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Preliminary phytochemical profiling and ovicidal potential of *Carica papaya* leaf extracts against the filarial vector C*ulex quinquefasciatus* (Diptera: Culicidae)

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Abstract
Mosquitoes play a predominant role in the transmission of many diseases. Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems. One such possibility is the use of botanicals which are readily biodegradable, nontoxic and show broad spectrum target specific activity. Therefore, the aim of the present study is to evaluate the ovicidal activity of extracts of *Carica papaya* leaf for their toxicity against the medicinally important vector mosquitoes (*Culex quinquefasciatus*). The ovicidal activity was observed after 48h of exposure. Maximum egg mortality was observed in chloroform extract followed by ethanol and petroleum ether extracts. Phytochemical screening of the leaf extracts showed the presence of bioactive compounds such as alkaloids, tannins, phenols, flavonoids, sterols, terpenoids, saponins, anthroquinones, proteins and quinones. To conclude the present investigation leads the path of exploration of *C. papaya* for eradication of selected important human vector mosquito, thereby, gaining a real momentum to include this plant product for intense mosquito control programme.

Keywords: Mosquito, *Culex quinquefasciatus,* plant extracts, *Carica papaya*, phytochemicals, ovicidal activity

1. Introduction
The Dipterian family Culicidae is sub divided into three subfamilies Anophelinae, Toxorhynchites and Culicinae. This includes more than 3400 species of mosquitoes [1]. On the basis of the fossil record, it is generally accepted that mosquitoes had evolved by the Jurassic approximately 210 million years ago [2].Our world today is still plagued by a myraid of ailments or diseases and a number of these diseases are caused by organisms which are vector borne. Mosquitoes which also serve as vectors of diseases to human have a worldwide distribution. They live throughout the world apart from the Antarctic regions [3].
Culex quinquefasciatus is an obligatory ecto-parasite vector, since it plays a major role in the transmission of the nocturnal periodic form of *Bancroftian filariasis* all over the world [4]. Vector borne diseases also results in school absenteeism, loss of productivity, aggravation of poverty, high costs for health care and a burden on public health services [5]. Some of the most debilitating diseases caused by mosquitoes are malaria, dengue, lymphatic filariasis, Japanese encephalitis, leishmaniasis, onchocerciasis, schistomiasis and trypanosomiasis [6]. Despite centuries of control efforts, mosquito borne diseases are flourishing worldwide. With a disproportionate effect on children and adolescents, these conditions are responsible for substantial global morbidity and mortality. Vector-borne diseases continue to inflict high morbidity and mortality, particularly in the resource constrained developing countries [7].
Targeting mosquito larvae and egg is more desirable than controlling adults because they are concentrated in a relatively small area [8]. The frequent use of insecticidal sprays and other chemical compounds to kill mosquitoes has lead to a destabilization of the ecosystem and has affected the natural environment. Insecticides resistance is also increasingly becoming a challenge in many vector control activities. One major drawback of chemical insecticides is that once introduced into the systems, they may remain there forever or for a very long duration. Thus they pose a threat to life and help insects to develop resistance against them [9].

To evade these problems, a major emphasis has been made on the use of natural plant based ovicides which could be a safe alternative to the synthetic insecticides [10]. Phytochemicals are the chemicals produced by various parts of the plants. These bioactive constituents of plants are steroids, terpenoids, carotenoids, flavonoids, alkaloids, tannins and glycosides. These compounds have various activities such as antimicrobial and antibacterial some have been reported to exhibit haemolytic and foaming activity [11]. Botanical insecticides are powerful with lesser side effects and degrading after sometime reducing the change to develop resistance against it. These benefits have renewed interest in exploiting the pest control potential of plants. Thus, the effort towards mosquito control continues to be an important strategy in preventing the mosquito borne diseases [9].

