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Evaluation of insecticidal effects of *Citrus limon* and *Cannabis sativa* on *Aedes aegypti* adult

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ABSTRACT
Plant based insecticides are safe, ecofriendly and cost-effective. These are the better alternatives to chemical insecticides as they are biodegradable and do not harm biota or humans. Mosquitoes are the major vectors which cause some life-threatening diseases like malaria, dengue, yellow fever and filariasis. Present study aims to evaluate LC_{50} of Citrus limon and Cannabis sativa plant extracts individually and synergistically in 24 hours and 48 hours to control dengue vector Aedes aegypti. The result revealed that individual LC50 of C. limon and C. sativa are respectively was 430.8 ppm and 617.4 ppm in 24 hours and 296.6 ppm and 508.1 ppm in 48 hours. But when the synergistic effects of C. limon and C .sativa plant extracts were evaluated, the $LC_{50}$was found to be 321.4 ppm in 24 hours. and 286.7 ppm in 48 hours. So, the present study showed that the combination method of eco-safe plant extracts are more effective than individual plant extracts to control dengue vector A. aegypti.
Key words: Dengue, Aedes aegypti, insecticides, Citrus limon and Cannabis sativa, LC50.

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INTRODUCTION

Mosquitoes are considered as serious nuisance pests and vectors of many dreadful diseases both in urban and rural areas [1-3]. They are responsible for the spread of various vector borne diseases such as malaria, filariasis, Japanese encephalitis and dengue. They cause human death, suffering and impediment to economic development not only in India but all over the world [4-7]. To control adult mosquito population, various chemicals like DDT formerly used throughout the world. Later mosquitoes developed resistance to them; Hence, it is now banned in most of the developed countries. Since 1945, more than 15,000 compounds have been synthesized and combined in 35,000 insecticide mixtures to circumvent insect resistance [8-10]. Repellents are known to play an important role in controlling *A. aegypti* population. Major commercial repellents are prepared by using chemicals like allethrine, N-N-diethyl-m-toluamide (DEET), dimethylephthalate (DMP) and N-N-diethylemendelic acid amide (DEM) [11-13]. But they have not yielded long lasting results due to the development of resistance against these insecticides [6, 7].
Prolonged exposure to the chemical synthetic insecticides like Mortein, All-out, Odomos*etc.* leads to irritation, severe allergic dermatitis, systemic allergic reactions and large amounts causes nausea, vomiting, headache and other central nervous system disturbance in human beings [14, 35, 63]. It is reported that many of these chemical repellents are not safe for public use [36, 62] because of their unpleasant smell, oily feeling [36, 40] and potential toxicity [36, 12]. Due to the use of chemical repellents, breathing problems are the most common and frequently this condition is accompanied with headache or eye irritation or both. Eye irritation is the next common complaint and often it is accompanied with bronchial irritation, headache or skin reaction. Cough, cold and running nose are accompanied with fever or sneezing. Some people suffer from wheezing and asthma. People who do not have asthma become asthmatic, even after discontinuing the use of repellents. People suffer from pain in the ear and throat. Due to the use of DEET based creams, users suffer from rashes, black spots or in some cases skin turns black or oily and cause itching [52].

Resistance of mosquitoes to insecticides, including organochlorine, organophosphate, carbamates and pyrethroids, was considered to be a recent revolutionary adaptation to environmental changes [5, 64]. Recently, stress is being given on eco-friendly means to control *Aedes* mosquito [18, 19]. In contrast with synthetic repellents, natural products are usually simple, cost-effective and accessible to communities with minimal external input [20, 21].

