyllene, caryophyllene oxide, myristicin, alpha/beta-asarone and citral (Kacaniova et al. 205). The GC-MS analysis of EO, obtained from *P. amboinicus leaves* + *M. fragrans* contains compounds Terpinen-4-ol 8.60%, Phenol, 2-methyl-5-(1-methylethyl)- 36.23%, Thymol 39.04%, Caryophyllene oxide 3.84%, Safrole 2.76%. The GC-MS analysis of EO, obtained from *T. ammi* + *M. fragrans* contains (-)-Terpinen-4-ol - 7.56%, Thymol 61.96%, Caryophyllene 3.75%, Caryophyllene oxide 7.19%. In our previous study for *T. ammi* essential oil chemical compostion Terpinen-4-ol (2.16%) and thymol (93.58%) were the primary components of *T. ammi* essential oil (Pandiyan et al. 2019).

Larvicidal activity for individual essential oil

This study's larvicidal effects are consistent with recent studies that highlight the strong mosquito-control potential of plant-derived essential oils. In our study *P. amboinicus* leaf essential oil contain higher quantity of thymol content, with combination of other chemical constitute it was displayed remarkable larvicidal activity against *Ae. aegypti* mosquito late 3rd instar larvae, LC_{50} and LC_{90} values of 47.34 and 74.63 ppm respectively. Carvacrol higher quantity contain same plant essential oil shown larvicidal effects against *Ae. aegypti* larvae with LC_{50} and LC_{90} values of 28.52 and 49.55ppm respectively (Pereira APM et al. 2021). Michele et al. (2011) reported carvacrol-based larvicidal activity with an LC_{50} of 58.9 µg mL^{-1}, but Huang et al. (2019) reported LC_{50} and LC_{90} values of 42.90 µg mL^{-1} and 61.47 µg mL^{-1} at 24 hours against *Ae. aegypti* larvae, with carvacrol and β-caryophyllene as the major ingredients. *M. fragrans* fruit EO our result for mosquito *Ae. aegypti* larvae LC_{50} 38.14 and LC_{90} 53.22ppm. Carolina and Maman reported 110.1µg/mL against *Ae. aegypti* larvae, and Gomes et al., the maximum larval mortality was recorded with EO *M. fragrans* (LC_{50} = 28.2 and LC_{90} = 41.7 microgram mL^{-1}). Larvicidal activity of *M. fragrans* essential oil is comparable with recent research using nanoliposomes and nanoemulsions, which exhibit higher toxicity against *Aedes* species (Gomes et al. 2019; Silva et al. 2025; Ali et al. 2025a)

In our result *H. suaveolens* leaves EO for mosquito *Ae. aegypti* larvae LC_{50} 45.78 and LC_{90} 60.86ppm. Previous research *H. suaveolens* leaves EO larvicidal activity against *Ae. albopictus* LC_{50} 240.3ppm at 24hrs (Conti et al. 2012). The current findings, however, are consistent with those of Moola et al. (2022), who demonstrated considerable, dose-dependent larval mortality against *Ae. aegypti*, with maximum lethality at 100ppm, respectively. Despite variances in geographic origin and experimental conditions, the close agreement across these studies confirms the long-term larvicidal activity of *H. suaveolens* essential oil. In our current study of *A. calamus* rhizome EOs for *Ae. aegypti* larvae LC_{50} 23.71 and LC_{90} 60.86 ppm. These results are more harmful than those reported Manzoor et al*., A. calamus* EO against *Ae. aegypti* larvae LC_{50} 99.41 ppm (Manzoor et al. 2013).

In a similar vein, *Citrus limon* leaf essential oil demonstrated potent larvicidal activity against *Ae. aegypti* larvae, with values of 43.34 ppm and 63.01ppm. Gomes et al. (2018) found *C. limon* peel essential oil to be effective against *Ae. aegypti* larvae (LC_{50}=15.5ppm), while Campolo et al. (2016) reported an LC_{50} value of 117.69 ppm against *Ae. albopictus* after 48 hours. Previous research found varying toxicity depending on plant section and species. The larvicidal activity of *C. limon* leaf oil is characterized by high proportions of neral (22.98%), geraniol (4.82%), and citral (27.55%), which is similar to recent findings by Ali et al. (2025b), who reported strong lemon essential oil toxicity against *Ae. aegypti* larvae with an LC_{50} value of 33.43 ppm, associated with β-pinene (26.8%) and limonene (43.1%) as major constituents. Overall, the current results are in line with worldwide patterns that validate essential oils as strong, environmentally friendly larvicidal treatments with encouraging potential for controlling mosquito vectors.

Chemical interactions between main and minor ingredients have a significant impact on larvicidal efficacy, as seen by the interaction effects seen various essential oil combinations. The main components of Rosmarinus officinalis essential oil, 1,8-cineole and camphor, have been demonstrated to work in concert to increase insecticidal efficiency (Tak et al. 2016). This is just one example of the reported synergistic interactions among essential oil components. In a similar vein, myristicin and safrole can work in concert with pesticides to increase their harmful effects, according to Casida (1970). Significant amounts of myristicin (18.12%) and safrole (6.43%) were found in the current study's Myristica fragrans essential oil, which may help to explain the strong synergistic effects shown when this oil was mixed with other essential oils.

Binary combination effects

Based on similar larvicidal bioassay protocols, binary combinations were assessed using CTF, CSC, techniques against *Ae. agypti* mosquito late 3rd instar larvae. Seventeen binary combinations showed significant synergistic larvicidal effects in the CTF and CSC techniques. (Table 3; Figure 4). In CTF method for *A. calamus* with *I. verum* IA (CTF=-54.7)*, H. suaveolens* AH (CTF=-35.14)*,* and *C. limon* AC (CTF = -27.8) observed antagonistic larvicidal effect, only one combination *C. limon* with *H. suaveolens* showed (CTF=4) additive larvicidal effect in CTF method. While CSC method IA combination shown antagonistic larvicidal effect ($\chi2$ = 4.49) and other three combination AH ($\chi2$=1.39), AC ($\chi2$=0.67), CH ($\chi2$=0.86) shown no effect on larvicidal. (Table 3; Figure 5).

Using Wadley's method among 21 binary combinations tested against *Ae. agypti* mosquito late 3rd instar larvae, only four combinations R value more than 1.5, IM (R=1.61), TM (R=1.94), PM (R=1.97), and MC (R=1.72) these combinations showed synergistic larvicidal effect. Only one combination IA (R=0.41) showed antagonistic larvicidal effect. Remaining seventeen combinations observed additive larvicidal effect R value between 0.5 to 1.5 (Figure 6; Table 6).

These patterns of interaction were further validated by the Ting–Chao Chou (TCC) approach. IP, IA, AC, IH, TA, TH, PA, PH, AC, AH, and CH showed antagonistic larvicidal effect (CI value more than 1 at 0.5 Fa). Remaining ten binary combinations shown synergistic larvicidal effect (CI value less than 1 at 0.5 Fa) (Figure 7 and 8a). IM, PM, IC, and TM combinations showed high synergistic interactions, while IA, IH, and TH showed strong antagonistic effects. The remaining combinations indicated additive or weak antagonistic effects, according to polygonogram analysis (Figure 8b).

When combined, thymol and carvacrol have more antibacterial activity, antioxidant, anti-inflammatory, and anticancer capabilities against breast cancer than when used separately (Kissels et al. 2017; Taibi et al. 2024). Increased microbial cell membrane rupture is linked to this synergy. Carvacrol was only found in trace amounts in the individual essential oils of *P. amboinicus* (0.74%) and *M. fragrans* in the current study. However, in their binary combination, carvacrol increased significantly to 36.3%, which may explain the strong synergistic effect seen for the *P. amboinicus* + *M. fragrans* mixture. According to in vitro research, asarone may adversely affect biological activity in specific mixtures because combinations of asarone isomers with flavonoids can show antagonistic interactions that reduce cytotoxic effects when compared to individual compounds (Thomas et al. 2019). In line with this finding, the combination of *A. calamus* and *I. verum* essential oils in the current investigation showed antagonistic effects using all four interaction assessment techniques.

Ternary combination effects

All ternary combinations showed additive effects in ternary mixture analysis, according to Wadley's technique (Figure 9; Table 9), indicating little interaction complexity under this paradigm. With 18 of the 35 ternary combinations (ITM, ITC, IPM, IAM, IMC, IMH, TPM, TPH, TAM, TAC, TMC, TMH, TCH, PAH, PMC, PMH, PCH, and MCH) demonstrating synergistic effects and the remaining 17 combinations displaying antagonistic effects, the TCC method, on the other hand, revealed greater interaction diversity (Figure 10). These results collectively show that binary combinations, especially when assessed using CTF, CSC, and TCC techniques, typically result in stronger and more reliable synergistic larvicidal effects than ternary combinations. These findings are consistent with recent research, which shows that interactions between monoterpenes and sesquiterpenes improve the larvicidal efficiency of essential oil blends. Similar binary-mixture synergy was seen in monoterpene blend systems (Sittichok et al. 2025) and against *Anopheles gambiae* (Wangrawa et al. 2022).

While Wadley's R-value (R>1.5) is used for interpretation, the current study's use of the Chou-Talalay CI approach is consistent with recent CI-based synergy models for terpenoid nanoemulsions and thymol-eugenol systems (Lucia et al. 2020). The chemicals 1,8-cineole, geranial, trans-anethole, and cinnamaldehyde exhibit greater toxicity when used together rather than separately, which is consistent with the high synergistic effects observed in mixes comprising *M. fragans* (Duarte et al. 2024; Sittichok et al. 2025). Overall, these comparisons demonstrate the potential of essential oil mixtures for mosquito larvae control and show that the combination effects observed in this study are consistent with the global synergy patterns identified for these mixtures. When compared to individual essential oils, the most successful binary combinations (PM and TM) were created at their respective LC_{50} ratios, resulting in noticeable changes in the chemical profile. The higher larvicidal activity seen in the PM and TM combinations is likely owing to the redistribution and enrichment of oxygenated monoterpenes and phenylpropanoids in these binary mixes.

Soft-gel formulation

Formulation-based delivery systems have recently gained popularity as a means of improving the stability, controlled release, and field use of biolarvicides. Water-based neem oil emulsions have been shown in previous field-oriented studies to effectively suppress Cx. quinquefasciatus, An. stephensi, and Ae. aegypti breeding in water storage tanks, pools, and containers for up to two to three weeks (Batra et al. 1998). Neem oil emulsions have been reported to exhibit prolonged larvicidal activity and enhanced persistence under experimental conditions (Anjali et al. 2012; Jadhav et al. 2021). In a same spirit, it has been demonstrated that chitosan-based hydrogels, like ChitH@Bti-Y, may maintain Bacillus thuringiensis israelensis (Bti) activity for prolonged periods of time while causing rapid larval mortality, reaching total larval death in 12 hours (Piazzoni et al. 2022; Negri et al. 2023). Chitosan hydrogels' brittle structure and biocompatibility encourage larval recruitment and make it easier for active chemicals to be released during feeding, underscoring its potential as environmentally friendly biolarvicide delivery systems.