The determination of the phytochemical constituents of plant extracts are essential in order to ensure the reliability of pharmacological and clinical research, to understand their bioactivities and possible side effects of active compounds and to enhance product quality control. Thus with regard there is much to be explored [12]. Searching for eco-friendly pesticide molecules from natural sources has become an important research these days. Nature is providing innumerable bioactive molecules for the well-being of mankind. Hence biological control method using plants would be a good approach in mosquito control program.

2. Materials and Methods

2.1 Origin and laboratory maintenance of the mosquito colonies

Mosquitoes used in the present study were *Culex quinquefasciatus*. Individuals were reared for several generations in the Department of Zoology, Nirmala College for Women, Coimbatore by Hay infusion method under laboratory conditions.

2.1.1 Collection of test materials

Fully developed fresh leaves of the plant *C. papaya* were collected from the natural habitat of Coimbatore locale, Tamil Nadu, India.

2.1.2 Preparation of leaf and flower powder

Fresh leaves were collected, washed in water and dried under shade at room temperature for 2 to 3 weeks and were powdered using an electric pulverizer. Fine powder was obtained by sieving.

2.1.3 Preparation of extracts

10g of the leaf powder was weighed using an electronic balance and were subjected to extraction[13,14]. Petroleum ether extraction was followed by chloroform and ethanol extraction in their increasing order of polarity. The leaf extracts thus obtained were concentrated by distillation and dried by evaporation in a water bath. The residue thus obtained was used for further bioassays.

2.2 Ovicidal Bioassay

Ovicidal activity was assessed by the slightly modified method of Su and Mulla[15]. The egg raft/eggs of *Cx. quinquefasciatus* were collected from Department of Zoology, Nirmala College for Women, Coimbatore. The *C. papaya* leaf extracts were diluted in the appropriate solvents to achieve various concentrations ranging from 100 to 300 ppm. Eggs of the mosquito species (100 nos.) were exposed to each concentration of *C. papaya* leaf extracts. After treatment the eggs from each concentration were individually transferred to distilled water cups for hatching assessment after counting the eggs under microscope.

Each experiment was replicated three times along with appropriate control. The hatch rates were assessed 48 h after treatment and counts were made every 24 h after exposure until the test was terminated. The hatch rates were assessed by the following formula.

$$\% \text{ of egg mortality} = \frac{\text{Mortality at treatment} - \text{Mortality at control}}{100 - \text{Mortality at control}} \times 100$$

2.3 Statistical analysis

The data of bioassay studies were also subjected to One Way Analysis of Variance (ANOVA) as described by Panse and Sukhatme [16]. The egg mortality data were subjected to probit analysis [17].

2.4 Phytochemical screening

2.4.1 Qualitative analysis

Preliminary phytochemical screening of leaf extract of selected plant was carried out using the standard procedures of Raman [18].

Test for Alkaloids

- **Mayer's test**

A fraction of extract was treated with Mayer's test reagent (1.36 g of mercuric chloride and 5 g of potassium iodide in 100 ml of water) and observed for the formation of cream coloured precipitate.

- **Wagner's test**

A fraction of extract was treated with Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml water) and observed for the formation of reddish brown colour precipitate.

- **Hager's test**

A few ml of extract was treated with Hager's reagent (saturated aqueous solution of picric acid) and observed for the formation of prominent yellow precipitate.

Test for Tannins

- **Acetic Acid Test**

The extract was treated with acetic acid solutions and observed for the formation of red colour solution.

- **Dilute HNO_3 Test**

The extract was treated with diuted HNO_3. The extract turns from reddish to yellow colour which indicates the presence of tannins.

Test for Phenols

- **Ferric chloride test**

The fraction of extract was treated with 5% ferric chloride and observed for the formation of deep blue or black colour

- **Liebermann's test**

The extract was heated with sodium nitrite, added H_2SO_4 solution diluted with water and excess of dilute NaOH was added and observed for the formation of deep red or green or blue colour.

Test for Flavonoids

- **NaOH test**

A small amount of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange colour.

- **H_2SO_4 test**

A fraction of the extract was treated with concentrated H_2SO_4 and observed for the formation of orange colour.

