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Research Article

Characterisation and Evaluation of Anti-Larval Properties of Aromatic Plants Against *Aedes aegypti*

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ABSTRACT

Piper nigrum, *Curcuma longa*, *Cinnamomum verum*, *Syzygium aromaticum* are the most commonly used spices for its flavour, aroma etc. Methanolic extracts of the four plant samples were tested against *Aedes aegypti* mosquito larvae which later develops as the vector for dengue virus that affects immunologically compromised individuals. The efficiency of the plant extracts were studied with respect to the third and the fourth instar larvae stages of *Aedes aegypti* mosquito. The larvicidal activity of the four plant extracts were conducted at 5%, 10% and 15% concentrations. *Syzygium aromaticum* showed the highest larvicidal capability when compared to the other three extracts with 100% mortality at all the concentration with fourth instar *Aedes aegypti* larvae and 100% mortality at 10% and 15% concentration with third instar *Aedes aegypti* larvae. *Curcuma longa* showed the next better activity with 38.4%, 84.6% and 100% of mortality at 5%, 10% and 15% of extract concentration respectively for third instar *Aedes aegypti* larva. Extracts of *Curcuma longa* showed 76.9%, 100% and 100% mortality respectively with fourth instar *Aedes aegypti* larva. *Piper nigrum* and *Cinnamomum verum* extracts exhibited higher anti-larval activity in fourth instar stages compared to third instar larva. The GC-MS analysis of partially purified plant extracts concluded the presence of Caryophyllene (RT: 13.830), alpha humulene (RT: 14.300), germacrene D (RT: 14620), p-cumenol (RT: 10.921) Oxime benzaldehyde (RT: 13.045) that confer larvicidal activity in extracts of *Piper nigrum*, *Curcuma longa*, *Cinnamomum verum* and *Syzygium aromaticum* respectively. The toxicity of the extracts (1%, 5%, 10% and 15%) tested on *Danio rerio* did not show any adverse effect. The study also standardised a gel formulation comprising larvicidal compounds (1%) using petroleum ether and their allergic nature was assessed by skin irritability and sensitivity assay. This research can be extended to identify the essentials of the commonly used plant materials as a promising mosquito repellent.

Keywords: Larvicidal activity, GC-MS, *Danio rerio*, sensitivity assay, *Piper nigrum*, *Curcuma longa*, *Cinnamomum verum*, *Syzygium aromaticum.*

INTRODUCTION

Mosquitoes, one of the most adaptive and opportunistic species of animal kingdom are known to spread intense and 'hard to understand' type of diseases, which has become the scope of current research of vector control. Some of the most prevalent disease in the Asian countries are Dengue, Malaria, Chikungunya, Zika virus, Yellow fever etc., in which various deadly pathogens are spread via mosquito as its most suitable vector (Malavige., 2007). The mosquitoes have a typical life-cycle with few changes in shape, function, and habitat (Wayne J. Crans., 2004). During their course of development, the larvae feeds on microorganisms and minute organic substances and develop gradually from first instar stage to fourth instar stage. When the larva has attained its full development and has reached the fourth instar stage, the metamorphosis is initiated for the fourth instar larva to enter pupal stage. Pupae further takes few days and develop into a flying adult. The complete life cycle lasts for about 10 days at room temperature, it also depends on other factors such as feeding, temperature, relative humidity, salinity of water etc (Wayne J. Crans., 2004). The vectors are highly epidemic and their geographical distribution is oriented at the equator thereby affecting Asian and pacific regions (Vaddadi Srinivas *et al*., 2015). It is also studied that the climatic and ecological factors have strong influence on the development, survival of mosquito which is directly proportional to the transmission kinetics of the disease they spread (Paul Reiter., 2001).

As per the study conducted by Pemola Devi *et al*., 2006, the global temperature with rise upto 3.5°C, increases the survival chances of many diseases which are mainly vector- borne and is reported around the world. Other factors that increases the survival of mosquito are improper sanitisation, agricultural practices, urbanisation, irrigation, and deforestation. With respect to *Aedes aegypti*, the important adaptation they have acquired is to withstand desiccation of their egg even under stressed condition thus making it a difficult task to control their replication. Female *Aedes aegypti* mosquitoes lay its eggs on the inner walls of containers thereby withstand desiccation for several months depending on the microclimate and stable states of environment such as temperature, humidity etc., hypothetical condition that even if the studies eradicate its

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Table 1: Phytochemical test results.

Plant extract	*Curcuma longa*	*Piper nigrum*	*Cinnamomum verum*	*Syzygium aromaticum*
Alkaloid	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Flavonoids	+	+	+	+
Phenol	+	+	+	+
Steroids	+	+	+	+
Protein	+	+	+	+
Carbohydrates	+	+	+	+

Table 2: Major anti-larval compounds present in the plant samples.

Sl.no	peak#	R time	I time	F time	Area	Area %	Compound	Plant Source
1	7	13.83	13.76	13.88	59441	4.11	Caryophylle-ne	*Syzgium aromaticum, Piper nigrum*
2	9	14.30	14.23	14.33	32713	2.26	Alpha Humulene	*Piper nigrum*
3	12	14.61	14.57	14.69	49875	3.45	Germacrene -D	*Piper nigrum*
4	5	13.04	13.02	13.07	45100	6.81	Oxime Benzaldehyde	*Cinnamomum verum*
5	6	10.92	10.89	10.97	10741	1.34	p-cumenol	*Curcuma longa*

Table 3: DPPH Assay for Antioxidant activity.