Both water volume and larvae density had a significant impact on the larvicidal effectiveness of the soft-gel capsule-like formulation in the current investigation. There were very slight differences in fatality rates amongst the three mosquito species (*Cx. quinquefasciatus, An. stephensi*, and *Ae. aegypti*). One capsule in one liter of water resulted in 100% larval mortality after 24 hours and continued to be effective after a second introduction of larvae, while additional reuse at 24 and 48 hours showed decreased activity. Complete mortality was likewise attained in 24 hours in 2 liters of water, however larvicidal efficacy decreased after 48 hours, suggesting little residual activity. Complete death was postponed to 48 hours in greater amounts (3–4 L), and following larval reintroduction, efficacy significantly declined. While two capsules of the same capacity produced 100% mortality in 48 hours, a single capsule of 5 L took 72 hours to reach full mortality, it shown in graph Figure-11. These findings show that the soft-gel capsule formulation's larvicidal efficiency is dose and dilution dependent, and that repeated dosing or improved capsule-to-water ratios may be necessary for long-term field effectiveness. All of these results support the idea that chemical interactions and formulation techniques are crucial for improving the usefulness of larvicides based on essential oils for controlling mosquito vectors.

Conclusion

The larvicidal activity of essential oils derived from seven medicinal plants against *Aedes aegypti* was assessed in this study. The LC_{50} values of all effective oils were less than 50 ppm. When tested separately, *Acorus calamus* and *Myristica fragrans* demonstrated greater larvicidal effects, which is in line with the highest concentration of asarone and terpinene-4-ol found by GC-MS. Several binary mixes outperformed single oils when combined. Larval mortality was clearly improved in combinations of *P. amboinicus* with *M. fragrans* and *T. ammi* with M. fragrans. Thymol, carvacrol, and caryophyllene oxide were found in these combinations by GC-MS analysis, which may have contributed to the observed activity. The combination of *T. ammi* and *M. fragrans* was chosen for formulation and made into soft-gel capsules based on oil yield and biological activity. Complete larval mortality was the outcome of this formulation. Under lab circumstances, full mortality was likewise attained in 24 hours with 1 capsule in 2 liters of water. The findings suggest that, with the right formulation, essential oil mixtures might be regarded as viable substitutes for traditional larvicides.

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Table 1a: GC-MS Chemical composition of *Plectranthus ambonicus* leaf essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	Terpinen-4-ol	$C_{10}H_{18}O$	154.1	5.36	1206	1.75
2	Carvacrol	$C_{10}H_{14}O$	150.1	7.0	1309	0.74
3	Thymol	$C_{10}H_{14}O$	150.1	7.21	1323	74.93
4	Caryophyllene	$C_{15}H_{24}$	204.2	9.12	1454	7.07
5	1,1,4,8-tetramethyl-cis,cis,4,7,10-cycloundecatriene	$C_{15}H_{24}$	204.2	9.58	1488	2.34
6	β-Selinene	$C_{15}H_{24}$	204.2	10.03	1522	3.46
7	4,11-selinadiene	$C_{15}H_{24}$	204.2	10.14	1530	0.69
8	Caryophyllene oxide	$C_{15}H_{24}O$	220.2	11.31	1624	5.94
9	Humulene-1,2-epoxide	$C_{15}H_{24}O$	220.2	11.63	1651	1.45
10	14-Hydroxycaryophyllene	$C_{15}H_{24}O$	220.2	12.31	1709	1.63

Table 1b: GC-MS Chemical composition of *Acorus calamus* root essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	Anethole	$C_{10}H_{12}O$	148.1	7.02	1310	0.89
2	cis-Methyl isoeugenol	$C_{11}H_{14}O_2$	178.1	9.46	1479	2.04
3	Shyobunone	$C_{15}H_{14}O$	220.2	10.08	1526	0.88
4	Cadina-3,9-diene	$C_{15}H_{24}$	204.2	10.45	1555	0.27
5	Isoshyobunone	$C_{15}H_{24}O$	220.2	10.56	1563	0.51
6	Elemicin	$C_{12}H_{16}O_3$	208.1	10.76	1579	0.82
7	γ-Asarone	$C_{12}H_{16}O_3$	208.1	11	1598	2.85
8	Alpha -Asarone	$C_{12}H_{16}O_3$	208.1	11.7	1657	85.51
9	Dehydroxy-isocalamendiol	$C_{15}H_{24}O$	220.2	11.91	1675	0.51
10	ent-Germacra-4(15),5,10(14)-trien-1β-ol	$C_{15}H_{24}O$	220.2	12.25	1703	0.3
11	β-Asarone	$C_{12}H_{16}O_3$	208.1	12.29	1707	3.55
12	(3bR,4S,7aS)-7,7,8,8-Tetramethyloctahydro2,3bmethanocyclopenta[1,3]cyclopropa[1,2]benzen-4-ol	$C_{15}H_{24}O$	220.2	12.4	1717	0.54
13	Acoramone	$C_{12}H_{16}O_4$	224.1	13.09	1777	0.27
14	Isocalamenediol	$C_{15}H_{26}O_2$	238.2	13.23	1789	1.06

Table 1c: GC-MS Chemical composition of *Myristica fragrans* seed essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	(Z)-Sabinene hydrate	$C_{10}H_{18}O$	154.1	3.73	1101	1.19
2	Linalool	$C_{10}H_{18}O$	154.1	4.05	1121	1.65
3	Terpinen-4-ol	$C_{10}H_{18}O$	154.1	5.36	1206	27.13
4	alpha- Terpineol	$C_{10}H_{18}O$	154.1	5.54	1217	3.76
5	(+)-Borneol acetate	$C_{12}H_{20}O_2$	196.1	7.03	1311	1.34
6	Safrole	$C_{12}H_{20}O_2$	162.1	7.08	1314	6.43
7	3-Methyl-4-isopropylphenol	$C_{10}H_{14}O$	150.1	7.17	1320	1.96
8	Citronellol acetate	$C_{12}H_{22}O_2$	198.2	7.93	1371	1.17
9	α-Terpinene	$C_{10}H_{16}$	136.1	7.96	1373	0.93
10	alpha-Cubebene	$C_{15}H_{24}$	204.2	8.02	1377	0.56
11	Eugenol	$C_{10}H_{12}O_2$	164.1	8.09	1381	0.52
12	Geranyl acetate	$C_{10}H_{12}O_2$	196.1	8.38	1401	1.2
13	Copaene	$C_{15}H_{24}$	204.2	8.45	1406	2.13
14	Methyl eugenol	$C_{11}H_{14}O_2$	178.1	8.71	1425	4.32
15	Caryophyllene	$C_{15}H_{24}$	204.2	9.11	1454	2.16
16	cis-alpha-Bergamotene	$C_{15}H_{24}$	204.2	9.24	1463	0.81
17	cis-Methyl isoeugenol	$C_{11}H_{14}O_2$	178.1	10.01	1520	0.72
18	beta-Bisabolene	$C_{15}H_{24}$	204.2	10.21	1536	0.34
19	Myristicine	$C_{11}H_{12}O_3$	192.1	10.4	1551	18.12
20	Cadina-1(10),4-diene	$C_{15}H_{24}$	204.2	10.45	1555	1.15
21	Elemicin	$C_{12}H_{16}O_3$	208.1	10.76	1579	14.41
22	Guaiol	$C_{15}H_{26}O$	222.2	11.42	1633	0.24
23	Isoelemicin	$C_{12}H_{26}O_3$	208.1	11.94	1677	0.42
24	Tetracosane	$C_{24}H_{50}$	338.4	21.84	2421	1.34
25	Hexacosane	$C_{26}H_{54}$	366.4	24.73	2624	1.65
26	Heptacosane	$C_{27}H_{56}$	380.4	26.13	2726	1.31

Table 1d: GC-MS Chemical composition of *Citrus limon* leaf essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	Linalool	$C_{10}H_{18}O$	154.1	4.06	1122	3.73
2	cis-2-Menthenol	$C_{10}H_{16}O$	152.1	4.66	1161	0.36
3	(+)-(E)-Limonene oxide	$C_{10}H_{16}O$	152.1	4.72	1165	0.49
4	(R)-(+)-Citronellal;	$C_{10}H_{18}O$	154.1	4.85	1173	1.66
5	alpha- Terpineol	$C_{10}H_{18}O$	154.1	5.54	1217	0.55
6	Decanal	$C_{10}H_{20}O$	156.2	5.65	1224	1.03
7	2-Isopropenyl-5-methylhex-4-enal	$C_{10}H_{16}O$	152.1	5.99	1245	1.03
8	cis-Geraniol	$C_{10}H_{18}O$	154.1	6.04	1248	4.05
9	Neral	$C_{10}H_{16}O$	152.1	6.27	1263	22.98
10	Geraniol	$C_{10}H_{18}O$	154.1	6.42	1272	4.82
11	Citral	$C_{10}H_{16}O$	152.1	6.72	1291	27.55
12	(R)-Lavandulol	$C_{12}H_{20}O_2$	196.1	6.86	1299	0.35
13	Epoxy-linalooloxide	$C_{10}H_{18}O_3$	186.1	6.9	1302	0.62
14	Safrole	$C_{10}H_{10}O_2$	162.1	7.08	1314	0.39
15	Carvacrol	$C_{10}H_{14}O$	150.1	7.18	1321	0.69
16	1,8-Diazacycloundecan-2,9-dione	$C_9H_{16}N_2O_2$	184.1	7.23	1324	0.8
17	Limonen-1,2-diol	$C_{10}H_{18}O_2$	170.1	7.81	1363	2.76
18	Geranic acid	$C_{10}H_{16}O_2$	168.1	7.91	1369	1.28
19	Nerol acetate	$C_{12}H_{20}O_2$	196.1	8.1	1382	2.09
20	Yamogi alcohol	$C_{10}H_{18}O$	154.1	8.31	1396	0.37
21	Geranyl acetate	$C_{12}H_{20}O_2$	196.1	8.38	1401	8.69
22	β-Elemen	$C_{15}H_{24}$	204.2	8.65	1420	1.08
23	Caryophyllene	$C_{15}H_{24}$	204.2	9.11	1454	2.3
24	β-Chamigrene	$C_{15}H_{24}$	204.2	10.14	1530	0.58
25	beta-Bisabolene	$C_{15}H_{24}$	204.2	10.21	1536	0.39
26	14-Hydroxycaryophyllene	$C_{15}H_{24}O$	220.2	10.65	1570	0.33
27	Caryophyllene oxide	$C_{15}H_{24}O$	220.2	11.31	1624	4.76
28	Isospathulenol	$C_{15}H_{24}O$	220.2	11.8	1665	1.28
29	Neointermedeol	$C_{15}H_{26}O$	222.2	12.15	1695	1
30	(-)-Spathulenol	$C_{15}H_{24}O$	220.2	12.31	1709	0.35
31	alpha-Bisabolol	$C_{15}H_{26}O$	222.2	12.42	1718	0.25
32	Isospathulenol	$C_{15}H_{24}O$	220.2	12.83	1754	0.22
33	Phytol	$C_{20}H_{40}O$	296.3	17.75	2135	0.39
34	3,7-Nonadien-2-ol, 4,8-dimethyl-	$C_{11}H_{20}O$	168.2	19.14	2233	0.77