Test for Sterols

- **Liebermann-Burchard test**

Extract (1 ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added and observed for the formation of dark pink or red colour.

Test for Terpenoids

- **Liebermann-Burchard test**

Extract (1 ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added and observed for the formation of dark green colour.

Test for Saponins

- **Foam Test**

The extract or dry powder was vigourosly shaken with water and observed for the formation of persistent foam.

Test for Anthraquinones

- **Borntrager's test**

About 50 mg of powdered extract was heated with 10% ferric chloride solution and 1ml concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia and observed for the formation of pink or deep red colouration of aqueous layer.

Test for Proteins

- **Ninhydrin test (Aqueous)**

The extract was treated with aqueous ninhydrin and observed for the presence of blue colour, indicating the presence of amino acid or purple colour indicating the presence of protein.

- **Ninhydrin (Acetone)**

Ninhydrin was dissolved in acetone and the extract was treated with ninhydrin and observed for the formation of purple colour.

- **Biuret test**

The extract was heated in distilled water and filtered. The filtrate was treated with 2% copper sulphate solution, 95% ethanol and potassium hydroxide and observed for the formation of pink ethanolic layer.

- **Test for Quinones**

A small amount of extract was treated with concentrated HCl and observed for the formation of yellow colour precipitate.

4. Results

4.1 Ovicidal Activity

The efficacy of various extracts of plant *Carica papaya* may vary from one another on the basis of their toxic effects. The various extracts are namely petroleum ether, chloroform and ethanol. The egg rafts of *Cx. quinquefasciatus* were treated with selected doses of plant extracts at different concentrations say 100, 150, 200, 250, and 300 ppm. Along with those concentrations, a control was also maintained. Throughout the experiment, egg hatchability was found to be 100% in the control but egg mortality was observed as 100% in certain ppm of extracts. The results of ovicidal activity are presented in presented in the Fig 1 and results of probit analysis for egg mortality data is presented in Table 1.

4.1.1 Leaf Extracts of *Carica Papaya*

The maximum ovicidal activity was noted in chloroform extract of *C. papaya* leaf. Egg hatchability was found to be totally inhibited at concentrations ranging from 200-300 ppm. At 150 ppm 3.33%, 1.66% and zero egg hatchability was observed at 48 h, 72 h and 96 h respectively (Figure 1 a, b, c).

The moderate ovicidal activity was noted in ethanol extract of *C. papaya* leaf. Egg hatchability was found to be totally inhibited by higher concentrations of 250-300 ppm. At 200 ppm 1.66% hatchability was recorded at 72 h (Figure 1b) and egg hatchability was inhibited completely at 96h.

However minimum ovicidal activity was exhibited by petroleum ether extract, in which total inhibition of egg hatchability was found at 300 ppm throughout the study period. At 200-250 ppm zero percentage egg hatchability was recorded at 96 h (Figure 1c).

4.1.2 Egg Mortality

Maximum egg mortality was observed in chloroform extract followed by ethanol and petroleum ether extracts (Figure 1d). In chloroform extract 100% egg mortality was exhibited at 200-300 ppm and its LC_{50} value was 42.58 ppm and regression equation was Y= -0.22+3.20X. The UCL LC_{50} of chloroform extract was 90.04 ppm and LCL LC_{50} value was recorded as 20.13 ppm. The χ^2 value and SE value were 0.53 and 2.19 respectively (Table 1).

Moderate egg mortality was observed in ethanol extract in which 100% egg mortality was exhibited at 250-300 ppm. 95% egg mortality was recorded at 200 ppm and its LC_{50} value was 100.46 ppm and regression equation was Y= -4.15+4.72X. The UCL LC_{50} of ethanol extract was 90.04 ppm and LCL LC_{50} value was noted as 75.44 ppm. The χ^2 value was 0.31 and SE value was 1.28.