Fumigants are also commonly used to drive mosquitoes from houses. Chemicals released from burning plants play an important role in repelling mosquitoes [22, 23]. The burning of plants releases insecticidal and irritant chemicals [23, 20]. It has been shown that the steam extracts of plants, *Mentha piperata* and *O. sanctum* have knock down effect [24, 25]. It is therefore unsurprising that many plants containing repellent volatiles will repel mosquitoes when burned. In Africa, the use of traditional fumigants is widespread. The most commonly used plants in Africa include *Azadirachta indica* (neem), *Hyptis* spp. (bushmint family), *Ocimum* spp. (basil family), *Corymbia*spp. (formerly Eucalyptus spp.) and *Daniellia oliveri* against *An. gambiae* throughout South-East Asia. In Srilanka, 69% of families burn neem kernels and leaves to repel mosquitoes along with coils despite all houses being regularly sprayed with residual insecticides [26, 27]. Moreover, smoke production lowers humidity by reducing the moisture carrying capacity of the air. This makes mosquitoes susceptible to dessication and reduces sensory input because mosquito chemoreceptors are more responsive in the presence of moisture [28, 29]. They can be grouped into major chemical categories like nitrogen compounds alkaloids, terpenoids, phenolics *etc*. Though these compounds were used against phytophagous insects, many are also effective against mosquitoes and other biting Diptera [30, 31]. The pests rarely develop resistance against pesticides of plant origin. However, a lot of plant materials are required to get a small amount of phytopesticides. So, the combination of different plant extracts would be useful. The synergistic effect of plant extracts on mosquito which are significantly more than the effect of their individual plant extracts [32, 33]. The introduction of synergists in mosquito control could be of great benefit both economically and ecologically, thereby, reducing the cost and increasing toxicity of a given treatment. *V. negundo, Z. Officinalis, O. sanctum* extracts [1] *etc.* are reported to be used in synergism against mosquito larvae. Most households in developing world rely on personal protection measures of limited effectiveness such as burning of mosquito coils or leaves [34], mosquito mat, using mosquito net and traditional neem leaf burning [35]. Coils were traditionally made with finely ground Pyrethrum Daisy *C. cinerariiefolium* (Compositae) flowers mixed with coconut husks [10].

Hanif *et al.*, [18] reported that the mixtures of secondary metabolites or natural essential oils combinations were found to be very effective against the pest management. These essential oils are used in many preparations such as pharmaceutical industries, food, beverage and chemical industries. These showed the comprehensive effect as antimicrobial and antioxidant properties and the insecticidal activities against *C. maculatus* respectively.

There is a need of further investigation of the synergistic approach of plant products. So, in this present study, *C. sativa* and *C. limon* plants were screened and their ethanolic extracts were prepared to study the effects on *A. aegypti* adult mosquitoes. The toxic effects of the plant extracts and their combinations were observed.

MATERIAL AND METHODS

Study Location

The study was conducted at St. John's College, which is located in Agra, Uttar Pradesh, India, and covers an area of around 21.20 hectares. In an elevation of 175 metres above mean sea level, it may be found at 27°11' 30" N to 27°11' 41.44" N and 77°00'18" E to 77°59'49" E. [5, 6]. The institute is home to a wide variety of flora and wildlife due to its vast size. Natural medicinal plants are an important part of the institute's flora, which may be found in the institute's large open spaces (Figure 1).