Sl.no.	Extract	Absorbance at 517nm	Antioxidant percentage
1	*Curcuma longa*	0.043	74±0%
2	*Syzygium aromaticum*	0.042	75±0%
3	*Piper nigrum*	0.086	47±0%
4	*Cinnamomum verum*	0.088	48±0%

population, at some point the eggs that has been laid can still desiccate upon favourable condition such as in case of a flood. This peculiar feature of *Aedes aegypti* has enabled it to spread vector borne disease throughout vast geographical distribution (Paul Reiter., 2001). Which makes dengue one of the most dreadful and commonly spread disease in the Asian countries. *Aedes aegypti* which is a day biting mosquito is able enough to spread the four serotypes of dengue virus (Soares Pinheiro *et al*., 2017 and Paul Reiter., 2001). Illness begins with a sudden appearance of fever and that also show a variety of signs and symptoms such as intense headache, severe pain behind the eyes, pains of the muscles and joints, nausea, vomiting, as well as skin rash. Fatality rates if untreated can rise up to 50 %.(Paul Reiter., 2001). There has been no evidence of vaccines against the dengue virus. Hence, biocontrol of vector is the only possible solution.

Individuals with compromised immune system tend to get affected much easier with this disease. Implication of pesticides to retard the growth or control of vector was used widely. Some of the commonly used pesticides have been reported to show adverse effects such as toxic encephalopathy on children. Due to the frequent use of DEET (N,N-diethyl-m-toluamide), allethrin, resmethrin , pyrethoid that are known to cause many side effects such as respiratory trouble, headache, asthma and much more adverse effects are also reported (E H Roland., 1985). This study was conducted on natural products so as to bring about a positive effect on mosquito repellent activity using plant based products such that these natural products can bring no adverse effect on human with respect to respiratory disorders etc. Though some plant products are known to cause allergic reaction in some sensitive individuals, not all the products are harmful similar to the one used in chemical repellents.

Plant extracts of *Piper nigrum, Curcuma longa, Cinnamomum verum, Syzygium aromaticum* showed promising anti-oxidant activity. These plants are known for many other properties apart from antioxidant and anti-larval such as *Piper nigrum* of the family Piperaceae, often called the 'King of Spices', is a universal table condiment used to flavor all types of cuisines worldwide (Mathew *et al*., 2001). *Curcuma longa*, of the family Zingiberaceae, is one of most essential spices used all over the world particularly in the countries of the east (Subrata Mallick., 2014). The plant has been credited with interesting pesticide properties against insects and fungi of agricultural significance, including repellent properties against mosquito species. *Syzygium aromaticum* of the family Myrtaceae have many biological properties (Susheela P., 2016). The twigs as well as barks of *Cinnamomum verum* belonging to the family Lauraceae are abundantly consumed in Asia as a spice and also used as traditional medicine for treating few conditions and diseases. (Xin Chao Liu *et al.*, 2014). The essential extracts of this plant extract has also been reported for larvicidal activity against several mosquitoes such as *Aedes, Culex, and Anopheles* (Sumangala K., 2009). Our research focused on developing a natural, phyto material that could

Table 4: Bioassay using *Danio rerio.*

Concentration	*Curcuma longa*	*Syzygium aromaticum*	*Piper nigrum*	*Cinnamomum verum*
	Percentage mortality (%)			
1%	0	0	0	0
5%	0	0	0	0
10%	0	0	0	0
15%	0	0	0	0

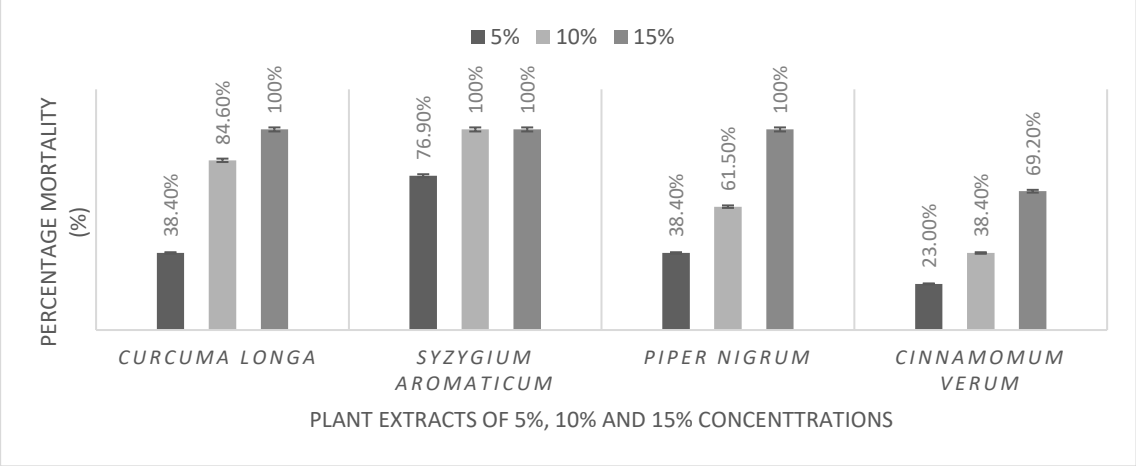

Figure 1: Chart indicating anti-larval activity against third instar larvae.