Minimum egg mortality was shown in petroleum ether extract. 100% egg mortality was observed at 300 ppm.94.99% of egg mortality was recorded at 250 ppm (Figure 1d) and its LC_{50}value was noted as 96.25 ppm and regression equation was Y= -2.68+3.87X. The UCL $LC_{50}$of petroleum ether extract was 110.60 ppm and LCL LC_{50} value was observed as 83.76 ppm. The χ^2 value and SE value were 4.03 and 0.92 respectively.

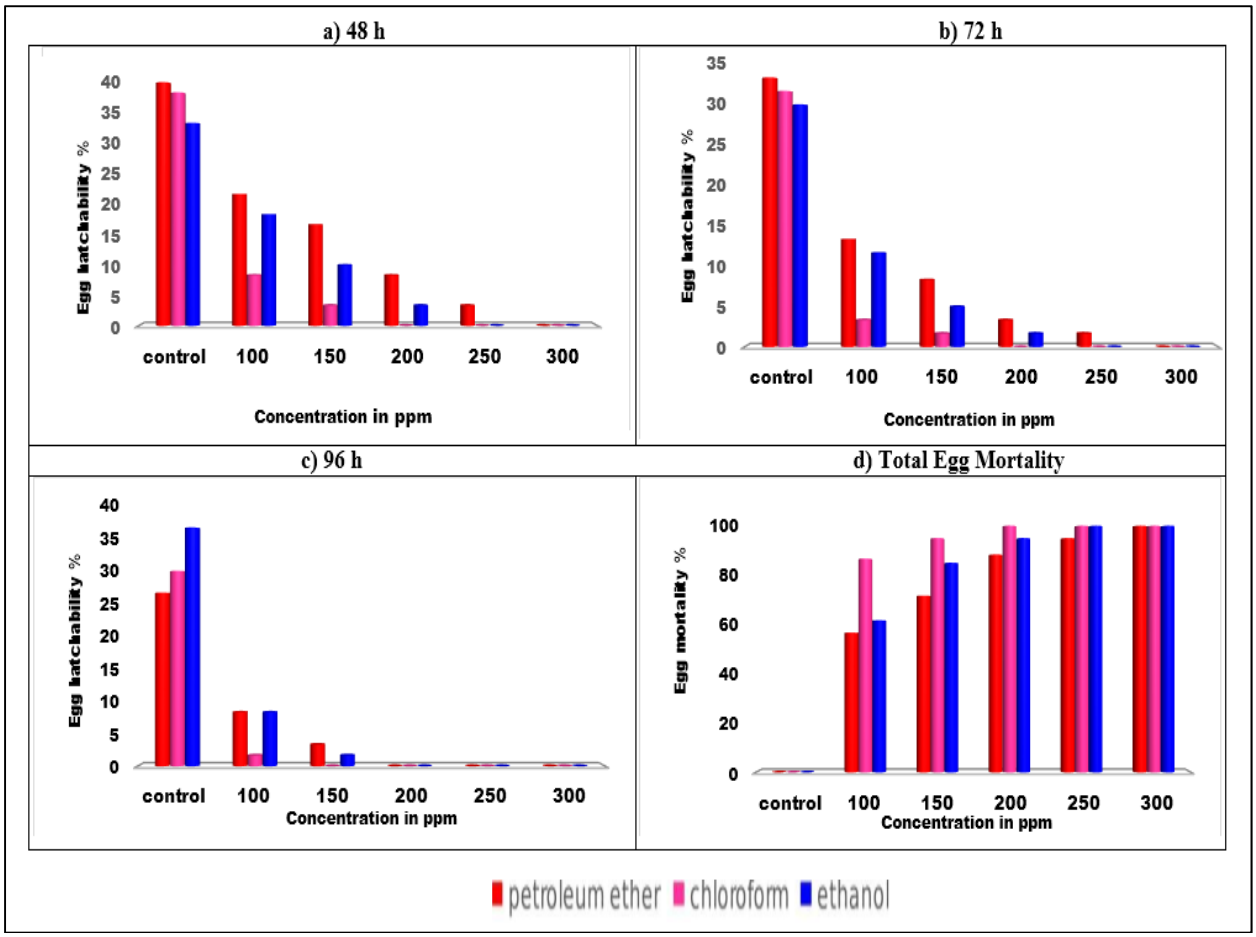

Fig 1: Effect of *C. papaya* leaf extracts on egg hatchability of *Cx. Quinquefasciatus*