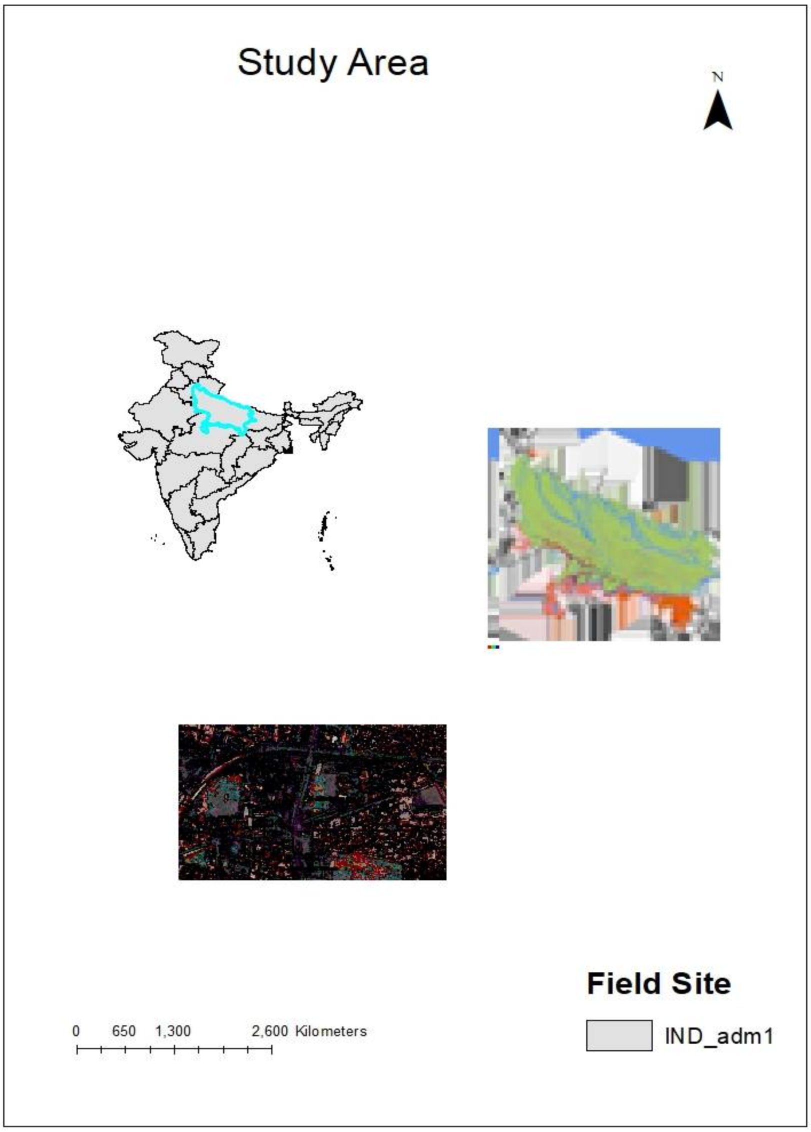

Figure 1. Map of the Study area.

Detail of Medicinal plants:
Various locally available plant species were screened from the vicinity of St. John's College area. Among them, *Citrus limon* and *Cannabis sativa* were chosen for toxicity bioassay due to their insecticidal properties.
2.2 *Preparation of plant extracts:* 150 gms. of fresh leaves of *C. sativa* and *C. limon* were collected in the morning, washed under tap water and air dried for few days. The plant materials were ground with the help of grinder into fine powder. Ethanolic extracts of all the plants were prepared with the help of Soxhlet apparatus [6, 7].
2.3 *Preparation of stock solutions:* 10% stock solutions of *C. sativa* and *C. limon* were prepared. On the other hand combination of plant extracts were made in 1:1 ratio.
2.4 *Preparation of different concentrations: different* concentrations of individual as well as combinations of plant extracts were taken. The details are given below-
100, 200, 300, 400, 500, 600, 700 ppm. of *C.limon;*
100, 250, 400, 550, 700, 800, 1000, 1150 ppm. of *C. sativa* were taken.
While in regard to combinations of plant extracts :
100, 120, 140, 160, 180, 200, 220 ppm. each of *C. limon* + *C. sativa.*

Toxicity assay on mosquitoes:

Experimental:

a) Cages were made by tight plastic and cotton soaked with the above-mentioned individual concentrations (ppm.) each of the individual plant extract was kept.

b) Cotton soaked with combinations of plant extracts from different concentration, mentioned above were also kept.

Control:

Separate sets of plastic cages without any treatment.

The above sets containing 10 adults at 40±2^{0}C at room temperature with sugar syrup supplement were kept. Mortality was recorded after 24 and 48 hrs. Triplicate sets were maintained and observations recorded and tabulated.

Statistical analysis

In toxicity assay, data recorded was subjected to log probit analysis to obtain the lethal concentration value (LC) using Origin 8 software. These computer-generated programme provided LC values with appropriate regression line and slope. LC_{50} was chosen as value of the activity [5, 15]. Safe concentration was recorded, below which larvae and adults remained alive. For making the combinations, the concentrations were made below safe concentration using the formula i. e.,

$$Safe \quad concentration(SC) = 48 \quad hrs.LC_{50} \times A \times S \qquad where \quad A = 0.3 \tag{3.2}$$

$$S = \frac{24 \quad hrs. \quad LC_{50}}{48 \quad hrs. \quad LC_{50}} \tag{3.3}$$

Synergistic factors (SF) were also calculated using the formula –

$$SF = \frac{LC_{50} \quad value \quad of \quad the \quad insecticide \quad alone^{*}}{LC_{50} \quad value \quad of \quad the \quad insecticide \quad with \quad the \quad assumed \quad synergist} \tag{3.4}$$

(Values of SF > 1 indicate synergism and SF < 1 indicate antagonism) (George *et al*., 2005) respectively.