Table 1e: GC-MS Chemical composition of *Hyptis suaveolens* leaf essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	(Z)-Sabinene hydrate	$C_{10}H_{18}O$	154.1	3.73	1101	1.13
2	Pinocarvone	$C_{10}H_{14}O$	150.1	5.17	1194	0.77
3	Terpinen-4-ol	$C_{10}H_{18}O$	154.1	5.36	1206	3.35
4	p-cymenol	$C_{10}H_{14}O$	150.1	5.42	1209	3.57
5	trans-5-Isopropyl-6,7-epoxy-8-hydroxy-8-methylnonan-2-one	$C_{13}H_{24}O_3$	228.2	5.55	1218	1.94
6	(1R)-(-)-Myrtenal	$C_{10}H_{14}O$	150.1	5.69	1226	2.02
7	trans-Ascaridol glycol	$C_{10}H_{18}O_2$	170.1	6.78	1294	0.43
8	p-Cymen-7-ol	$C_{10}H_{14}O$	150.1	7.07	1313	0.81
9	9-Ethylbicyclo(3.3.1)nonan-9-ol	$C_{10}H_{20}O$	168.2	7.6	1349	0.9
10	(1S,2S,4S)-4-Isopropyl-1-methylcyclohexane-1,2,4-triol;	$C_{10}H_{20}O_3$	188.1	9.84	1507	0.48
11	β-Selinene	$C_{10}H_{24}$	204.2	10.03	1522	1.64
12	Ageratriol	$C_{15}H_{24}O_3$	252.2	10.9	1590	2.47
13	Spatulenol	$C_{15}H_{24}O$	220.2	11.22	1616	2.24
14	Caryophyllene oxide	$C_{15}H_{24}O$	220.2	11.32	1624	36.11
15	Humulene-1,2-epoxide	$C_{15}H_{24}O$	220.2	11.63	1650	3.1
16	Caryophylla-4(12),8(13)-dien-5.beta.-ol	$C_{15}H_{24}O$	220.2	11.93	1676	0.68
17	Neointermedeol	$C_{15}H_{26}O$	222.2	12.15	1694	2.13
18	14-Hydroxycaryophyllene	$C_{15}H_{24}O$	220.2	12.31	1708	4.49
19	5-Hydroxy-10,10-dimethyl-6-methylenebicyclo[7.2.0]undecan-2-one	$C_{14}H_{22}O_2$	222.2	12.49	1724	1.63
20	5,5,8a-Trimethyldecalin-1-one	$C_{13}H_{22}O$	194.2	12.99	1768	0.32
21	alpha-Cyperone	$C_{15}H_{22}O$	218.2	13.28	1793	0.49
22	cis-Z-alpha-Bisabolene epoxide	$C_{15}H_{24}O$	220.2	13.86	1840	1.13
23	Abitatriene	$C_{20}H_{30}$	270.2	17.25	2100	9.64
24	Methandriol 3-Acetate	$C_{22}H_{34}O_3$	346.3	19.53	2260	1.26
25	9(11)-Dehydrotestosterone	$C_{19}H_{26}O_2$	286.2	19.86	2283	0.66
26	Totara-8,11,13-triene-7-β,13-diol	$C_{20}H_{30}O_2$	302.2	20.69	2340	5.06
27	Dehydroabietol	$C_{20}H_{30}O$	286.2	21.62	2405	2.23
28	Delta-9(11)-Methyltestosterone	$C_{20}H_{28}O_2$	300.2	25.35	2669	1.82

Table 1f: GC-MS Chemical composition of *Plectranthus ambonicus* leaf + *Myristica fragrans* seed, mixed essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	(Z)-Sabinene hydrate	$C_{10}H_{18}O$	154.1	4.15	1128	0.74
2	Terpinen-4-ol	$C_{10}H_{18}O$	154.1	5.35	1205	8.6
3	alpha- Terpineol	$C_{10}H_{18}O$	154.1	5.53	1216	0.98
4	4,11-Dimethyl-8-(propan-2-yl)-5,12-dioxatricyclo[9.1.0.04,6] dodecan-7-ol	$C_{15}H_{26}O_3$	254.2	6.56	1281	0.63
5	Carvacrol	$C_{10}H_{14}O$	150.1	7.01	1309	36.23
6	Safrole	$C_{10}H_{10}O_2$	162.1	7.08	1314	2.76
7	Thymol	$C_{10}H_{14}O$	150.1	7.18	1321	39.04
8	Methyl eugenol	$C_{11}H_{14}O_2$	178.1	8.71	1425	0.55
9	Caryophyllene	$C_{15}H_{24}$	204.2	9.11	1454	1.17
10	Humulene	$C_{15}H_{24}$	204.2	9.58	1488	0.46
11	β-Selinene	$C_{15}H_{24}$	204.2	10.03	1522	1.07
12	Myristicin	$C_{11}H_{12}O_3$	192.1	10.4	1551	2
13	Elemicin	$C_{12}H_{16}O_3$	208.1	10.76	1579	1.25
14	Caryophyllene oxide	$C_{15}H_{24}O$	220.2	11.3	1623	3.84
15	4a-Methyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	$C_{11}H_{18}$	150.1	15.07	1938	0.68

Table 1g: GC-MS Chemical composition of *Trachyspermum ammi* seed + *Myristica fragrans* seed, mixed essential oil:

S. No	Molecule	Mol. Formula	Mol. weight	Retention time	Retention index	% of compound
1	Terpinolene	$C_{10}H_{16}$	136.1	4	1118	1.11
2	(Z)-Sabinene hydrate	$C_{10}H_{18}O$	154.1	4.15	1128	0.86
3	Terpinen-4-ol	$C_{10}H_{18}O$	154.1	5.36	1206	7.56
4	alpha- Terpineol	$C_{10}H_{18}O$	154.1	5.54	1217	0.91
5	Carvacrol	$C_{10}H_{14}O$	150.1	7.01	1309	0.85
6	Safrole	$C_{10}H_{10}O_2$	162.1	7.08	1314	2.86
7	Thymol	$C_{10}H_{14}O$	150.1	7.21	1323	61.96
8	Methyl eugenol	$C_{11}H_{14}O_2$	178.1	8.71	1425	0.42
9	Caryophyllene	$C_{15}H_{24}$	204.2	9.12	1454	3.75
10	1,1,4,8-Tetramethyl-cycloundeca-4Z,7Z,10Z-triene.	$C_{15}H_{24}$	204.2	9.58	1488	1.36
11	β-Selinene	$C_{15}H_{24}$	204.2	10.03	1522	2.7
12	Selinene	$C_{15}H_{24}$	204.3	10.14	1530	0.53
13	Myristicin	$C_{11}H_{12}O_3$	192.1	10.4	1551	1.44
14	Elemicin	$C_{12}H_{16}O_3$	208.1	10.76	1579	0.91
15	Caryophyllene oxide	$C_{15}H_{24}O$	220.2	11.31	1624	7.19
16	Humulene-1,2-epoxide	$C_{15}H_{24}O$	220.2	11.63	1651	1.73
17	14-Hydroxycaryophyllene	$C_{15}H_{24}O$	220.2	12.31	1709	0.97
18	Rosefuran	$C_{10}H_{14}O$	150.2	14.6	1902	0.41
19	4a-Methyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	$C_{11}H_{18}$	150.1	15.07	1938	1.18
20	Asteriscunolide A	$C_{15}H_{18}O_3$	246.1	15.43	1965	1.31

Table 2: Individual essential oil larvicidal activity against *Ae. aegypti* larvae

Plant	LC_{50} in ppm (95% CI)	LC_{90} in ppm (95% CI)	Chi-square χ^2	Df
*I. verum**	41.30 (39.57–42.87)	59.04 (56.11–63.05)	0.256	3
*T. ammi**	39.48 (38.21–40.57)	51.57 (49.46 – 54.00)	2.99	3
P. amboinicus	47.343 (45.244-49.480)	74.629 (69.345-82.247)	3.109	3
A. calamus	23.71 (18.89 - 429.33)	48.500 (37.79- 74.21)	7.60	3
C. limon	43.343 (42.024-44.672)	63.006 (59.749-67.448)	3.058	5
M. fragrans	38.145 (36.906-39.352)	53.224 (50.352-57.468)	1.274	3
H. suaveolens	45.781 (44.302-47.118)	60.863 (58.356-64.373)	4.935	3

CI=Confidential Intervals, Df = Degree of freedom; * larvcidal data taken from our previous study (Pandiyan et al. 2019).

Table 3: Larvicidal activity at LC_{25} dose against *Ae. aegypti* larvae for comparision of CTF and CSC method

Sl. No.	Compound		Pure compound % mortality at LC_{25} concentration		Binary Combination (AB)		CTF method		CSC method	
	A	B	A	B	Expected mortality %	Observed mortality %	CTF	Effect	$\chi 2$	Effect
1	*I. verum*	*T. ammi*	26.67	27.33	39.58	100	85.2	P	92.23	S
2	*I. verum*	*P. amboinicus*	26.67	19.67	33.97	100	115.8	P	128.3	S
3	*I. verum*	*A. calamus*	26.67	13	29.1	1.67	-54.7	AG	4.49	AG
4	*I. verum*	*M. fragrans*	26.67	41	49.62	100	47.8	P	51.15	S
5	*I. verum*	*C. limon*	26.67	13.33	29.32	100	150	P	170.3	S
6	*I. verum*	*H. suaveolens*	26.67	11.67	28.1	90.67	136.5	P	139.3	S
7	*T. ammi*	*P. amboinicus*	27.33	19.67	34.15	100	112.8	P	126.9	S
8	*T. ammi*	*A. calamus*	27.33	13	29.31	52.67	30.6	P	18.62	S
9	*T. ammi*	*M. fragrans*	27.33	41	49.66	100	46.3	P	51.02	S
10	*T. ammi*	*C. limon*	27.33	13.33	29.55	100	146.2	P	167.9	S
11	*T. ammi*	*H. suaveolens*	27.33	11.67	28.34	89	128.2	P	129.8	S
12	*P. amboinicus*	*A. calamus*	19.67	13	26.24	58	77.5	P	38.44	S
13	*P. amboinicus*	*M. fragrans*	19.67	41	48.74	100	64.8	P	53.91	S
14	*P. amboinicus*	*C. limon*	19.67	13.33	26.51	74	124.2	P	85.07	S
15	*P. amboinicus*	*H. suaveolens*	19.67	11.67	25.17	100	219.1	P	222.4	S
16	*A. calamus*	*M. fragrans*	13	41	46.98	84	55.5	P	29.17	S
17	*A. calamus*	*C. limon*	13	13.33	22.91	19	-27.8	AG	0.67	NE
18	*A. calamus*	*H. suaveolens*	13	11.67	21.46	16	-35.14	AG	1.39	NE
19	*M. fragrans*	*C. limon*	41	13.33	32.05	100	84	P	144.0	S
20	*M. fragrans*	*H. suaveolens*	41	11.67	31.07	100	89.9	P	152.9	S
21	*C. limon*	*H. suaveolens*	13.33	11.67	21.67	26	4	A	0.86	NE

CTF = Co-Toxicity factor, P/S = Potentiation/Synergism, A/No = Additive effect, AG = Antagonism, NE=No effects

Table 4: Binary combination of essential oil in 1:1 ratio (their individual LC_{50} values) larvicidal activity against *Ae. aegypti* larvae