Table 1: Lethal concentration of leaf extracts of *C. papaya* against eggs of *Cx. quinquefasciatus*

Solvents Used	Log LC_{50}	Log LC_{70}	Log LC_{90}	LC50 (ppm)	LC_{70} (ppm)	LC_{90} (ppm)	Regression Equation	95% Confidence Limits				χ^2	SE
								UCL (ppm)		LCL (ppm)			
								LC_{50}	LC_{90}	LC_{50}	LC_{90}		
Petroleum ether	1.98	2.11	2.31	96.25	131.47	206.22	Y=-2.68+3.87	110.60	231.29	83.76	183.87	4.03	0.92
Chloroform	1.62	1.79	2.02	42.58	62.04	106.82	Y=-0.22+3.20	90.04	130.87	20.13	87.19	0.53	2.19
Ethanol	1.93	2.05	2.21	87.05	112.43	162.67	Y+=-4.15+4.72	100.46	179.86	75.44	147.12	0.31	1.28

(χ^2) - Chi square

4.2 Phytochemical screening

Phytochemicals screening of extracts of *C. papaya* leaf were carried out to test the presence of secondary metabolites such as alkaloids, tannins, phenols, flavonoids, sterols, terpenoids, saponins, anthroquinones, proteins and quinones by using standard procedures described by Raman[18]. Results of phytochemical analysis are shown in the Table 2.

Chloroform extract of *C. papaya* which showed maximum ovicidal activity exhibited the presence of secondary metabolites such as tannins, phenols, flavonoids, sterols, terpenoids, saponins, anthroquinones and proteins. Moderate ovicidal activity was observed in ethanol extract showed the presence of alkaloids, flavonoids, phenols, terpenoids, proteins, quinones and saponins. Minimum ovicidal activity observed was in petroleum ether extract showed the presence of phytochemical such as saponins, phenols, flavonoids and quinones.

Table 2: Phytochemicals present in the extracts of *C. papaya* leaf

Sl. No.	Constituents	*Carica papaya* leaf		
		Petroleum ether extract	Chloroform extract	Ethanol extract
1	Alkaloids	-	-	+
2	Tannins	+	+	-
3	Phenols	+	+	+
4	Flavonoids	+	+	+
5	Sterols	-	+	-
6	Terpenoids	-	+	+
7	Saponins	+	+	+
8	Anthroquinones	-	+	-
9	Proteins	-	+	+
10	Quinones	+	-	+

(+) Presence (-) Absence

5. Discussion

Due to indiscriminate use of synthetic chemicals to control the mosquitoes in the natural habitats, they have developed strong resistance to almost all the chemicals. Moreover, chemical pesticides gradually altered the behavior of non-target organisms. Thus, in the context, the world scientific community is intensively searching for the alternative mosquitocidal agent preferably from plants available in nature. Today the environmental safety of an insecticide is considered to be of important milestone in the field of pest control in general and vector control programme in particular. An insecticide must not cause high mortality in target organisms in order to be acceptable [19].

In the present study freshly laid eggs obtained from the general stock of mosquitoes were tested for their hatching ability in relation to the different concentrations of chloroform, ethanol and petroleum ether extract of *C. papaya*. Chloroform extract of *C. papaya* exerted 100% mortality throughout the study period at 250-300 ppm. Similar findings were recorded by Govindarajan and Sivakumar [20], that showed that, methanol extract of *Asparagus racemosus* exerted 100% mortality at 375, 300 and 225 mg/l against *Cx. quinquefasciatus, Ae. aegypti and An. stephensi* respectively.