* LC_{50} value of maximum effective plant product.

RESULTS

The insecticidal activities were observed due to treatment with ethanolic plant extracts of *C. limon* and *C. sativa* were shown in Table 1 and 2. *Citrus limon:* The LC_{50} value of the leaf extract of *C. limon* on*A. aegypti* at 24 hrs. was found to be 430.8ppm., while at 48 hrs. it was observed to be 296.6 ppm. Respectively (Figure 3). On the other hand 185.2 ppm. concentration was found to be safe for adult mosquitoes to survive (Table 1). Maximum 97% mortality in the mosquitoes after 24 hrs. at 700 ppm. and 93% mortality in 48 hrs. at 600 ppm. were further observed from table 1. However, in control, no mortality was observed (Table 1, Figure 3). The LC_{50} value of the leaf extract of *C. sativa* on *A. aegypti* at 24 hrs. was found to be 617.4 ppm., while at 48 hrs. it was observed to be 508.1ppm. respectively (Figure 2). On the other hand, 129.2 ppm. concentration was found to be safe for the mosquitoes to survive (Table 1). Maximum 97 % mortality in the adult after 24 hrs. at 1150 ppm. and 93% mortality in 48 hrs. at 1000 ppm. were further observed Table 1. However, in control, no mortality was observed (Table 1, Figure 2). Further the effects of combination of plant extracts on *A. aegypti* adult mosquito: The synergistic effect on *A. aegypti* was observed when plant extracts were combined in equal proportion (1:1) below the level of their safe concentration limits. They were shown in Table 2 and. Moreover, the combination of plant extracts of *C. limon* and *C. sativa* showed synergistic effect. The LC_{50}at 24 hrs. was 321.4 ppm and at 48 hrs. 286.7 ppm. (Figure 4). On the other hand, 96.4 ppm. concentration was found to be safe for the mosquitoes to survive Table 2 . In the present study the effect of combination of plant extracts were found more effective than the individual plant extracts on the *A. aegypti* adults. The mortality exhibited by the combinations of plant extracts on *A. aegypti* show the possibility of using them as mosquito control agents.

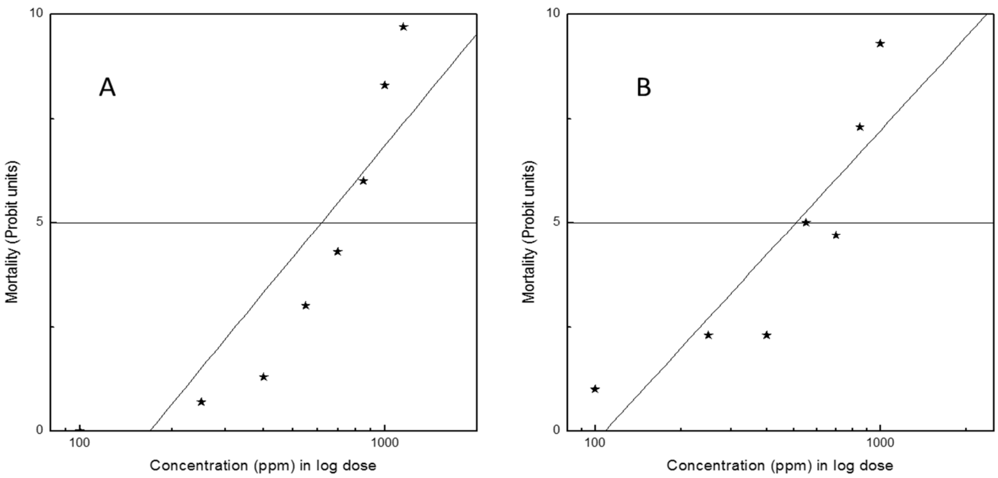

Figure 2.Effect of *C. sativa* on *A. aegypti* mosquito in 24 hrs. (A) and 48 hrs. (B).