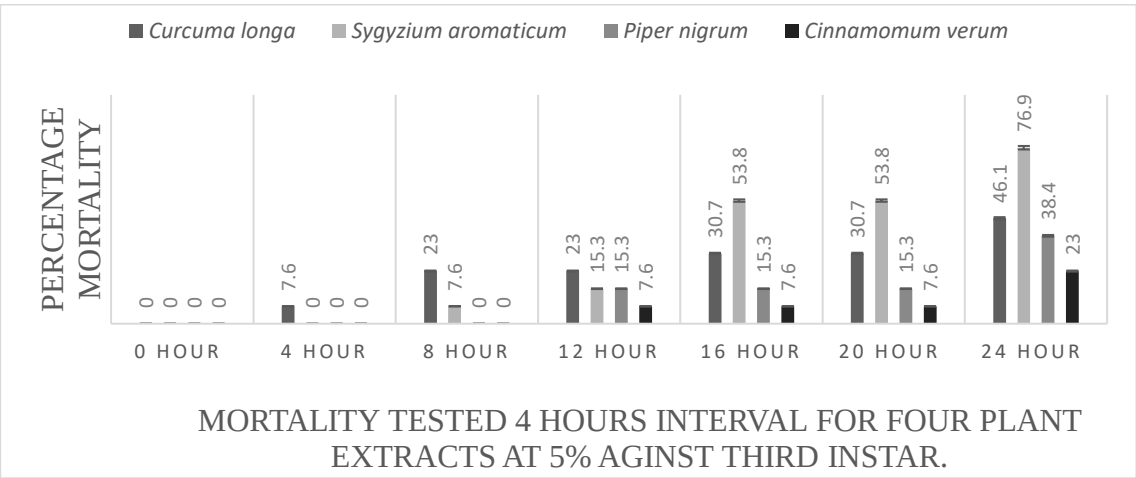

Figure 1a: Graphical representation of larval mortality (THIRD INSTAR) at a time interval of 4hours with 5% concentration of the extract.

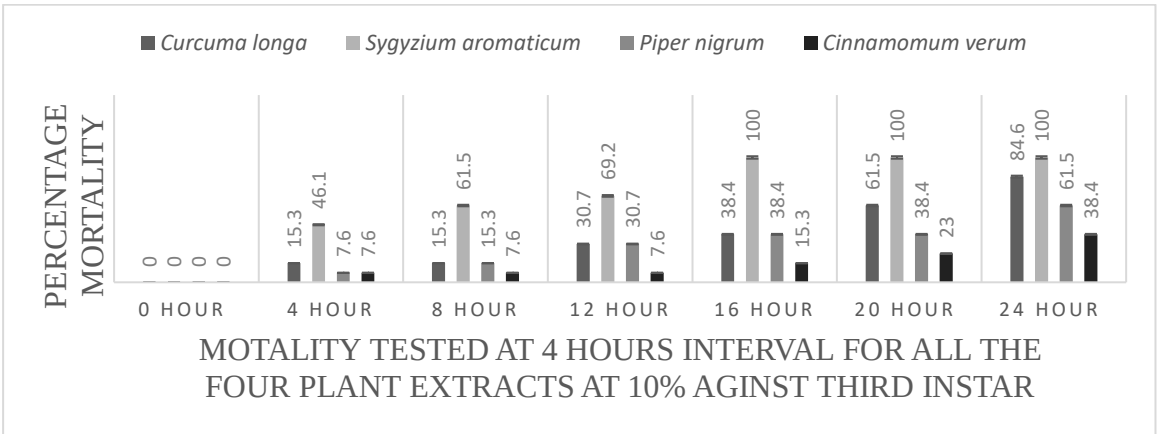

Figure 1b: Graphical representation of larval mortality (THIRD INSTAR) at a time interval of 4hours with 10% concentration of the extract.

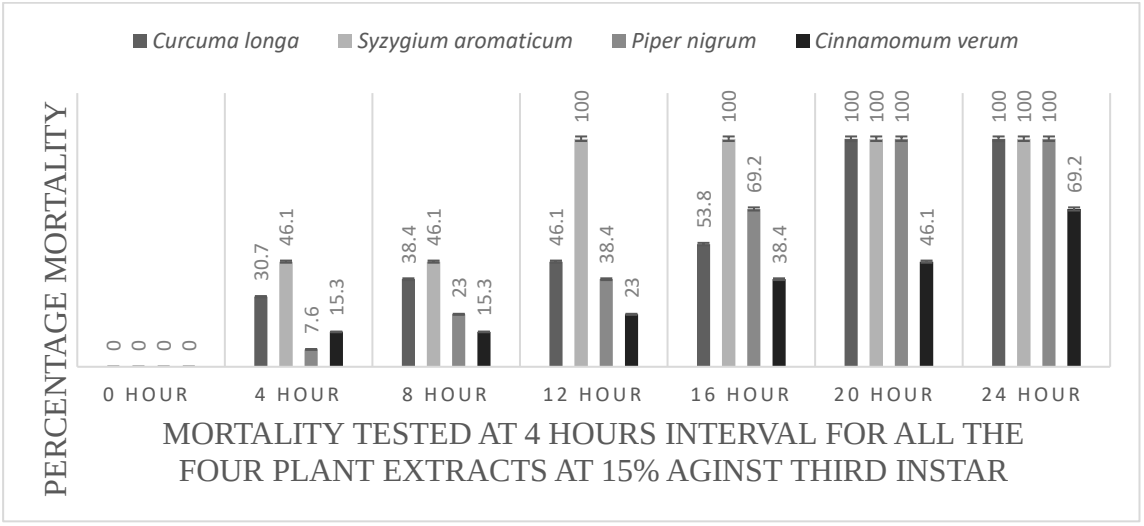

Figure 1c: Graphical representation of larval mortality (THIRD INSTAR) at a time interval of 4hours with 15% concentration of the extract