Binary combination	**LC_{50} in ppm (95% CI)**	**LC_{90} in ppm (95% CI)**	**Chi-Square**	**Df**
BC_1-IT	34.274 (32.873-35.544)	51.183 (47.55-57.293)	4.985	3
BC_2-*IP*	51.704 (50.648-52.727)	63.650 (61.486-66.753)	4.694	3
BC_3-IA	78.406 (77.127-79.715)	92.530 (90.024-95.923)	2.260	3
BC_4-IM	24.573 (23.897-25.246)	32.492 (31.067-34.481)	1.061	3
BC_5-IC	32.018 (31.225-32.726)	39.656 (38.443-41.318)	3.594	3
BC_6-IH	69.208 (66.776-71.343)	95.061 (90.283-102.386)	3.092	3
BC_7-*TP*	34.813 (33.762-35.711)	45.009 (43.276-47.569)	3.704	3
BC_8-TA	41.500 (40.463-42.408)	52.592 (50.367-56.212)	0.629	3
BC_9-TM	19.979 (18.599-21.224)	38.529 (35.246-43.413)	2.371	4
BC_{10}-TC	28.739 (27.305-30.011)	44.138 (41.357-48.287)	3.753	3
BC_{11}-TH	77.795 (76.888-78.640)	87.991 (86.442-90.070)	1.617	4
BC_{12}-PA	44.191 (43.092-45.181)	56.370 (53.916-60.318)	1.937	3
BC_{13}-PM	21.709 (20.887-22.442)	30.633 (29.027-33.030)	2.568	3
BC_{14}-PC	36.023 (34.427-37.507)	54.734 (51.319-59.746)	3.947	3
BC_{15}-PH	85.696 (84.745-86.576)	95.410 (93.791-97.665)	2.485	3
BC_{16}-AM	27.922 (26.706-29.060)	44.054 (41.266-48.068)	6.560	4
BC_{17}-AC	46.121 (44.733-47.460)	62.770 (59.571-67.558)	1.796	3
BC_{18}-AH	64.147 (63.533-64.779)	70.903 (69.683- 72.585)	0.081	3
BC_{19}-MC	23.652 (23.053-24.221)	29.660 (28.696-30.924)	2.453	3
BC_{20}-MH	32.018 (31.225-32.726)	39.656 (38.443-41.318)	3.594	3
BC_{21}-CH	56.719 (55.426-58.056)	71.972 (69.106-76.003)	0.318	3

CI=Confidential Intervals, Df = Degree of freedom

Table 5: Binary combination effect calculation in Ting-Chao Chou method

Sl. No.	Compound	Ratio	m	Dm	r	CI values		Effect
						@ 0.5 Fa	@ 0.9 Fa	
1	I	-	6.27	41.14	0.997	-	-	-
2	T	-	8.88	39.4	0.984	-	-	-
3	P	-	4.74	46.88	0.989	-	-	-
4	A	-	3.27	22.19	0.978	-	-	-
5	M	-	6.33	38.12	0.997	-	-	-
6	C	-	5.61	43.23	0.992	-	-	-
7	H	-	8.1	45.81	0.98	-	-	-
8	IT	1.0:0.956	5.2	34.276	0.972	0.85	0.96	S
9	IP	0.869:1	10.331	51.476	0.982	1.17	0.96	AG
10	IA	1:0.57	11.72	77.69	0.981	2.47	1.8	AG
11	IM	1:0.92	7.74	24.49	0.996	0.62	0.58	S
12	IC	0.953:1	8.32	29.73	0.92	0.7	0.63	S
13	IH	0.9:1	6.79	69.1	0.989	1.59	1.61	AG
14	TP	0.869:1	8.998	34.76	0.973	0.81	0.72	S
15	TA	1:0.601	8.9	41.51	0.997	1.36	1.12	AG
16	TM	1.0:1.0	3.31	19.9	0.987	0.51	0.74	S
17	TC	0.91:1	6.05	28.79	0.961	0.7	0.73	S
18	TH	0.86:1	18.92	77.52	0.992	1.82	1.58	AG
19	PA	1:0.5	8.72	44.43	0.99	1.29	0.94	AG
20	PM	1:0.8	6.18	21.68	0.991	0.51	0.49	S
21	PC	1:0.9	5.47	35.87	0.978	0.8	0.78	S
22	PH	1:0.96	20.81	85.54	0.987	1.84	1.31	AG
23	AM	1:0.62	5	27.67	0.968	0.92	0.87	S
24	AC	1:0.55	7.02	45.96	0.989	1.42	1.15	AG
25	AH	1:0.52	18.86	63.97	0.993	1.9	1.35	AG
26	MC	0.88:1	9.93	23.56	0.994	0.58	0.5	S
27	MH	0.83:1	11.8	31.93	0.962	0.76	0.67	S
28	CH	0.95:1	9.12	56.86	0.998	1.28	1.17	AG

m = "shape" of the median-effect plot, Dm = The dose that produces 50% effect such as LC_{50}. r = the linear correlation coefficient. CI = Combination Index, S = Synergism; A = Additive Effect; AG = Antagonism; @ 0.5 Fa = fraction affected at 50%, @ 0.9 Fa = fraction affected at 90%.

Table 6: Wadley's and Ting-Chao Chou synergism calculation method for binary combination

Binary combination	Observed LC_{50} value in ppm	Expected LC_{50} value in ppm	Wadley's method		Ting-Chao Chou method	
			R	Effect	CI values @ 0.5 Fa	Effect
BC_1-IT	34.274	40.39	1.18	A	0.85	S
BC_2-*IP*	51.704	44.42	0.86	A	1.17	AG
BC_3-IA	78.406	32.5	0.41	AG	2.47	AG
BC_4-IM	24.573	39.62	1.61	S	0.62	S
BC_5-IC	32.018	42.25	1.32	A	0.70	S
BC_6-IH	69.208	43.54	0.63	A	1.59	AG
BC_7-*TP*	34.813	43.51	1.25	A	0.81	S
BC_8-TA	41.500	31.59	0.76	A	1.36	AG
BC_9-TM	19.979	38.81	1.94	S	0.51	S
BC_{10}-TC	28.739	41.41	1.44	A	0.70	S
BC_{11}-TH	77.795	42.63	0.55	A	1.82	AG
BC_{12}-PA	44.191	35.62	0.81	A	1.29	AG
BC_{13}-PM	21.709	42.84	1.97	S	0.51	S
BC_{14}-PC	36.023	45.44	1.26	A	0.80	S
BC_{15}-PH	85.696	46.66	0.54	A	1.84	AG
BC_{16}-AM	27.922	30.93	1.11	A	0.92	S
BC_{17}-AC	46.121	33.52	0.73	A	1.42	AG
BC_{18}-AH	64.147	34.74	0.54	A	1.90	AG
BC_{19}-MC	23.652	40.74	1.72	S	0.58	S
BC_{20}-MH	32.018	41.96	1.31	A	0.76	S
$BC2_1$-CH	56.719	44.56	0.79	A	1.28	AG

CI = Combination Index, @ 0.5 Fa = fraction affected at 50%, S=Synergism, A = Additive effect, AG = antagonism

Table 7: Ternary combination of essential oil in 1:1:1 ratio (their individual LC_{50} values) larvicidal activity against *Ae. aegypti* larvae

Sl. No	Combination	LC_{50} in ppm (95% CI)	LC_{90} in ppm (95% CI)	Chi-Square	df
1	TC1-ITP	51.63 (50.25-52.99)	69.88 (66.69-74.40)	5.69	4
2	TC2-ITA	51.20 (50.54-51.82)	59.78 (58.43-61.63)	2.61	5
3	TC3-ITM	35.028 (34.318-35.793)	44.273 (42.529-46.717)	3.806	4
4	TC4-ITC	33.992 (33.272-34.629)	40.616 (39.630-41.926)	4.555	3
5	TC5-ITH	43.937 (42.385-45.713)	66.772 (61.741-74.427)	5.033	4
6	TC6-IPA	52.328 (50.39-54.01)	76.55 (71.55-84.98)	3.92	4
7	TC7-IPM	36.212 (35.364-37.092)	47.798 (45.598-50.992)	2.043	4
8	TC8-IPC	49.654 (47.805-51.756)	73.053 (67.837-80.840)	1.430	3
9	TC9-IPH	51.349 (49.333-53.636)	80.419 (72.174-96.965)	5.149	3
10	TC10-IAM	38.60 (37.74-39.53)	48.46 (46.56-51.08)	3.283	3
11	TC11-IAC	47.427 (46.205-48.679)	61.949 (59.199-65.855)	4.795	3
12	TC12-IAH	54.52 (52.81-56.43)	75.95 (71.20-83.16)	3.279	3
13	TC13-IMC	30.612 (29.425-31.704)	43.897 (41.568-47.250)	2.064	3
14	TC14-IMH	43.653 (42.843-44.378)	51.335 (50.156-52.920)	4.362	3
15	TC15-ICH	43.872 (42.216-45.601)	65.522 (61.105-72.079)	2.636	3
16	TC16-TPA	48.158(44.15-51.26)	75.60 (70.95-82.62)	3.60	2
17	TC17-TPM	34.899 (34.175-35.642)	43.530 (41.831-46.028)	2.648	3
18	TC18-TPC	53.142 (51.117-54.986)	75.115 (71.406-80.357)	4.981	3
19	TC19-TPH	42.610 (40.962-44.298)	63.408 (59.410-69.121)	4.908	3
20	TC20-TAM	30.609 (29.285-31.924)	49.815 (46.215-55.081)	6.655	4
21	TC21-TAC	34.592 (33.556-35.643)	47.532 (44.910-51.497)	0.226	3
22	TC22-TAH	45.542 (43.537-47.786)	73.153 (66.833-83.004)	0.668	3
23	TC23-TMC	39.073 (37.621-40.618)	58.719 (54.312-65.765)	3.805	3
24	TC24-TMH	42.690 (41.803-43.537)	52.146 (50.555-54.323)	1.598	3
25	TC25-TCH	42.785 (41.691-43.865)	54.914 (52.730-57.951)	2.974	3
26	TC26-PAM	37.978(36.114-39.915)	64.321 (58.560-73.389)	0.316	3
27	TC27-PAC	50.872(47.329-54.945)	109.371 (94.739-133.438)	3.279	3
28	TC28-PAH	39.321(38.686-39.987)	46.131(44.912-47.744)	3.846	3
29	TC29-PMC	38.847 (37.751-40.010)	53.869 (51.008-57.941)	5.528	4
30	TC30-PMH	39.514 (38.605-40.508)	51.528 (49.202-54.809)	2.633	4
31	TC31-PCH	47.665(46.358-49.179)	62.955 (59.544-67.973)	3.640	3
32	TC32-AMC	48.37 (46.15-50.96)	81.63 (74.19-92.73)	2.441	4
33	TC33-AMH	42.73(40.27-45.64)	80.23 (70.60-96.32)	3.875	3
34	TC34-ACH	47.549(45.540-49.745)	73.973 (68.338-82.299)	5.215	3
35	TC35-MCH	44.603 (43.595-45.605)	55.853 (53.908-58.487)	0.869	3