As in the present study, the chloroform extract showed excellent ovicidal activity at higher ppms, it may be compared with the research of Samidurai *et al* [21] in which the methanolic crude leaf extracts of *Pemphis acidula* exhibited 100% ovicidal activity against *Cx. quinquefasciatus* at 500 ppm. Similarly Rajkumar and Jebanesan [22] studied ovicidal activity of *Moschosma polystachyum* leaf extract against *Cx. quinquefasciatus* and observed 100% egg mortality at 100 ml/l. Mullai and Jebanesan [23] have reported complete ovicidal activity at 300 ppm for methanol, benzene, petroleum ether and ethyl acetate extracts of *Citrullus pubescens* against *Cx. quinquefasciatus*. This was in concordance with the results of present study in which petroleum ether of *C. papaya* showed 100% mortality at 250-300 ppm

Broadbent and Pree [24] reported that when eggs were directly exposed to higher concentrations of the compounds, more chemicals entered the egg shell, which affected the embryogenesis. Exposure time also has a crucial role in causing toxicity. At the present study, in the chloroform extract, 86.66% mortality was observed at 100 ppm which gradually increased to 100% as the concentration was increased. Similar observations were recorded by Kuppusamy and Murugan [25] and Govindarajan [26] who stated that longer time exposure periods also facilitate increased penetration of the compounds into the egg shells. Shorter duration of treatment was decisively inferior to longer exposure to insecticides at the egg stage. Smith and Salkeld [27] reported differences in susceptibility to ovicides to occur due to differential rates of uptake, penetration through the chorion, conversion to active inhibitor, detoxification and failure of the toxicant to reach the target.

The lowest hatchability recorded in chloroform, petroleum ether and ethanol extract was 1.66% with no egg hatchability being observed in concentrations ranging from 200-300 ppm, and 250-300 ppm respectively. Similarly aqueous extract of *Leucas aspera* was found to be ovicidal against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* at 500 and 1000 ppm [28]. In the present study of ethanol extract, zero percent hatchability was recorded at 96h in 200ppm. Zero percentage hatchability was observed throughout the study period in 250-300 ppm concentrations.

In petroleum ether, at 96 h of the study period, in the concentration of 200-250 ppm 100% ovicidal activity was observed. 100% ovicidal activity was noted throughout the study period at the concentration of 300 ppm. Similar work was done by Dhanasekaran *et al* [29], in which at 300 ppm of ethanolic leaf extract of *Celosia argentea*, *Anthocephalus cadamba*, *Gnetum ula*, *Solena amplexicaulis* and *Spermacoce hispida* showed 100% ovicidal activity against *Ae. aegypti, An. stephensi* and *C. tritaeniorhynchus*. Roni *et al* [30] carried out ovicidal activity of ethyl acetate, aqueous solution, ethanol leaf extracts *Nerium oleander* against *An. stephensi* at 100, 150, 200, 250 and 300 ppm. At a concentration of 100 ppm the percentage of hatchability was very high and nil hatchability was recorded when the concentration of extract was raised. In chloroform extract of *C. papaya* at 96h in 150 ppm concentration and at concentration ranging 200-300 ppm total arrest of egg hatchability was observed. Similar observations were recorded by Govindarajan *et al* [31] in which the methanol leaf extract of *Coccinia indica* imposed zero egg hatchability at 150 ppm against eggs of *Cx. quinquefasciatus*.

The three extracts of *C. papaya* were subjected to phytochemical screening. In chloroform extract, maximum number of phytoconstituents was present when compared to petroleum ether and ethanol. These variations may be due to a number of environmental factors such as climate, altitude, rainfall etc. as mentioned [32] and also may be due to methods of extraction. Saponins were present in the three extracts namely chloroform, petroleum ether and ethanol in the present study. It is said that saponins has antimicrobial activity [33]. Reports of Dhivya and Manimegalai [34] exhibited that ethanol extract of *Calotropis gigantea* flower extract showed the presence of phytochemical compounds like alkaloids, tannins, phenols, flavonoids, sterols, anthraquinone, proteins and quinones. Similar observations were found in the present study in which phytochemicals such as tannins, phenols, flavonoids, sterols, terpenoids, saponins, anthroquinones and proteins were present in chloroform extract and the ovicidal activity was found to be more effective in this extract. Phytochemicals may serve as suitable alterations to synthetic insecticides in future as they are relatively safe, inexpensive and are readily available throughout the world as reported by Bowers *et al* [35].