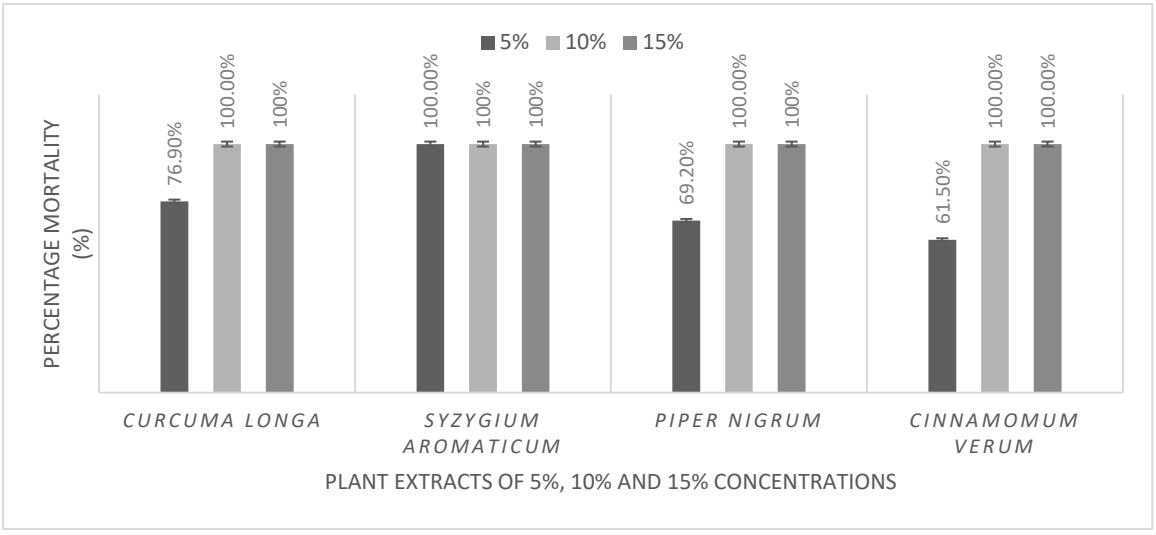

Figure 2: Chart indicating anti-larval activity against fourth instar larvae.

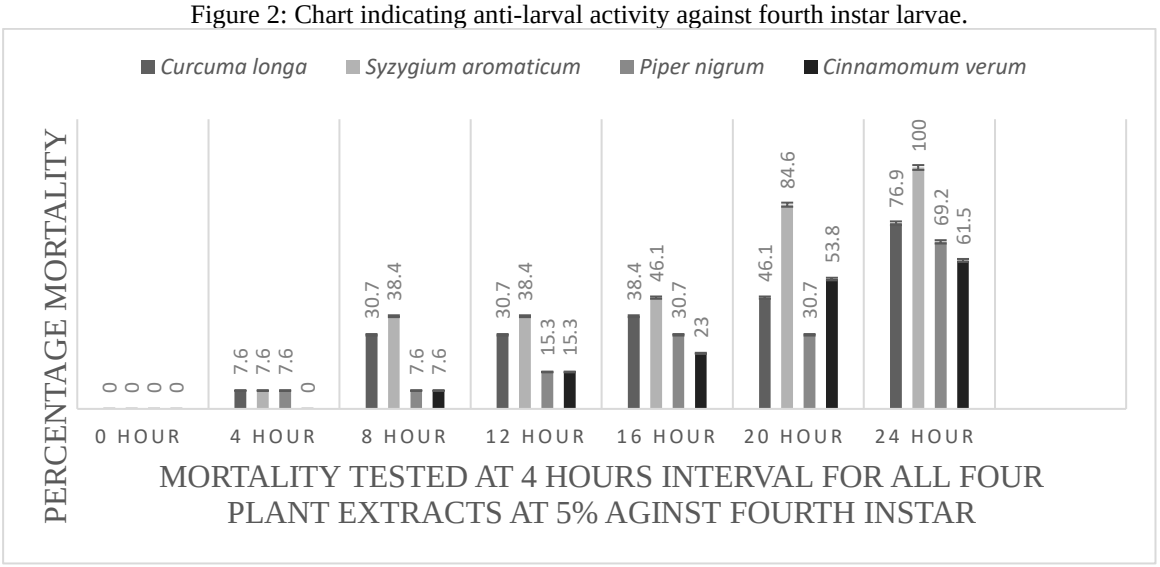

Figure 2a: Graphical representation of larval mortality (FOURTH INSTAR) at a time interval of 4hours with 5% concentration of the extract.