CI=Confidential Intervals; Df = Degree of freedom

Table 8: Ternary combination of essential oil in Ting-Chao Chou method

S. No	Combination	Ratio	m	Dm	r	CI values		Effect
						@ 0.5 Fa	@ 0.9 Fa	
1	I	-	6.27	41.14	0.997	-	-	-
2	T	-	8.88	39.40	0.984	-	-	-
3	P	-	4.74	46.88	0.989	-	-	-
4	A	-	3.27	22.19	0.978	-	-	-
5	M	-	6.33	38.12	0.997	-	-	-
6	C	-	5.61	43.23	0.992	-	-	-
7	H	-	8.10	45.81	0.980	-	-	-
8	ITP	0.32:0.31:0.37	7.02	51.39	0.982	1.209	1.165	AG
9	ITA	0.39:0.38:0.23	13.88	51.08	0.988	1.503	1.165	AG
10	ITM	0.35:0.33:0.32	9.32	35.0	0.990	0.884	0.818	S
11	ITC	0.33:0.32:0.35	12.53	33.83	0.983	0.820	0.703	S
12	ITH	0.33:0.31:0.36	5.257	44.06	0.984	1.046	1.190	AG
13	IPA	0.37:0.42:0.21	5.305	51.76	0.980	1.420	1.316	AG
14	IPM	0.32:0.37:0.30	7.699	36.26	0.992	0.862	0.779	S
15	IPC	0.31:0.36:0.33	5.510	49.69	0.998	1.135	1.133	AG
16	IPH	0.31:0.35:0.34	4.625	51.49	0.952	1.154	1.296	AG
17	IAM	0.4:0.23:0.37	9.857	38.795	0.9885	1.155	0.921	S
18	IAC	0.38:0.22:0.4	8.056	47.25	0.989	1.341	1.106	AG
19	IAH	0.37:0.21:0.41	6.353	54.56	0.992	1.765	1.50	AG
20	IMC	0.34:0.31:0.35	5.981	30.56	0.993	0.748	0.751	S
21	IMH	0.33:0.30:0.36	14.42	43.60	0.975	1.046	0.882	S
22	ICH	0.32:0.33:0.35	5.44	43.56	0.989	1.00	1.073	AG
23	TPA	0.36:0.43:0.21	3.050	41.97	0.926	1.169	1.531	AG
24	TPM	0.32:0.38:0.31	9.45	34.86	0.992	0.840	0.747	S
25	TPC	0.3:0.36:0.33	6.439	52.71	0.970	1.220	1.193	AG
26	TPH	0.3:0.36:0.34	5.75	42.17	0.981	0.957	1.015	S
27	TAM	0.39:0.23:0.38	4.76	30.41	0.977	0.921	0.968	S
28	TAC	0.37:0.22:0.41	6.70	34.60	0.999	0.997	0.904	S
29	TAH	0.36:0.22:0.42	4.49	45.67	0.998	1.286	1.428	AG
30	TMC	0.33:0.32:0.36	5.27	39.00	0.984	0.968	1.059	S
31	TMH	0.32:0.31:0.37	11.55	42.48	0.988	1.033	0.937	S
32	TCH	0.39:0.23:0.38	8.528	42.77	0.994	1.005	0.976	S
33	PAM	0.43:0.22:0.35	4.09	37.97	0.998	1.165	1.160	AG
34	PAC	0.41:0.21:0.38	2.86	51.60	0.983	1.389	1.806	AG
35	PAH	0.41:0.20:0.39	13.73	39.15	0.993	0.855	0.764	S
36	PMC	0.37:0.30:0.34	6.45	38.8	0.988	0.907	0.855	S
37	PMH	0.36:0.29:0.35	8.29	39.72	0.992	0.910	0.830	S
38	PCH	0.35:0.32:0.33	7.52	47.73	0.990	1.053	0.971	S
39	AMC	0.23:0.36:0.41	4.18	48.75	0.989	1.423	1.513	AG
40	AMH	0.22:0.36:0.42	3.36	42.90	0.986	1.246	1.508	AG
41	ACH	0.21:0.38:0.41	4.75	47.75	0.98	1.299	1.333	AG

42	MCH	0.30:0.34:0.36	9.78	44.46	0.996	1.049	0.939	S

m = “shape” of the median-effect plot, Dm = The dose that produces 50% effect such as LC_{50}. r = the linear correlation coefficient. CI = Combination Index, S = Synergism; A = Additive Effect; AG = Antagonism; @ 0.5 Fa = fraction affected at 50%, @ 0.9 Fa = fraction affected at 90%.

Table 9: Wadley's and Ting-Chao Chou synergism calculation method for ternary combination

Sl. No	Combination	Observed LC_{50}value in ppm	Expected LC_{50}value in ppm	Wadley's method		Ting-Chao Chou method	
				R	Effect	CI values @ 0.5 Fa	Effect
1	TC_1-ITP	42.773	51.63	0.83	A	1.209	AG
2	TC_2-ITA	34.827	51.2	0.68	A	1.503	AG
3	TC_3-ITM	39.677	35.028	1.13	A	0.884	S
4	TC_4-ITC	41.373	33.992	1.22	A	0.820	S
5	TC_5-ITH	42.185	43.937	0.96	A	1.046	AG
6	TC_6-IPA	37.514	52.328	0.72	A	1.420	AG
7	TC_7-IPM	42.364	36.212	1.17	A	0.862	S
8	TC_8-IPC	44.060	49.654	0.89	A	1.135	AG
9	TC_9-IPH	44.873	51.349	0.87	A	1.154	AG
10	TC_{10}-IAM	34.417	38.6	0.89	A	1.155	AG
11	TC_{11}-IAC	36.114	47.427	0.76	A	1.341	AG
12	TC_{12}-IAH	36.925	54.52	0.68	A	1.765	AG
13	TC_{13}-IMC	40.964	30.612	1.34	A	0.748	S
14	TC_{14}-IMH	41.776	43.653	0.96	A	1.046	AG
15	TC_{15}-ICH	43.473	43.872	0.99	A	1.00	AG
16	TC_{16}-TPA	36.908	48.158	0.77	A	1.169	AG
17	TC_{17}-TPM	41.757	34.899	1.20	A	0.840	S
18	TC_{18}-TPC	43.453	53.142	0.82	A	1.220	AG
19	TC_{19}-TPH	44.266	42.61	1.04	A	0.957	S
20	TC_{20}-TAM	33.811	30.609	1.10	A	0.921	S
21	TC_{21}-TAC	35.508	34.592	1.03	A	0.997	S
22	TC_{22}-TAH	36.318	45.542	0.80	A	1.286	AG
23	TC_{23}-TMC	40.357	39.073	1.03	A	0.968	S
24	TC_{24}-TMH	41.169	42.69	0.96	A	1.033	AG
25	TC_{25}-TCH	42.575	42.785	1.00	A	1.005	AG
26	TC_{26}-PAM	36.497	37.978	0.96	A	1.165	AG
27	TC_{27}-PAC	38.194	50.872	0.75	A	1.389	AG
28	TC_{28}-PAH	39.006	39.321	0.99	A	0.855	S
29	TC_{29}-PMC	43.044	38.847	1.11	A	0.907	S
30	TC_{30}-PMH	43.857	39.514	1.11	A	0.910	S
31	TC_{31}-PCH	45.553	47.665	0.96	A	1.053	AG
32	TC_{32}-AMC	35.097	48.37	0.73	A	1.423	AG
33	TC_{33}-AMH	35.908	42.73	0.84	A	1.246	AG
34	TC_{34}-ACH	37.605	47.549	0.79	A	1.299	AG
35	TC_{35}-MCH	42.456	44.603	0.95	A	1.049	AG

CI = Combination Index, @ 0.5 Fa = fraction affected at 50%, S=Synergism, A = Additive effect, AG = antagonism

Figure 1: Plant material and extracted essential oil: A. *Plectranthus amboinicus*, B. *Acorus calamus*, C. *Hyptis suaveolens*, D. Lemon oil, E. *Syzygium aromaticum*, F. *Illicium verum*, G. *Trachyspermum ammi*.

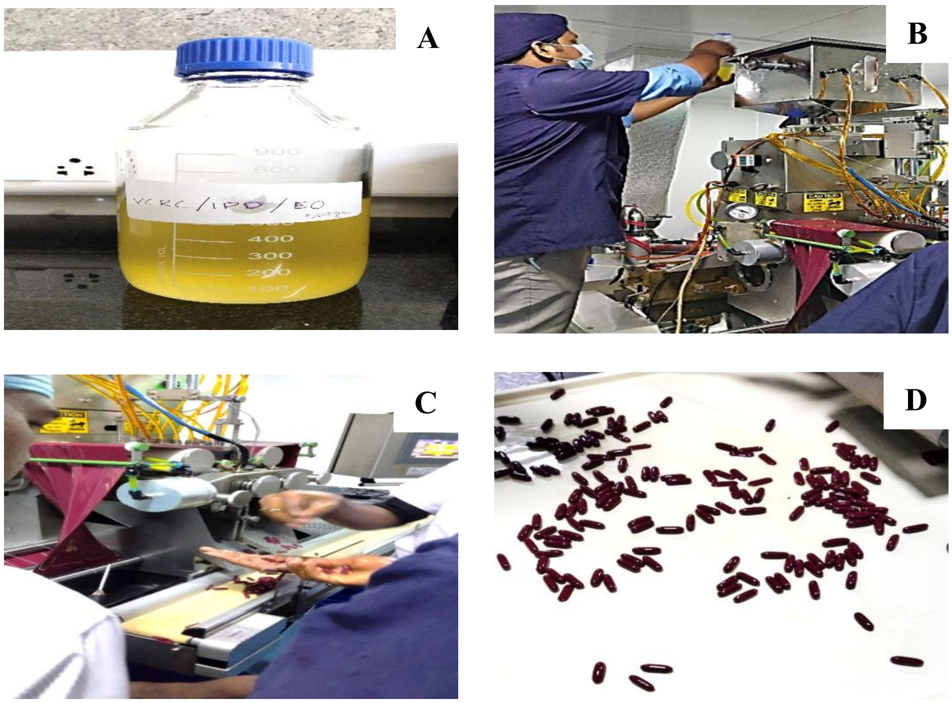

Figure 2: Preparation of Soft Gel Capsule Formulation: A. Prepared emulsion for the formulation, B. Addition of emulsion into the instrument, C. Soft gel capsules, D. Final soft gel capsules.

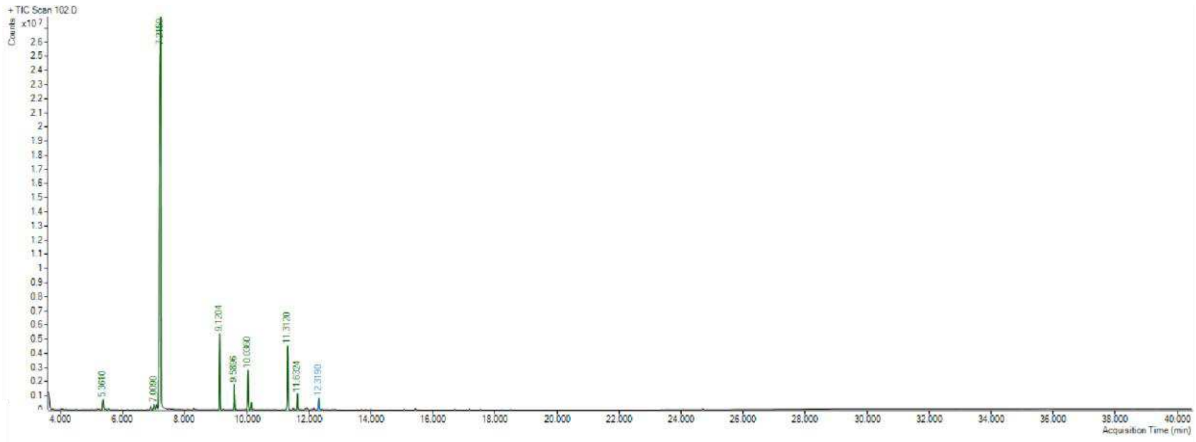

Figure 3a: GC Chromatogram – *Plectranthus ambonicus* leaf essential oil:

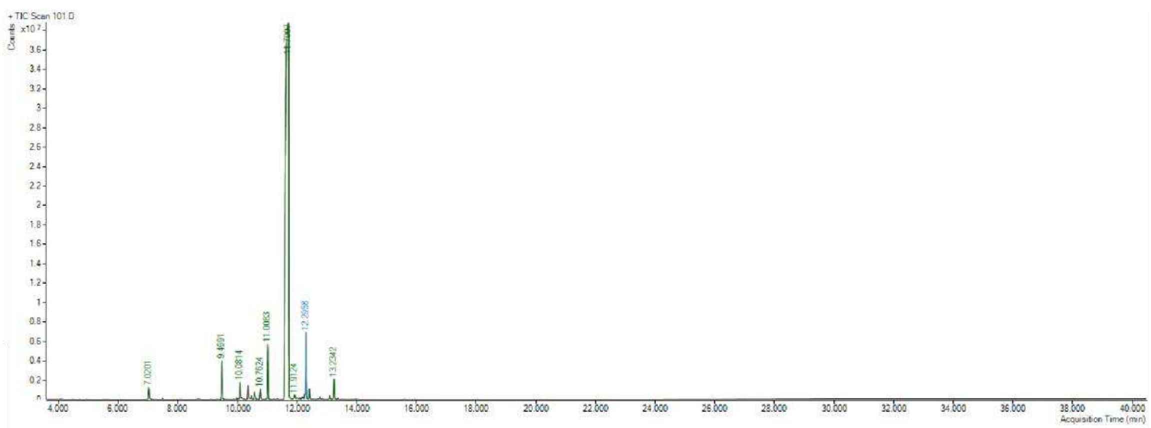

Figure 3b: GC Chromatogram – *Acorus calamus* root essential oil:

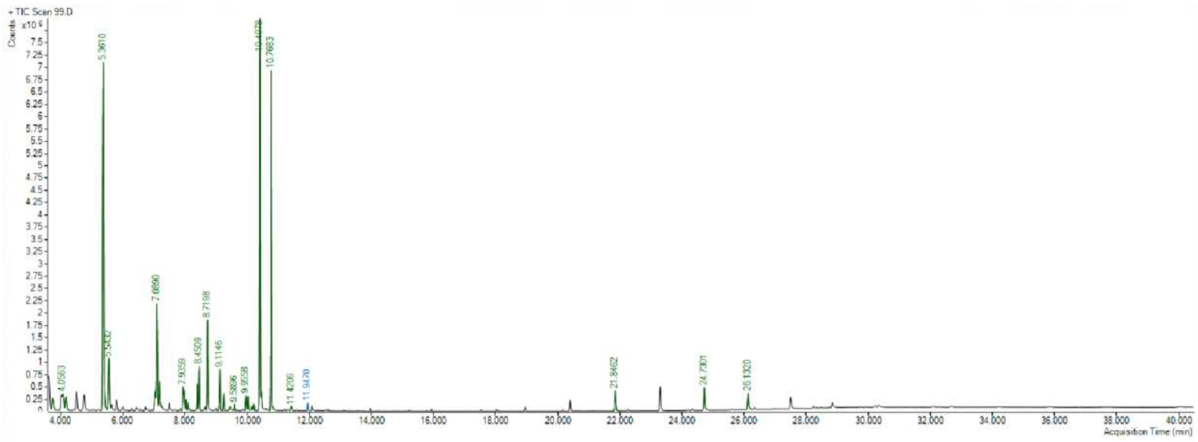

Figure 3c: GC Chromatogram – *Myristica fragrans* seed essential oil:

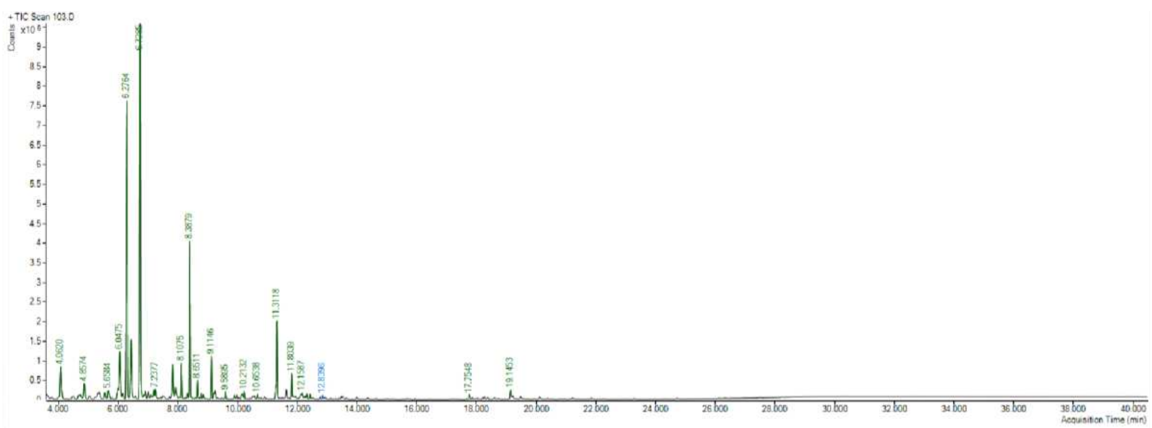

Figure 3d: GC Chromatogram – *Citrus limon* leaf essential oil:

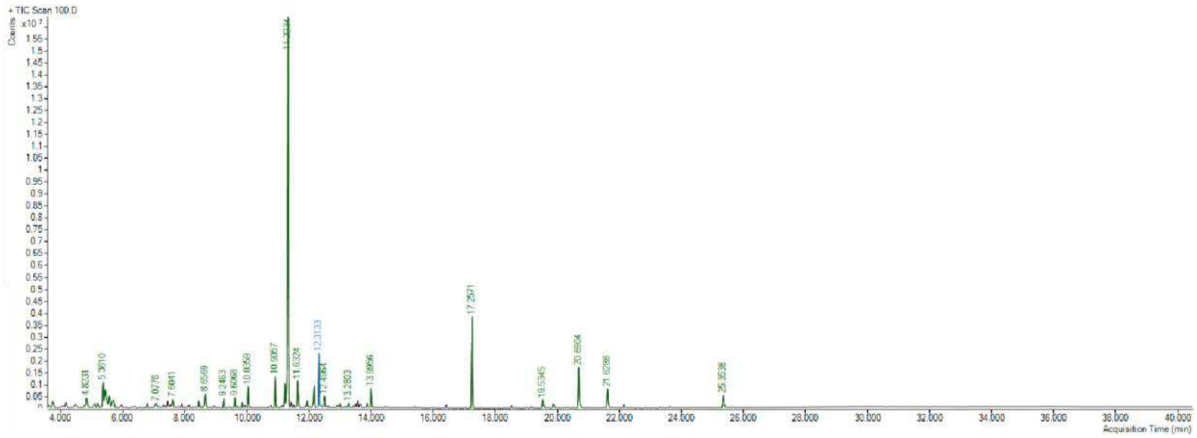

Figure 3e: GC Chromatogram – *Hyptis suaveolens* leaf essential oil:

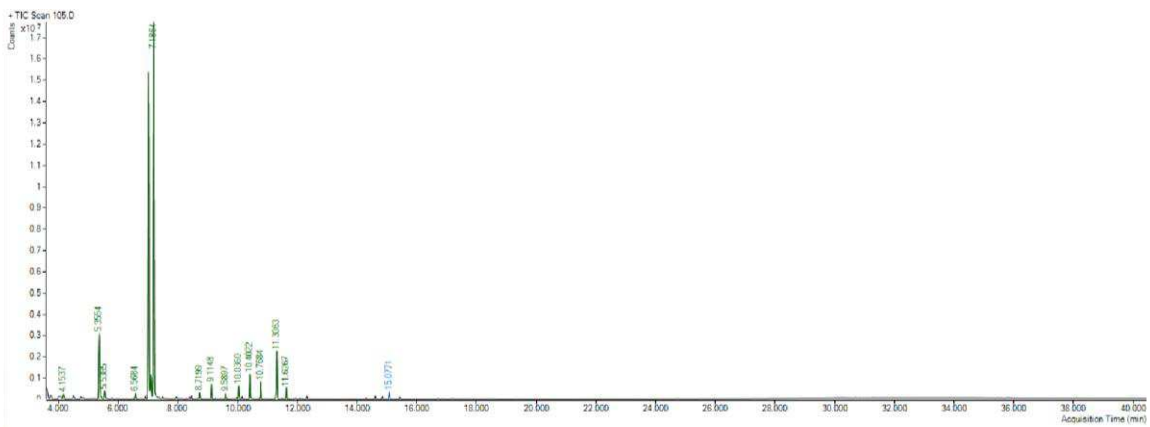

Figure 3f: GC Chromatogram – Essential oil combination of *Plectranthus ambonicus* leaf + *Myristica fragrans* seed

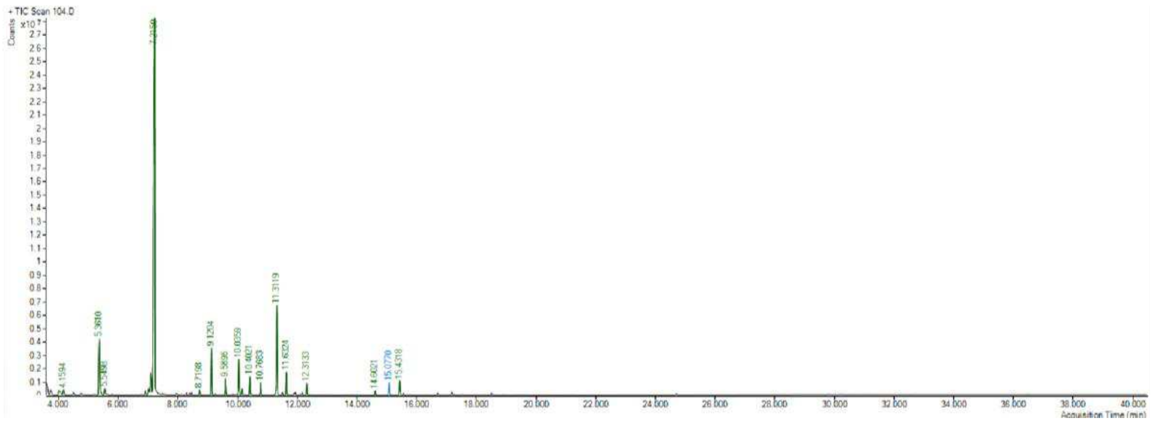

Figure 3g: GC Chromatogram – Essential oil combination of *Trachyspermum ammi* seed + *Myristica fragrans* seed