Phenols were observed in chloroform and ethanol extract of *C. papaya*. They are generally known to be important sources of potent insecticides, fungicides, bactericides and herbicides for pest control [36]. Phenolic compounds are synthesized in plants partly as a response to ecological and physiological pressures such as pathogen and insect attack [37]. The basic structural feature of phenolic compounds is an aromatic ring bearing methyl ester, hexadecanoic acid, eicosanoic acid, ethyl ester with more hydroxyl groups [38]. In the present study saponins, phenols, flavonoids, quinones and tannins were present in petroleum ether extract of *C. papaya*. Similar observation were noted by Gopieshkhanna and Kannabiran [39] in which phytochemicals such as saponins, carbohydrates, phytosterols, phenols, flavonoids and tannins were present in the plant extracts tested.

In the present study, chloroform extract of *C. papaya* showed effective ovicidal activity on eggs of *Cx. quinquefasciatus* which may be to due to the phytochemical constituents

present in them. Similarly Rajkumar *et al* [40] reported that the methanolic extract of *Coccinia indica* treated eggs exhibited an allayed hatchability and this may be due to the action of phytochemicals present in the extract. The extract may inhibit the hatchability of the eggs by interfering with their chorion. Eggs and egg shells treated with plant extracts become damaged probably due to endosmosis. After the initial phase of swelling, eggs become desiccated followed by shrinkage and death of larvae trapped within [28].

Secondary metabolites in plants are responsible for several biological activities in man and animals. There is a growing awareness in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. Screening active compounds from plants has lead to the invention of new medicinal drugs which have efficient protection and treatment roles against various diseases [41]. The environmental conditions sometimes have a direct effect on plant physiology and behavior. Stress responses in plants are dynamic and engage complex cross stalk at different regulatory levels. Plants might overcome these stresses through avoidance or tolerance, which includes metabolic adjustment through alteration of compatible solutes or secondary metabolites [42, 43]. Secondary compounds of plants may jointly or independently have activity against mosquito targets from their ovicidal and pupicidal activity against the adult and thereby inhibit growth activity [44]. Phytochemicals may also have potential uses as larvicides, repellents, ovicides and oviposition deterrents and growth and reproduction inhibitors [45, 46].

In the present study ethanol extracts of *C. papaya*, showed the presence of phytochemicals such as phenols, saponins, proteins, quinones and anthroquinones. Similarly Mathivanan *et al* [47] reported that preliminary screening of phytochemicals in *Ervatamia coronaria*, showed the presence of alkaloids, saponins, tannins, flavonoids and steroids. The bio active compounds in plants that induced larvicidal or adulticidal response might be from various compounds including phenolics, terpenoids, flavonoids and alkaloids as single compound or as joint compounds [48]. The plant based products might block the micropyle region of the egg, thereby preventing the exchange of gases, ultimately killing the embryo in the egg itself.

6. Conclusion

Finding an environment friendly insecticide for the control of mosquito vectors is considered to be of paramount importance to reduce the negative impacts caused by chemical insecticides to environment. The present study revealed the ovicidal activity of *C. papaya* against *Cx. quinquefasciatus* and therefore warrants a more thorough exploitation. As the plant of the present study is widely distributed, the commercial exploitation could provide an important step in the development of new novel plant based insecticide as one of the alternatives to expensive and environmentally harmful chemical insecticide. Further studies are necessary to elucidate the active compounds that are responsible for the ovicidal property of the plant and other possible biological activities of the plant for greener approach in *Culex* mosquito control programme. Therefore the present investigation lead the path of exploration of *C. papaya* for eradication of selected medically important human vector mosquitoes, thereby gaining a real momentum to include this plant product for intense vector control programme.

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