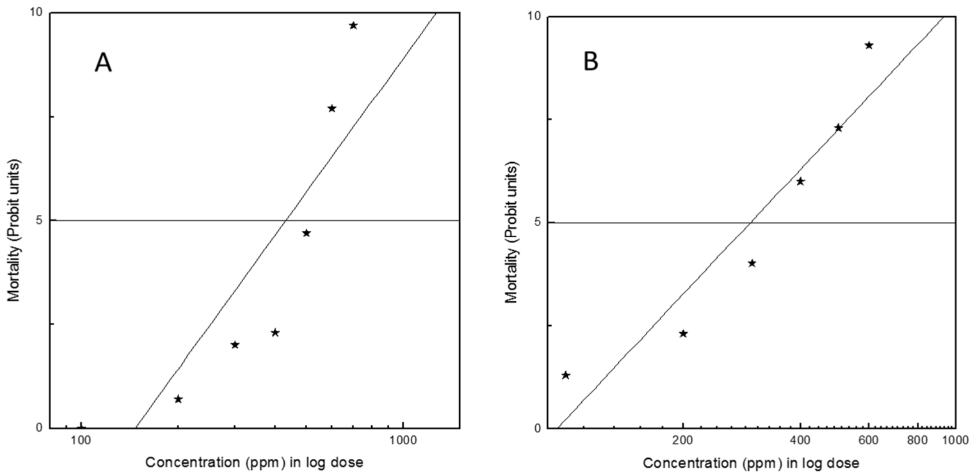

Figure 3. Effect of *C. limon* on *A. aegypti* mosquito in 24 hrs. (A) and 48 hrs. (B)

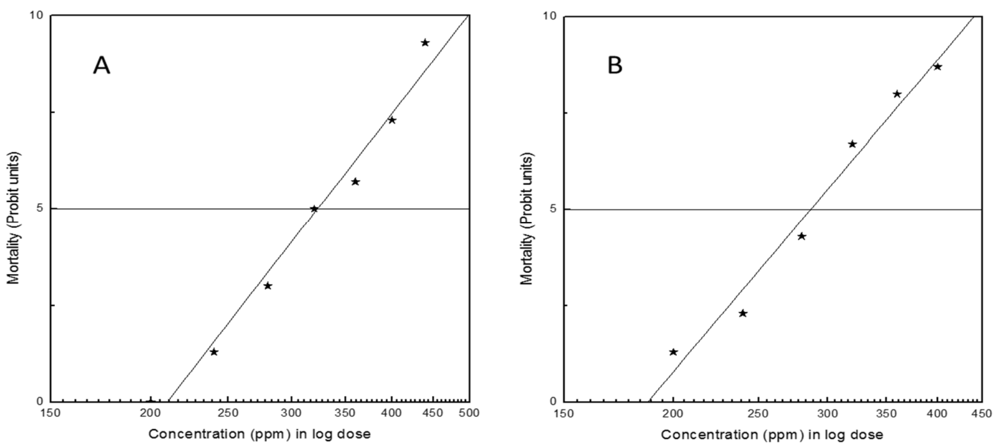

Figure 4. Effect of *C. limon* and *C. sativa* on *A. aegypti* mosquito in 24 hrs. (A) and 48 hrs. (B)

Table 1: LC_{50} and safe concentration (S.C.) of 2 different plants extracts on *A. aegypti* mosquito.

Plant	24 hr. LC_{50} (ppm)	Regression Equation	48 hr. LC_{50} (ppm)	Regression Equation	S. C. (ppm)
C. sativa	617.4	$Y = 8.9x - 19.8$	508.1	$Y = 7.5x - 15.2$	129.2
C. limon	430.8	$Y = 10.7x - 23.2$	296.6	$Y = 10.1x - 19.9$	185.2

Table 2: LC_{50}, Synergistic factor (S. F.) and safe concentration (S. C.) of the combination of plant extracts on *A. aegypti* mosquito

S. No.	Particulars	Values
1.	Combination	*C. limon + C. sativa*
2.	24 hr. LC_{50} (ppm)	321.4
3.	Regression Equation	*Y = 26.6x – 61.8*
4.	Synergistic factor (S. F.)	1.3*
1.	48 hr. LC_{50} (ppm)	*286.7*
2.	Regression Equation	*Y = 26.9x – 611.1*
3.	Synergistic factor (S. F.)	1.5*
4.	safe concentration (S. C.)	96.4

