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LARVICIDAL ACTIVITY OF TWO RUTACEAE SPECIES AGAINST THE VECTORS OF DENGUE AND FILARIAL FEVER

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Larvicidal

ABSTRACT

Mosquito control strategies have been primarily dependent on the use of synthetic chemical insecticides but its long-term stability and its tendency to bioaccumulate have fostered many environmental and human health concerns resulting in increase of resistance to chemical insecticides and rebounding vectorial capacity by mosquitoes. Botanical insecticides serve as suitable alternatives to synthetic ones as they are relatively effective, and are safe to environment, human, and animal life. In the present study, *Citrus sinensis* and *Murraya koenigii* leaf powders were tested separately on the third instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0%. Larval mortality was assessed after 24, 48, 72 and 96 hours and their respective LC₅₀ values for *C. sinensis* were 0.69, 0.54, 0.48 and 0.36% for *A. aegypti*; and 0.61, 0.53, 0.44 and 0.34% for *C. quinquefasciatus*. For, *M. koenigii*, it was 1.05, 0.73, 0.38 and 0.24%; and 0.54, 0.50, 0.32 and 0.22% for *C. sinensis* respectively. Amongst tested two botanicals, *C. sinensis* was found to be more active against *C. quinquefasciatus* in dose and time dependent manner and the impact of phytochemicals on expression of *C. quinquefasciatus* protein analysed by SDS-PAGE revealed that the phytochemicals from *C. sinensis* suppressed the expression of certain proteins present in *C. quinquefasciatus*. Hence, this study confirms and recommends that use of *C. sinensis* and *M. koenigii* safe and eco-friendly and could use as an alternative to synthetic pesticides in vector control.

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1 Introduction

Insect transmitted disease remains a major source of illness and death worldwide of which vector-borne diseases are responsible for 17% of the global burden of parasitic and infectious diseases (WHO, 2014). Mosquitoes are tremendous public health pests because of their predominance as marketers of potentially deadly pathogens of human beings and the annoyance of skin reactions caused by their bites. Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides (Elimam et al., 2009). Most of the mosquito control techniques have depended on the use of artificial chemical insecticides (Hemingway et al., 2006). However, the unfriendly effect of most of these synthetic chemical insecticides leads the insect pest managers of the world to comb for alternative ways of countering this disease causing insect (Ileke & Ogunbite, 2015). Also, the long-term stability of many of these chemical insecticides and their tendency to bioaccumulate in non-target organisms have fostered many environmental and human health concerns such as the threats faced due to resulting in increase of resistance to chemical insecticides and rebounding vectorial capacity by mosquitoes (Senthilkumar et al., 2008). Botanical insecticides may serve as suitable alternatives to synthetic ones in future, as they are relatively effective, and safe to environment, human, and animal life (Pitasawat et al., 2007; Borah et al., 2010). Research from all over the world have documented the effect of various phytochemical compounds against a wide range of mosquito species (Samuel et al., 2016; Pathak et al., 2018; Huang et al., 2019; Kaushik et al., 2019; Samuel et al., 2019; Nathan, 2020).

The Rutaceae family comprises of 150 genera and 1,600 species of trees, shrubs, and climbers distributed throughout the temperate and tropical regions of the world (Pollio et al., 2008). The important genera of this family are *Citrus*, *Fortunella*, *Murraya*, *Ptelea*, *Ruta* and *Zanthoxylum* (Siddique et al., 2012). Most of the Rutaceae plants are aromatic whose leaves, fruits or cotyledon in seeds contain a complex mixture of volatile aroma compounds (Aziz et al., 2010); which are used in perfumery, gastronomy and traditional medicine. In addition, various researchers have reported the presence of various secondary metabolites viz., alkaloids, coumarins, flavonoids, limonoids, and volatile oils from family Rutaceae, these metabolites are associated to different biological activities such as antimicrobial (Ali et al., 2008), anti-diarrhoeal (Mandal et al., 2010), anticholinesterasic (Cardoso-Lopes et al., 2010), antileishmanial (Andres et al., 2011), antiprotozoal (Severino et al., 2009), antioxidant (Wansi et al., 2006), and mosquito larvicidal (Rajkumar & Jebanesan, 2008; Arivoli & Samuel, 2011; Arivoli et al., 2015) properties. Consequently, this study was conducted to determine the larvicidal efficacy of *C. sinensis* and *M. koenigii* leaves as an alternative to synthetic insecticides for the management of *A. aegypti* and *C. quinquefasciatus*.

2 Materials and Methods

2.1 Plant collection and preparation of phytopowders

Mature and healthy leaves of *C. sinensis* and *M. koenigii* were collected from Nagercoil, Kanyakumari district, Tamil Nadu, India and identified taxonomically and confirmed at the Department of Botany and Research Centre, Nagercoil, Kanyakumari district, Tamil Nadu, India. In the laboratory, dechlorinated water was used to wash the leaves and thereafter these leaves were shade dried. Leaves of each plant (1Kg) was then powdered by an electric blender and was stored in air tight sterilized amber coloured bottles for bioassay.