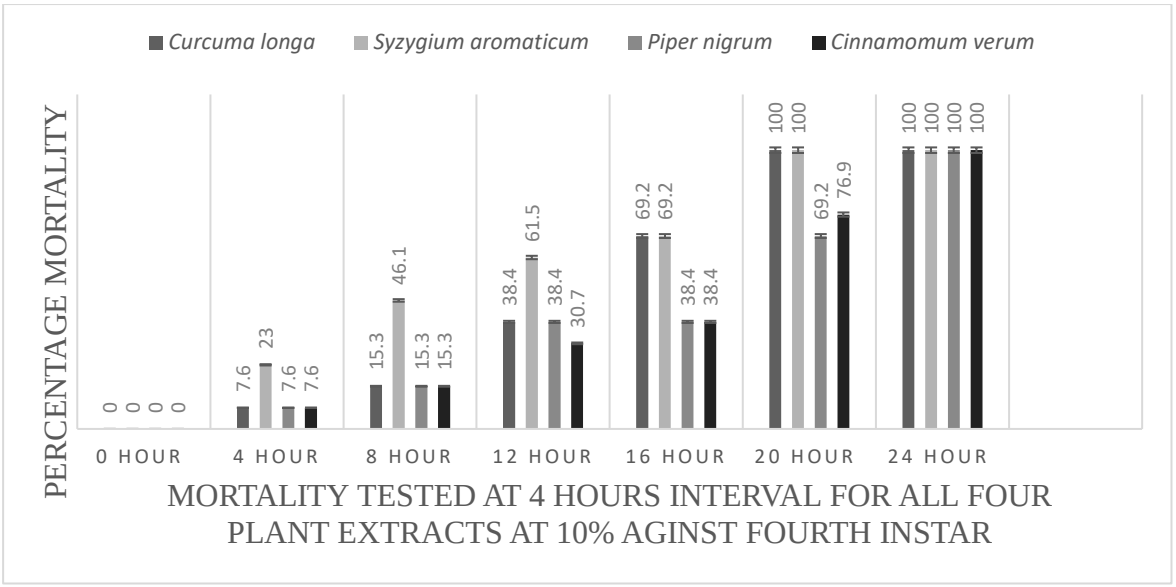

Figure 2b: Graphical representation of larval mortality (FOURTH INSTAR) at a time interval of 4hours with 10% concentration of the extract.

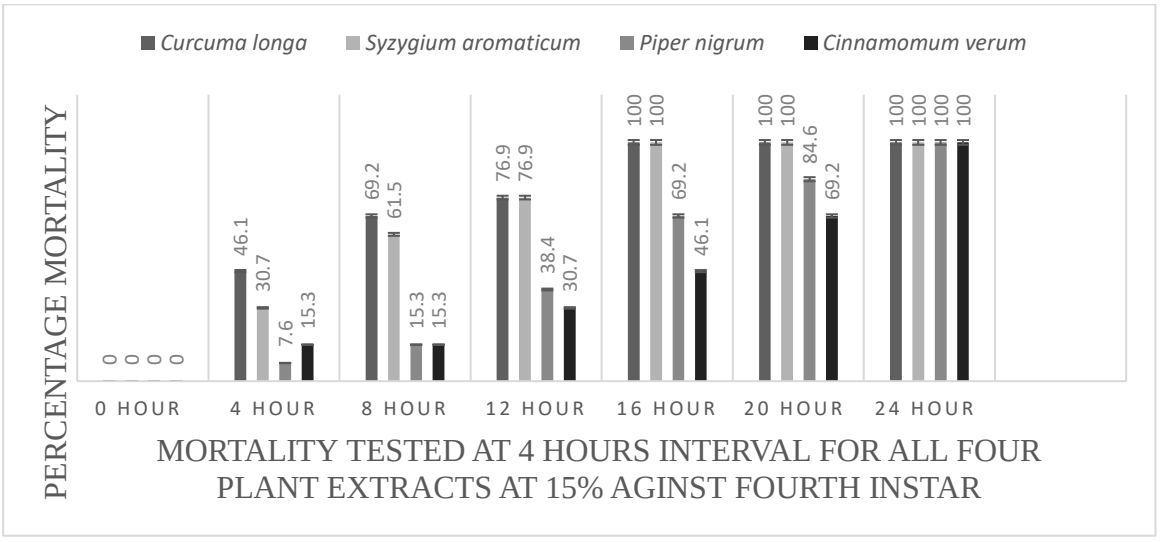

Figure 2c: Graphical representation of larval mortality (FOURTH INSTAR) at a time interval of 4hours with 15% concentration of the extract

be used as a repellent against the highly dangerous *Aedes aegypti* larvae.

MATERIALS AND METHODS

Ethnobotanical identification of plants

Piper nigrum was identified by its alternately arranged pattern on the stem, its leaves are almond shaped with tapering ends, and they are dark green in colour which has more shine on the top region than the bottom. *Syzygium aromaticum* leaves were identified due to the presence of their leathery, shiny texture on the upper side, when young, these leaves are bright pink in colour which on development turns into light glossy green. They are oblong shaped with a peculiar aroma whose base is generally spotted with black spots. *Curcuma longa* leaves were identified as they are alternatively arranged distichous with a dark green colour on top with prominent midrib and pale green at the bottom. They have thin blades which are elliptic to oblong and the presence of their prominent aroma (Velayudhan.,2012).*Cinnamomum verum* leaves were ovate to elliptical and arranged oppositely, their texture is stiff and bear three yellow veins from the base to the tip and the leaves are reddish when young (Jiofack.,2010). Every leaf has its own peculiar aroma making them distinct.

Preparation of plant extracts

The leaves of *Piper nigrum, Curcuma longa, Cinnamomum verum* and *Syzygium aromaticum* was collected from Ethno medicinal garden, Bangalore, India. Leaves were shade dried for ten days and was ground to coarse powder and preserved in dry containers at room temperature. 10 grams of the leaf powder was utilised for Soxhlet extraction using 100% methanol as solvent system (RNS Yadav., 2011). The extracted material was collected in the baby jars and stored at -4°C for further use. Three different concentrations of all the samples were prepared.