DISCUSSION

Mosquitoes are nuisance pests and vectors of many diseases both in urban and rural areas [35-42]. They cause human death, suffering and impediment to economic development not only in India but all over the world [43-45]. Mosquito borne diseases are one of the most prevalent public health problems in the developing countries. The diseases can be controlled by preventing mosquito bite using repellent, causing larval mortality and killing mosquitoes [46-47]. Mosquito control represents an important strategy for prevention of disease transmission and epidemic outbreaks. However, a high level of insecticide resistance has developed through chemical control of the vector and pests threatening the control strategies. To overcome these problems, it is necessary to search for alternative methods of vector control. The failure of chemical insecticides to control the insect and growing public concern for safe food and a healthy environment have catalyzed the search for more environmentally benign control methods for the management of the vectors. Many approaches have been developed to control mosquito menace. One such approach to prevent mosquito borne diseases is by killing mosquitoes at larval stage. The current mosquito control approaches are based mainly on synthetic insecticides. Even though they are effective, they created many problems like insecticide resistance [48-52], pollution and toxic side effects on human beings [53-56]. Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans and other advantages [43, 57]. This has necessitated the need for a systematic research and development of environmentally safe, biodegradable indigenous method for vector control. Many herbal products have been used as natural insecticides before the discovery of synthetic organic insecticides [9, 13]. The effects of botanical derivatives against mosquitoes have been reviewed by Senthil-Nathan [41], Sukumar *et al.* [56]. Undoubtedly, plant derived toxicants are a valuable source of potential insecticides [57, 58]. Many of the herbs and shrubs are found to have promising medicinal properties, mosquito larvicidal and mosquito repellent properties [59, 60]. Several workers have suggested various larvicidal plant species in the control of mosquitoes [61, 62].

C. Limon: Citrus is one of the most commonly consumed fruits and is also known as flavoring agent. The nature has provided it with elements that have mosquitocidal properties [43] and the oils from these are effective against dengue and malarial mosquitoes [63]. Citrus seeds and peel remain primary wastes. Many researchers have defined ways to utilize citrus peel wastes by producing flavonoids or pectin [58]. Sumroiphon *et al.* [57] reported that the LC_{50} of ethanolic extracts of *Citrus* seed on *A. aegypti* was 2267.71 ppm. and 2639.27 ppm. against *Cx. quinquefasciatus*in 24 hrs. The LC_{50} of local liquor extract of Citrus seed against *A. aegypti* was 6389.22 ppm. and 5611.66 ppm. against *Cx. quinquefasciatus*in 24 hrs. The LC_{50} of aqueous extract of Citrus seed on *A. aegypti* was 135,319.40 ppm. and 127,411.88 ppm. against *Cx. quinquefasciatus* in 24 hrs. of *C. limon* presented high mortality in various life stages.

Sarma *et al*., [47] observed the efficacy of peel and leaf essential oil of *Citrus aurantifolia* against *Aedes aegypti*. The insecticidal activities of leaf and peel essential oils of *Citrus aurantifolia* were conducted against (adults mosqito) *Aedes aegypti* at different concentrations (LC50 value of 5.26 and 17.71 ppm) for 72 hrs and also larvicidal activities of leaf and peel essential oils of *Citrus aurantifolia* were conducted at different concentrations (LC50 value of 188.59, 107.37, and 104.59 104.59 at different time intervals such as 24, 48 and 72 hrs and 128.81 ppm(24h) 106.77 ppm(72h) at different time intervals such as 24, 48 and 72 hrs and these essential oils were analysed by GC-MS that showed the presence of different constituent compounds.

Oshaghi *et al*. [43] observed that *C. limon* oil can be used as repellent against *An. Stephensi* since it showed 71.6 % repellency at 30 min. Yang *et al*. [60] noticed that *C. limon* peel oil contained the insecticidal properties against adults of *Cx. quinquenfasciatus.* However, during the present investigation, the LC $_{50}$ of *C. limon* ethanolic leaf extract were found to be 430.8 ppm. in 24 hrs. and 296.6 ppm. in 48 hrs. Silva *et al*. [54] used it as an ideal approach in public health management in regard to mosquito control which is further recommended through the present study based on the results obtained against the adult stage of *A. aegypti*. The insecticidal properties exhibited by leaf extract of *C. limon* were due to the major components i.e., limonene (3.2-75.2%) or linalool (1.4-47.3%) or *a*-pinene (up to 41.4%). *c*-Terpinene (up to 36.1%), sabinene (up to 30.3%), geranial (up to 22.6%), 1,8-cineole (up to 19.2%), neral (up to 16.1%), linalyl acetate (up to 18.5%), (*E*)-*a*-ocimene (up to 15.8%), and R-terpineol (up to 11.4%) *etc.* [35].