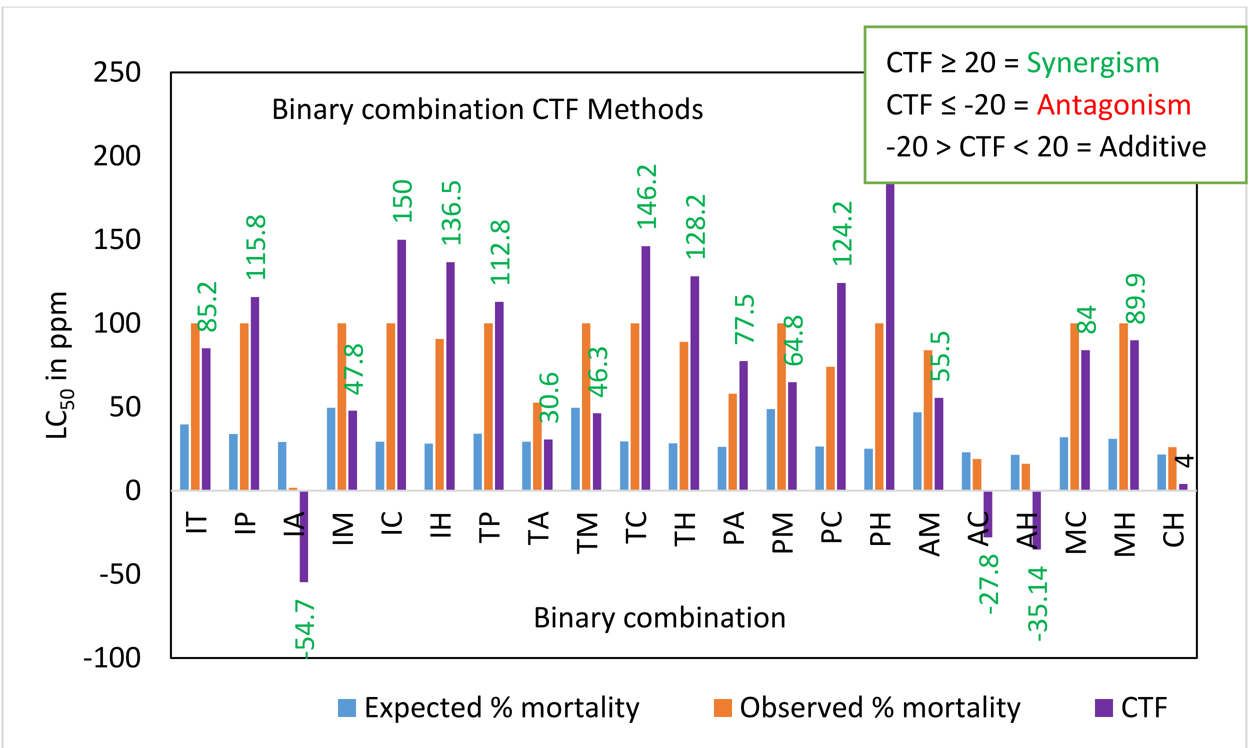

Figure 4: Binary combination method synergism calculation using Co-Toxicity factor (CTF) method.

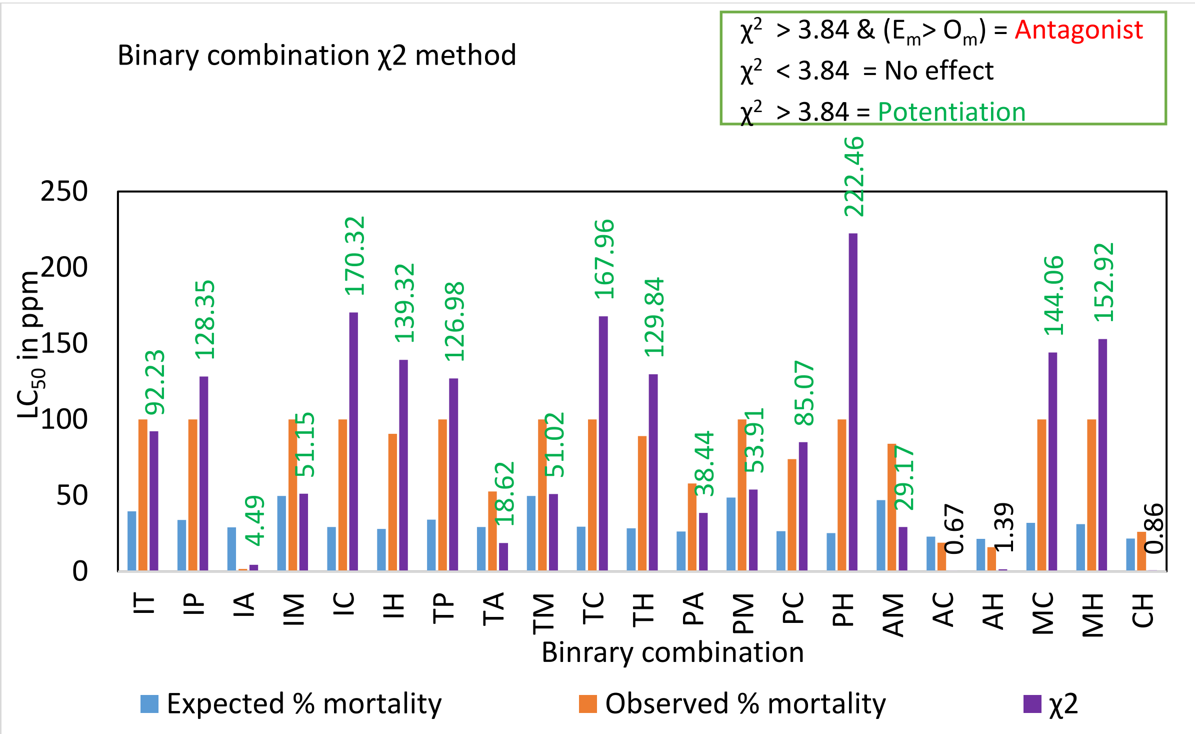

Figure 5: Binary combination method synergism calculation using Chi square comparison method (χ2) method.

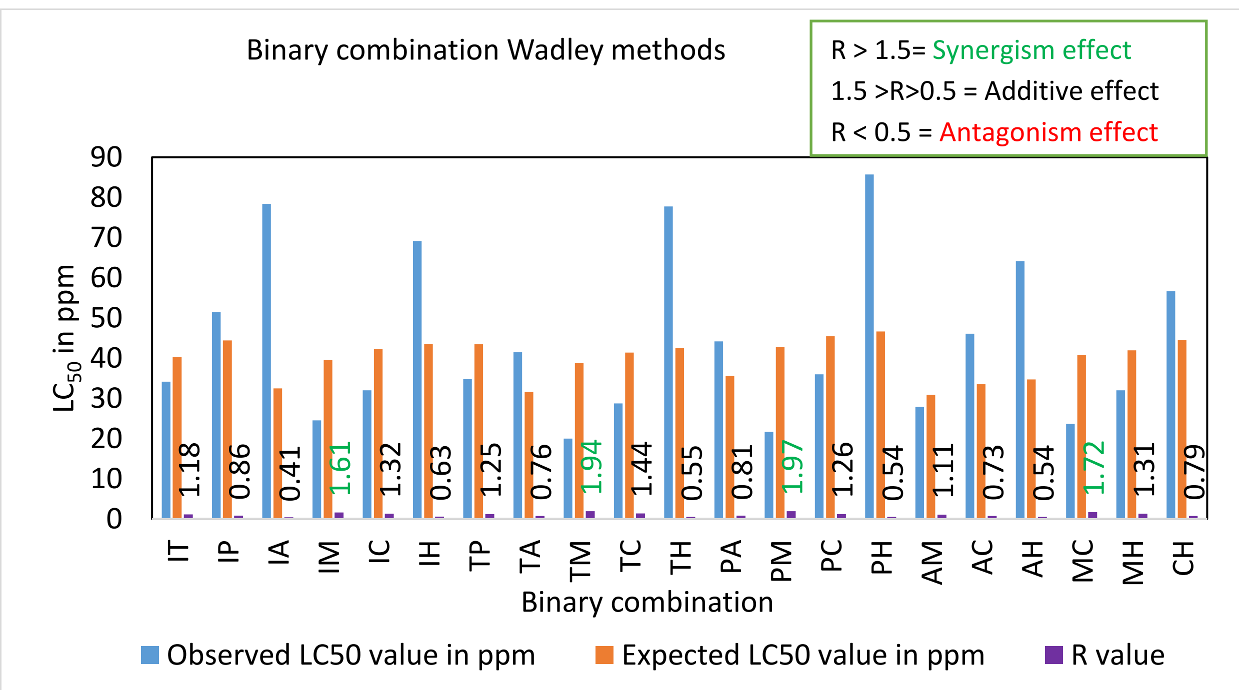

Figure 6: Binary combination method synergism calculation using Wadley's (R) method.

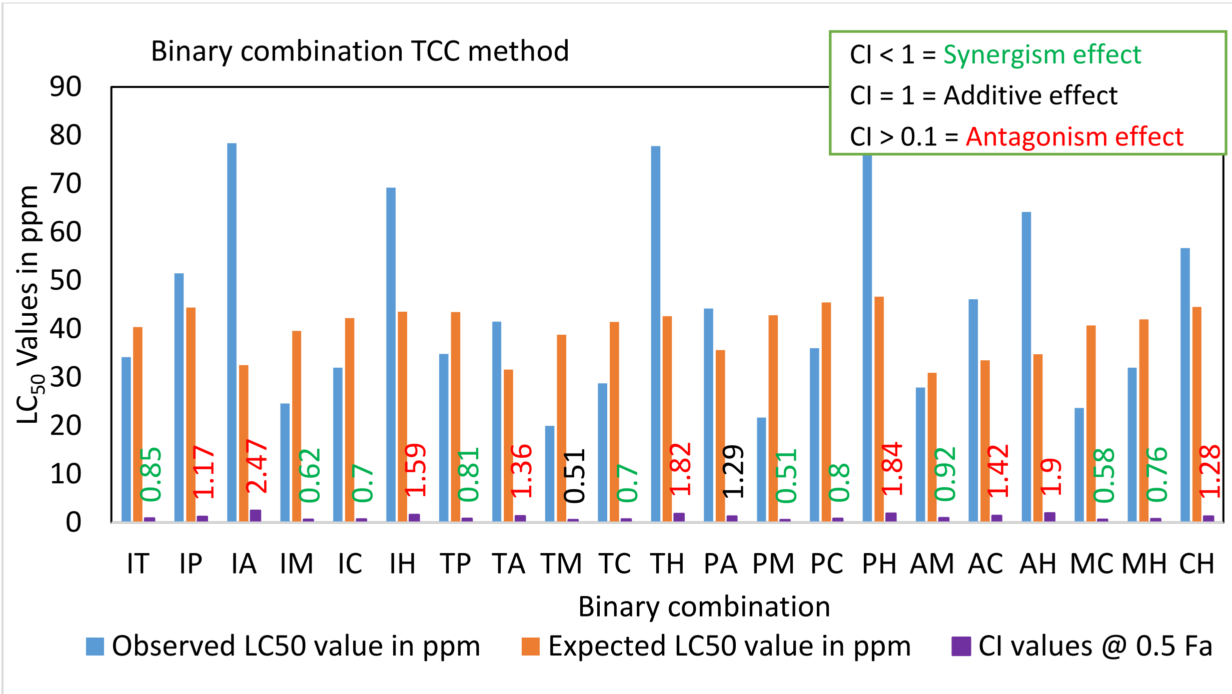

Figure 7: Binary combination method synergism calculation using Ting-Chao Chou method.