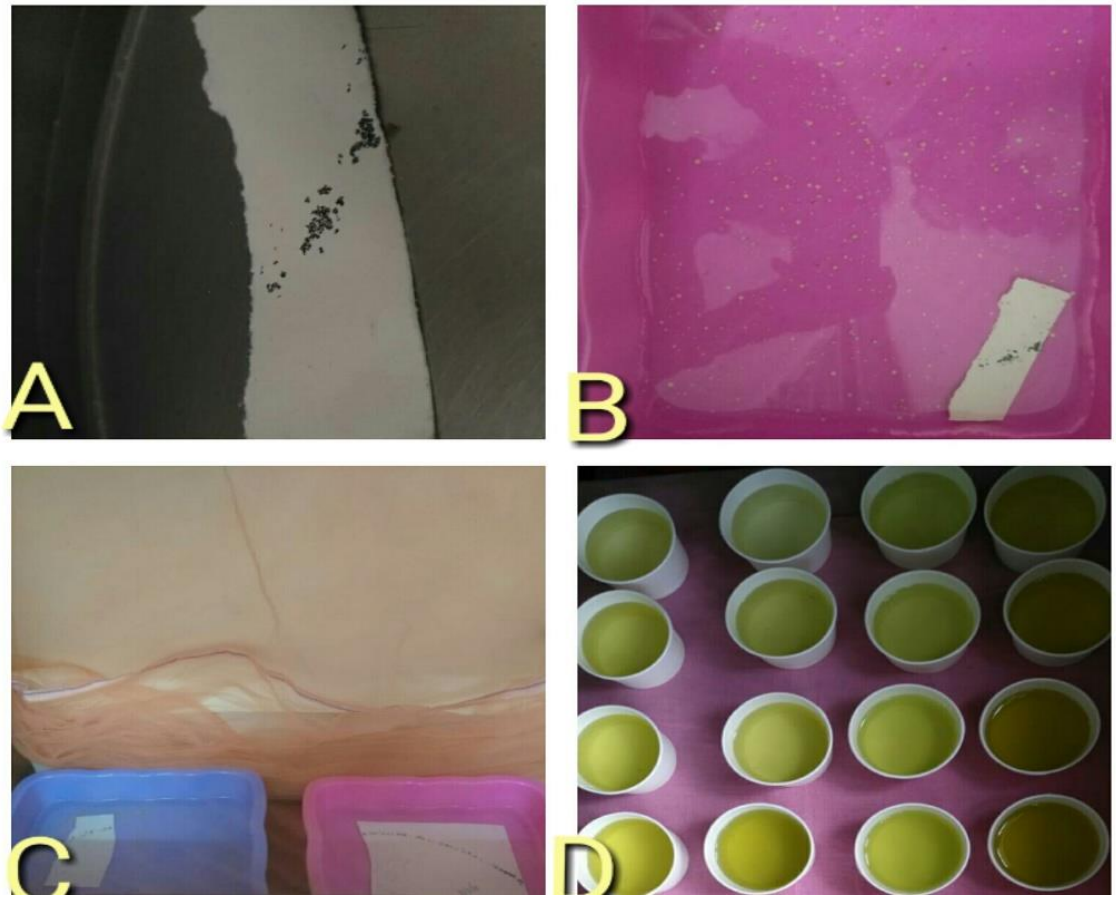

Figure 3: A: *Aedes aegypti* egg sheet, B: Egg sheet dipped in RO water and provided with diet, C: Setup was covered from top using net and preventing any intervention, D: plants arranged at increasing concentrations to study the effect of it on the larvae.

The concentrations designed for the study were 5%, 10% and 15% with distilled water. Experiments were repeated twice to confirm the reproducibility.

Phytochemical assessment

The bioactive compounds in the plant extracts were tested using standard qualitative phytochemical procedures. Test for alkaloids, flavonoids, tannins, saponins, proteins, steroids, phenols, carbohydrates were analysed (RNS Yadav., 2011). 2ml of extracts were used to test the phytochemical compounds using their respective reagents. Alkaloids were tested using Wagner's method, flavonoids using sodium hydroxide (Mohammad Amzad Hossain., 2013), tannins using lead acetate (Lemino Singh., 2013), saponins by conducting foam test, proteins using Millon's reagent, steroids using chloroform, sulphuric acid and acetic acid, phenols using ferric chloride and carbohydrates using Fehling's test (RNS Yadav., 2011).

GC-MS analysis

GC-MS analysis was conducted using the protocol of Praveen Kumar., 2010. The extract was processed and subjected to GC-MS analysis using GCMS-OQP2010SE, SHIMADZU. Column used was SH-Rxi. 5sil MS (30m×0.25mm ID×0.25µm df) Helium gas was the carrier gas at the speed of 1 ml/minute. The injection quantity used was 8µl and the injected temperature was programmed at 250℃, the ion source temperature was 200℃, the oven temperature was set from 60℃ (isothermal for 3 minutes) with an increase of 10℃/minute to 200℃, ending with an isothermal at 280℃. There was a solvent delay of 4-5 minutes. The compounds present in these extracts were sensed and were analysed using NIST 17 library.