Cannabis sativa: Ghosh *et al.* (2012) observed that *Cannabis sativa* leaf extract made in petroleum ether showed LC_{50} value to be 376.58 ppm. in 24 hrs. and 1316.09 ppm. in 48hrs. against *An. stephensi.* Maurya *et al.* [37] found that when the extract of *C. sativa* tested against *Anopheles stephensi,* LC_{50}within 24 hrs. was 15.58 and 8.04 ppm. in48 hrs. (Aina*et al*., 2009). Insecticidal properties of *C. sativa* were further observed against adult stage of *A. aegypti* though the literature of *C. sativa* as an adult mosquito control agent was not found. It was observed that the LC_{50} value of *C. sativa* was 617.4 ppm. at 24 hrs. and 508.1 ppm. at 48 hrs. The chemical compositions are Cannabigerol, cannabigerolic acid, cannabichromene, cannabichromenic acid, cannabicyclol (aka cannabipinol), cannabicyclolic acid, cannabicitran, cannabielsoic acids A and B, cannabinolic acid (neutral cannabinoid), cannabichromanon, cannabifuran, dehydrocannabifuran 2-oxo-[delta 3]-tetrahydrocannabinol, cannabigerol monomethyl ether, cannabidiol monomethyl ether, cannabinol methyl ether, propylcannabidiol (aka cannabidivarol &cannabidivarin), propylcannabinol (aka cannabivarol & cannabivarin), propyl-[delta 1]-THC (aka [delta 1]-tetrahydrocannabivarol & tetrahydrocannabivarin), propylcannabigerolpropylcannabicyclol propylcannabichromenemethyl cannabidiol (aka cannabidiorcol), methylcannabinol (aka cannabiorcol), methyl-[delta 1]-THC (aka [delta 1]-tetrahydrocannabiorcol), [delta 1]-tetrahydrocannabivarolic acid [15].

Synergistic effects of *C. sativa* and *C. limon*: *C. sativa* and *C. limon* have been considered as good synergists. Hence, they have been used as pest control agents. In the present study, the synergistic effects of *C. limon* and *C. sativa* plant extracts were evaluated. The LC50 found to be 321.4 ppm in 24 hr. and 286.7 ppm in 48 hr.

The present study has identified plants with insecticidal activities, thus indicating their potential for application in the control of the dengue vector, *A. aegypti*. The results of the present investigations revealed the broad-spectrum toxic properties of the tested botanical ethanolic extracts against the adult stage of *A. aegypti.* Screening and identification of effective compounds in plants available in north-eastern region of India will certainly bring more success towards the control of *A. aegypti.* The results of the present investigation revealed the broad-spectrum toxic properties of the tested botanical extracts against the adult stages of *Aedes aegypti.* Combined effect or synergistic effect of different combinations of plant extracts used had proved very advantageous in the control of this mosquito. Thus, the present synergistic approach can be used as an eco-safe popular combination of plant extracts tested, is recommended for management and control of *Ae. aegypti* where the synergistic mixtures can be incorporated into mosquito control programmes as well.

CONCLUSION

As the chemical pesticides are harmful to the environment, insecticides made from plant extracts and their combination are the best choice. The selected botanical products obtained from leaf extracts of *Cannabis sativa* and *Citrus limon* are some of the good mosquito control agents due to their effective insecticidal properties against *A. aegypti* adult stage. They are safe to the environment and human health than synthetic insecticides. Using them as substitutes would not only remedy the current chemical resistance problem of insect pests, but also help to reduce unnecessary loads of toxic chemicals on the environment when used as synergistic mixtures. Hence, are recommended for their application in control of *A. aegypti* mosquito.

Declaration of Competing Interest

The authors declare no conflict of interest.

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