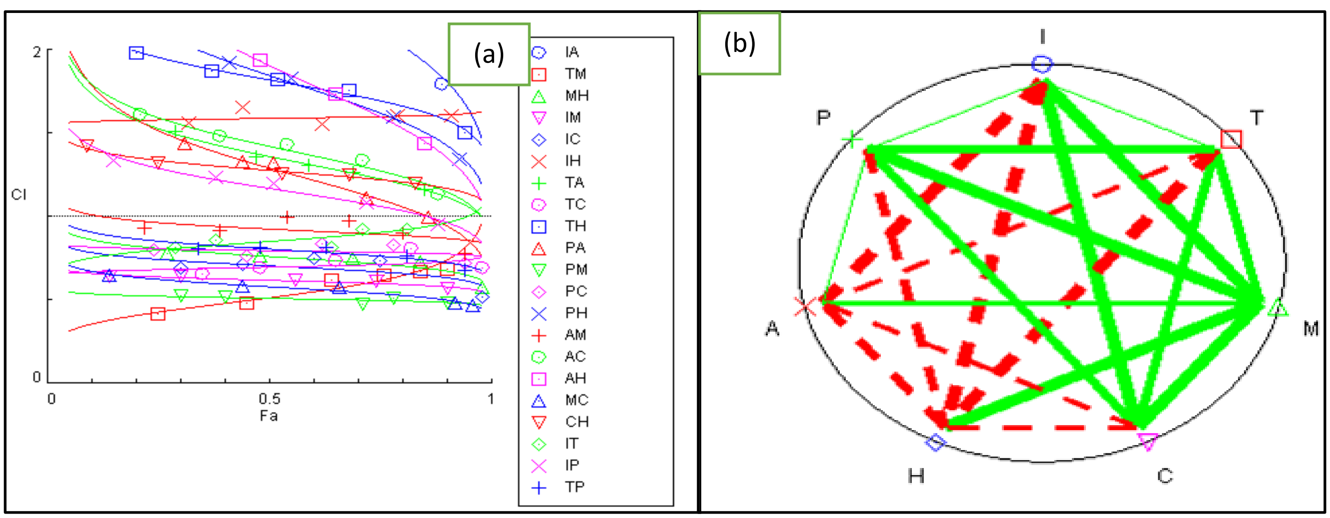

Figure 8: Binary combination method synergism calculation using Ting-Chao Chou method (a) Combination Index plot (b) Polygonogram.

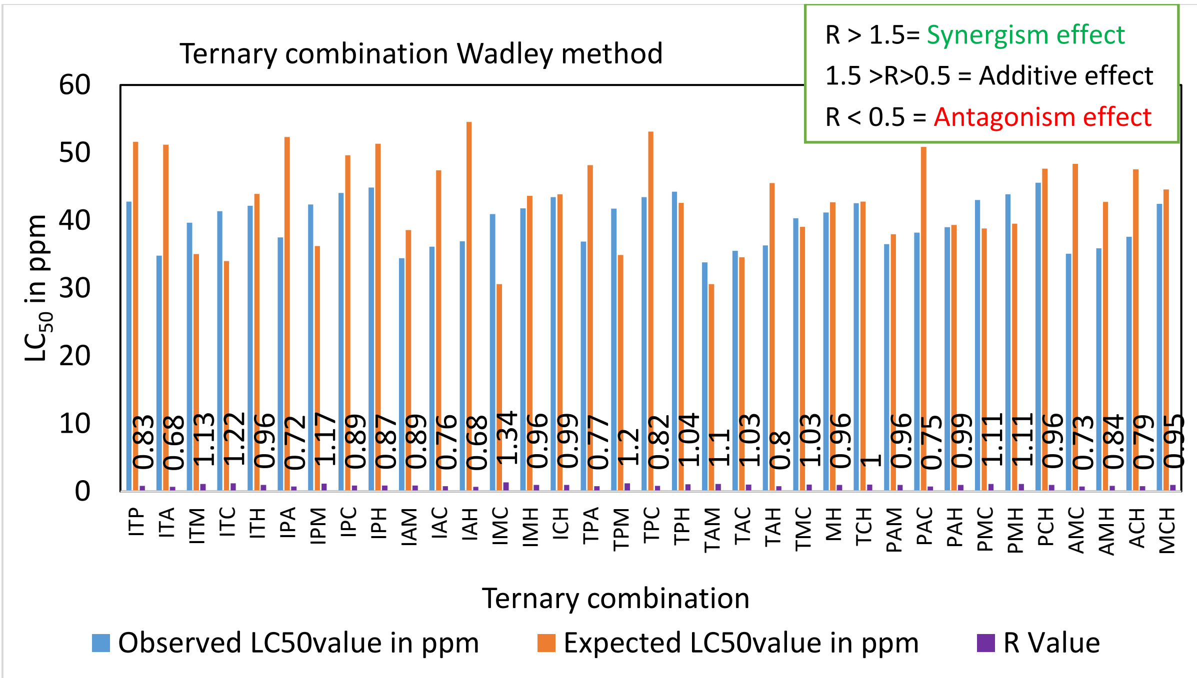

Figure 9: Ternary combination method synergism calculation using Wadley's (R) method.

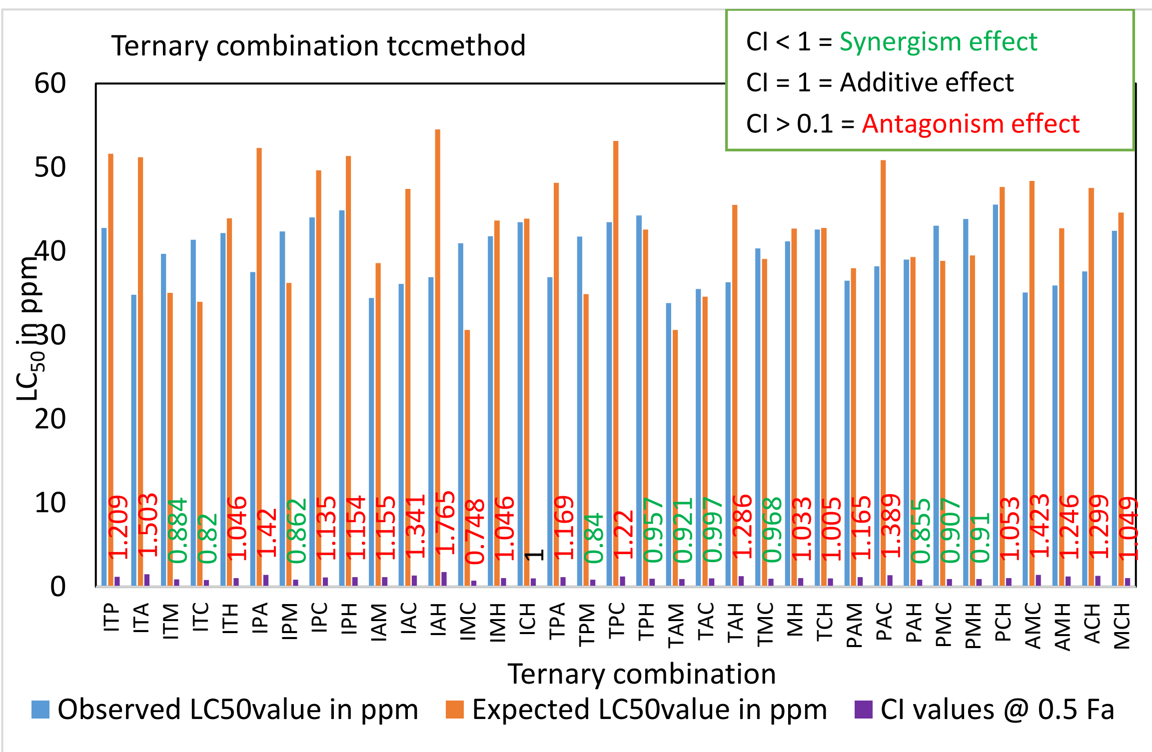

Figure 10: Ternary combination method synergism calculation using Ting-Chao Chou method.

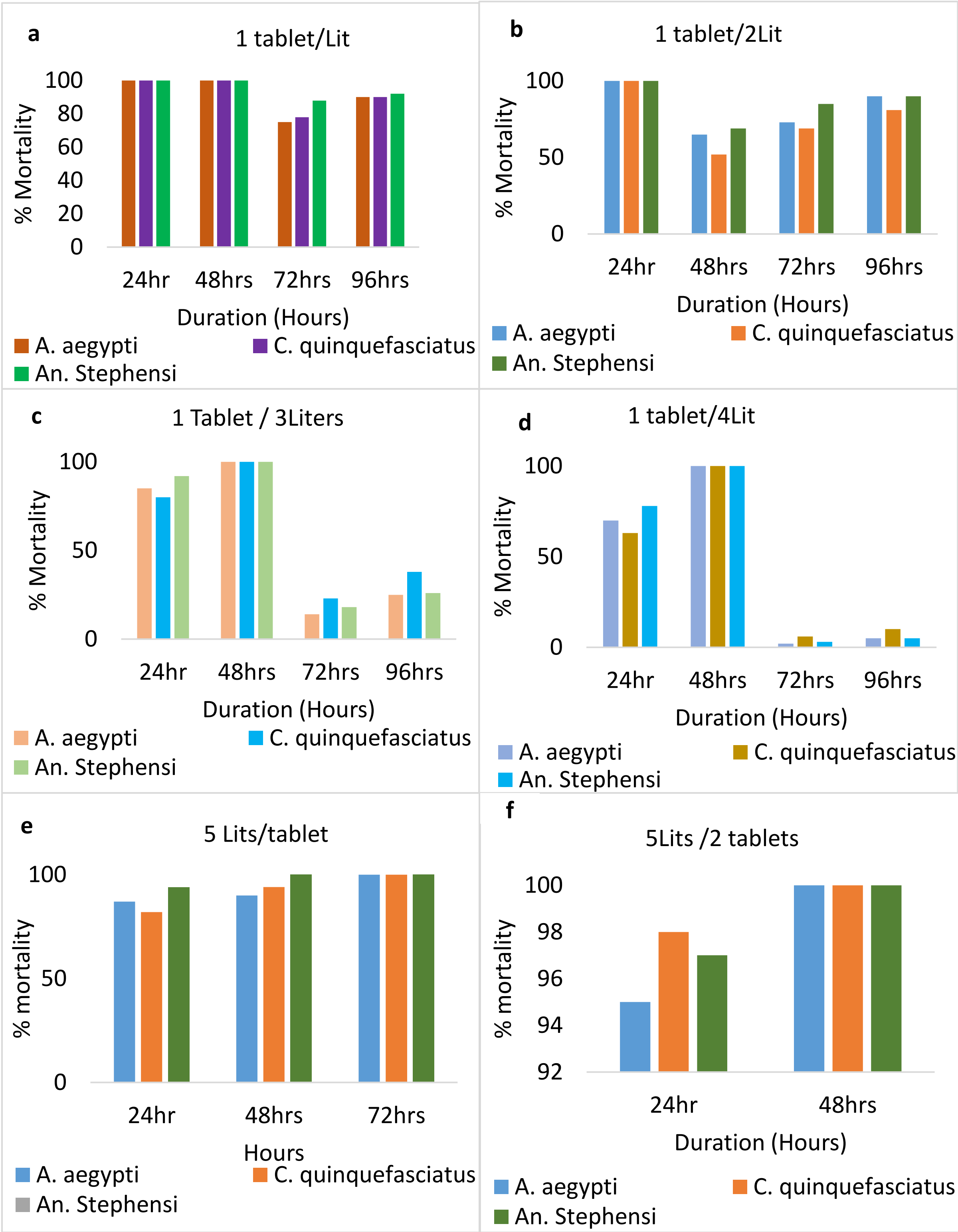

Figure 11: Percentage (%) mortality of *A. aegypti*, *An. stephensi* and *C. quinquefasciatus* - (a) 1 tablet in 1 liter of water, (b) 1 tablet in 2 liters of water, (c) 1 tablet in 3 liters of water, (d) 1 tablet in 4 liters of water, (e) 1 tablet in 5 liters of water, (f) 2 tablets in 5 liters of water.

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