Antioxidant activity (DPPH Assay)

Based on the method followed by Alagesan Paari *et al* (2011), the radical scavenging percentage was calculated using 15µl of each sample with 2 ml of methanol along with 1 ml of DPPH, shaked vigorously and incubated at an area with no light passage for 30 minutes the radical scavenging activity was detected at 517nm using spectrophotometer. Methanol along with 1 ml of DPPH was kept as the blank. The radical scavenging activity or the antioxidant capability was calculated using the following equation:

% of radical scavenging activity =

$$\frac{absorbance\ of\ DPPH - absorbance\ of\ extract}{absorbance\ of\ DPPH} \times 100$$

Collection and growing of Aedes aegypti eggs

The *Aedes aegypti* eggs were collected from the CRME (Centre for Research in Medical Entomology), Madurai, Tamilnadu. (Figure: 4B: A) The eggs were preserved in a dry container and stored at room temperature. Care was taken to avoid contact with water. These *Aedes* eggs were

developed to larval stages based on the procedure given by WHO (2016). The *Aedes aegypti* eggs were allowed to grow in trays with 500 ml reverse osmosis water at room temperature at controlled environment. Self-safety precautions were taken by using gloves and masks while handling the setup. Mosquito larvae were fed at intervals of 24 hours with a diet of 5% protein, 5% fat, 0.3% fibre, 145 mg of calcium and 1.5mg of vitamin E contained in the form of dry powder and dry yeast at 3:1 ratio. Cages and racks were supplemented for their growth and maintained in a separate animal breeding room with periodic cleansing of water. Controlled room temperature of 25℃ was maintained until *Aedes aegypti* attained third and fourth instar stages. (Figure: 4B- A, B, C and D)

Effect of extracts on larva

As per the WHO protocol, the larva was selected at third and fourth instar stages based on the changes in their size and variation in colour. The fourth instar *Aedes aegypti* larvae was differentiated from third instar *Aedes aegypti* larvae with the elongated body size and darker coloration of growing mid gut. The selected *Aedes aegypti larvae* was developed in a tray of size 30×20×4 cm of length, breadth and height respectively. Fully grown stages of 13 larvae were collected and transferred into 5%, 10% and 15% concentrations of the four plant extracts. *Aedes aegypti* larvae were exposed to the extracts for 24 hours and their mortality was noted for every 4 hours.

Preparation of formulation

To analyse the possibility to develop a repellent formulation against adult mosquito, a formulation was prepared using petroleum jelly. Extracts of *Piper nigrum, Curcuma longa, Cinnamomum verum* and *Syzygium aromaticum* was prepared using the following procedure: 1. 5 mg of petroleum jelly was weighed and was placed in a water bath and allowed for double boiling. The petroleum jelly was allowed to reach up to 75℃ at which it melts and changes its phase to liquid. To the melted jelly, 2 ml of the extract of three different concentrations (5%, 10% and 15%) was added and stirred continuously for 20 minutes. Care was taken throughout to avoid froth formation. This mixture was removed from the water bath and allowed to stand at room temperature until it solidifies into a gel form. The prepared formulation was used to check the skin sensitivity.

Sensitivity assay

Following the protocol of Ranasinghe *et al.,*2016 skin sensitivity assay was carried out on individuals with no other skin irritability issues with general medicines, food ingredients etc. Eight volunteers were made to sterilise their lower forearm region with alcohol and allowed to dry. After the alcohol was completely dried off, the prepared formulation was applied in small circular area and allowed to incubate for 30 minutes and the individuals were checked for any irritability in the form of redness, itchiness etc. Skin irritation and redness was ensured using a sensory score ranging from 1 to 5. One being no irritation and 5 indicating redness.

Bioassay using zebra fish

The toxicity of the plant extracts was tested on the zebra fish, *Danio rerio.* The fishes were grown in fish bowls of diameter of 15cm. The fish bowls were filled with 500 ml of reverse osmosis water. The setup was maintained at controlled room temperature at 25℃ supplemented with aerator. As per the protocol followed by Suwannee Promsiri., 2006, fishes were exposed to 1%, 5%, 10% and 15% concentration of *Piper nigrum, Curcuma longa, Cinnamomum verum* and *Syzygium aromaticum* extracts separately and observed for their survival at 24 hours.

RESULTS AND DISCUSSION

The extracts of *Piper nigrum, Curcuma longa, Cinnamomum verum, Syzygium aromaticum* (pepper, turmeric, cinnamon and clove respectively) were selected for the study as they are the most commonly used condiments in Asian countries for increasing the flavour of the traditional food materials, thus availability of these plant specimens is abundant in these countries. These plants are not only known for their flavours but also known for many other characters such as antioxidant, antibacterial etc. The usage of many synthetic materials for the purpose of eradication and management of mosquito have caused an increased number of accumulation of pollutants in the environment over the period of time has become a source of pollution. One such synthetic substance used to manage mosquito was DDT (Dichlorodiphenyltrichloroethane) which has now become one of the major source of pollution (Tenzing Gyalpo., 2012). Other such compounds are DEET (N, N-Diethyl-meta-toluamide), Scourge, Anvil, Permethrin, Malathion etc. Usage of natural compounds such as plant based derivatives would not cause a harmful effect on the environment since it is biodegradable. Natural substances such as plants, plant parts and plant secretory compounds have many natural anti-larval and mosquito repellent properties. Factors such as aroma of certain plants, acidic residues of certain fruits etc causes a tendency of aversion in mosquitoes towards these plants. Therefore the study was mainly conducted for assessing the larvicidal activity of four natural extracts of the selected plant samples such as *Piper nigrum, Curcuma longa, Cinnamomum verum, Syzygium aromaticum* was assessed. Three different concentrations: 5%, 10% and 15% of all the plant extracts showed higher control over fourth instar larvae compared to third instar larvae. According to Bruce A. Harrison.1973, the *Aedes aegypti* larvae develop complex after every moulting, therefore by the time they reach the fourth instar stage they become complex and their parts are much resistant to various environmental conditions whereas the present study when compared to the above data showed difference that the fourth instar larvae showed lesser resistance than the third instar larvae. The extracts of *Syzygium aromaticum* that showed 100% mortality in alcohol extract of clove along with onion, the highest anti-larval property in both the instars with 100% mortality in all the three concentrations in fourth instar and 100 % mortality in 10% and 15% in third instar, Susheela P., 2016 also reported similar pattern of larval mortality. Next to *Syzygium aromaticum* the highest anti-larval activity was observed in *Curcuma longa* with 100% mortality at 15% and 10% concentrations and 76.9% mortality at 5% in larva fourth instar whereas when

compared to third instar larva was found to be 100% only at 15% concentration along with 84.6% and 38.4% larval mortality at 10% and 5% respectively. Similar reports of mortality was observed by Viji.S., 2015 at 0.6mg/ml of *Curcuma longa* on *Aedes aegypti* larvae. The third highest mortality was seen in *Piper nigrum* with 100% mortality at 15% and 10% concentration in fourth instar just as in *Curcuma longa* whereas the 5% concentration showed only 69.2% mortality. The mortality rate at 5% and 10% concentrations of third instar was found to be 38.4% and 61.5% respectively. The least mortality in third instar larvae was exhibited by *Cinnamomum verum* with no 100% mortality. 23%, 38.4% and 69.2% mortality was observed at 5%, 10% and 15% respectively. However in fourth instar 100% mortality was noted for 10% and 15% concentration and only 61.5% mortality noted at 5%. This varied mortality rate may be due to the presence of less number of phyto compounds in the *Cinnamomum verum* extract as studied by the GC-MS analysis and also reported by Xin Chao Liu *et al.*, 2014. (Figure 1, Figure 2) The larval death was studied at varying time intervals of every 4 hours for 24 hours and the individual result is displayed in table 1a, 1b and 1c for 5%, 10% and 15% concentration of third instar respectively. Tables 2a, 2b and 2c displays the 5%, 10% and 15% concentrations studied on fourth instar larvae.

The most important anti-larval compounds found from the GC-MS analysis were Caryophyllene (RT: 13.830), p-cumenol (RT: 10.921), alpha humulene (RT: 14.300), germacrene D (RT: 14620), Oxime benzaldehyde (RT: 13.045) (Table 2). A study conducted by Praveen Kumar., 2010, the presence of high number of volatile phyto compounds from which the compounds specific to the anti-larval study was studied and the work was compared to the present study.

The plant extracts were examined for various phytochemicals present in them using standard qualitative analysis (RNS Yadav., 2011). Presence of alkaloids, flavonoids, tannins, proteins, steroids, carbohydrates, phenols and saponins were recorded in all the plant samples (Table 1). These are the eight basic phyto compounds that has various properties such as anti-bacterial, anti-larval, nutritional benefits etc and was assessed for its presence in the plants that is selected for the present study.

As per the protocol followed by Promsiri., 2006, bioassay was conducted with the zebra fish, *Danio rerio.* Zebra fish is a fresh water fish commonly found in Asian countries especially India and Pakistan. Its similarity at genetic level with humans, makes *Danio rerio* an important species of vertebrate study. The observations done at 4 different concentrations of 1%, 5%, 10% and 15%. The study indicated that the zebra fish showed strong potential of survival against the extracts, thus indicating that the extracts were safe at all the four ranges of extract concentration. Thus marking a biosafety line for the usage of these extracts. Table 4 indicates the zero percent mortality at all concentrations.

The formulation was prepared and the gel was tested on eight individuals (equal number of males and females).The individuals were on constant observation throughout the study, at every 10 minutes interval the individuals were checked to observe any irritabilities in the form of itchiness, redness etc. the individuals were also interrogated to check if they had any other symptoms such as nausea etc. It was concluded that none of them indicated any skin irritabilities or any other side effects of applying the formulated plant extract gel on their skin.

According to Uchenna J. Unachukwa., 2010, free radicals are atoms with odd number of electrons. It's become a recent boom to the field of research to find an alternative to the synthetic anti-oxidants used which are synthetically made with chemicals and that which cause side effects. DPPH assay is based on the antioxidants with DPPH and its ability to reduce the DPPH to DPPH-H and as a consequence the absorbance decreases. (Thomas J. Herald., 2012) The degree of discoloration from red to yellow indicates the scavenging capability of the extracts. This highest antioxidant capacity of the *Syzygium aromaticum* with 75%, followed by *Curcuma longa* (74%), *Piper nigrum* (48%) and least is the *Cinnamomum verum* (47%) (Table 3)

CONCLUSION

Variation in the result when compared to other studies might be due to difference in the climatic condition for the larval mortality study, different standards of chemicals used.

The outcome of present results concluded the larvicidal property of the natural extracts against *Aedes aegypti* over the conventional methods of using various chemicals which over the course of exposure has led to many invariable diseases and also has been a reason for creating more of resistance in the mosquitoes. The study has drawn various conclusions with respect to the anti-oxidative capability of the four extracts along with their compatibility with the environment as it did not lead to the mortality of the zebra fish. The extracts we have re reported to various phytochemicals that is essentially present in most of the economically aiding plants. By the method of GC-MS these extracts also showed a good amount of compounds that are anti-larval in nature which makes an impact of them over the chemicals as a method of removal of disease causing mosquito.

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