2.2 Phytochemical screening

The active phytochemical compounds in *C. sinensis* and *M. Koenigii* leaf powders were qualitatively determined using methods described by Harborne (1998) for alkaloids, glycosides, steroids, phenolics and carbohydrates, Van Burden & Robinson (1981) for tannins and proteins, Obadoni & Ochuko (2001) for saponins, Boham & Kocipai (1974) for flavonoids, and Okwu & Okwu (2004) for vitamins.

2.3 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is the most powerful tool for identifying functional groups present in plant extracts. Dried methanol extract powder (10mg) of each plant was encapsulated in 100mg of KBr pellet, in order to prepare translucent sample discs. The powdered samples were loaded in FTIR spectroscope (Shimadzu, Japan), with a scan range from 400 to 4000cm⁻¹ (Visveshwari et al., 2017).

2.4 Sodium Dodecyl sulphate – PolyAcrylamide Gel Electrophoresis (SDS - PAGE) analysis

SDS-PAGE is the most widely used analytical method to resolve separate components of a protein mixture. It is almost obligatory to assess the purity of a protein through an electrophoretic method. SDS-PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix. The technique is also a powerful tool for estimating the molecular weights of proteins. The molecular weight of the protein was estimated using a high molecular weight protein calibration kit (Merck, Bangalore, India) as markers. The molecular mass markers (expressed in Da) used were myosin (205,000), β -galactosidase (11,600), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20,100) and lysozyme (14,300).

Total protein was extracted by using acetone- TCA (Trichloro Acetic Acid) precipitation technique of Damerval et al. (1986) and the

estimation of protein was executed according to the methodology of Lowry et al. (1951). Every sample (0.5g) with a buffer (2mL) containing Tris (hydroxymethyl) aminomethane (Tris)-Glycine (pH 8.3) (50mM), sucrose (0.5M), EDTA (50mM), potassium chloride (0.1M), PMSF (2mM) and 0.1% (v/v) 2-mercaptoethanol was homogenized at 4°C in a chilled pestle and mortar. The homogenate was centrifuged at 14000 rpm for 10 minutes in a refrigerated centrifuge. The concentration of protein in supernatant samples were assessed according to the technique of Bradford (1976) and gels prepared as per the protocol adopted by Laemmli (1970). A separating gel (12.0%) was used for resolving the polypeptides which comprised of Tris- HCl (375mM), pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.4µLmL⁻¹ TEMED. For a stacking gel (4%) used to concentrate (stack) the polypeptides, it comprised of Tris-HCl (125mM), pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.5µLmL⁻¹ TEMED. The electrophoresis running buffer was made of Tris (25mM), glycine (192mM), SDS (0.1%) with pH 8.3, and was accomplished for 4 hours at 35mA. The gels were stained for two hours with Coomassie Brilliant Blue R-250 (Sigma) (0.25%) in 50% (v/v) methanol and (v/v) acetic acid (10%) and then destained with methanol and acetic acid until a clear background was obtained.

2.5 Vector mosquitoes

Insecticide free *A. aegypti* eggs and *C. quinquefasciatus* egg rafts were obtained from Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India. Cyclic generations of the above mentioned vector mosquitoes were maintained separately in two feet mosquito cages with a mean room temperature of 27±2°C and a relative humidity of 70-80% inside an insectary and the adults were fed on 10% glucose solution in water. Ovitrap were placed inside the mosquito cages for the female mosquitoes to oviposit eggs and the laid eggs were then transferred to the larval rearing chamber and were maintained in enamel larval trays. The larvae fed with larval food (dog biscuits and yeast in the ratio 3:1) on becoming pupae were transferred to plastic bowls kept inside another mosquito cage for emergence of adults.

2.6 Larvicidal bioassay

According to the guidelines of World Health Organization (WHO, 2005) with minor modifications, bioassays were performed on

twenty five F₁ generation of laboratory colonized third instar larvae of the above mentioned vector species at test concentrations of (w/v) 0.2, 0.4, 0.6, 0.8 and 1.0% by introducing them into glass beakers (250mL) containing 200mL of distilled water and test concentration. One per cent stock solution for each plant powder was prepared by dissolving 1g of each leaf powder in 100mL distilled water, from which the above mentioned concentrations were arrived. Third instar larvae formed the choice as the research sample, compared to first and second instar since they possessed a larger body size and are more adaptive to the environment; while fourth instar transforms to a pupa in approximately 48 hours (Marin et al., 2020). Control (distilled water) was run simultaneously and maintained separately. Bioassays were performed with five replicates for each concentration per trial with a total of three trials. Larval mortality was observed 24, 48, 72 and 96 hours after treatment and moribund larvae were recorded dead when they displayed no signs of movement when probed by a needle at their respiratory siphon.

2.7 Statistical analysis

Data was subjected to statistical analysis with significance set at 95% confidence in IBM SPSS Statistics v22 (SPSS, 2010). Per cent larval mortality was calculated and corrections for control mortality (5-20%) if required was carried according to Abbott's formula (Abbott, 1925) and then larval mortality were subjected to probit analysis. Analysis of variance (ANOVA) of larval mortality was performed to measure differences between treated bioassays and controls and at which doses in particular and the differences were considered significant at $P \leq 0.001$ level.

$$\frac{1 - n \text{ in T after treatment}}{n \text{ in C after treatment}} \times 100$$

Where, n is the number of larvae, T: treated and C: control.

3 Results

The qualitative phytochemical analysis of *C. sinensis* and *M. koenigii* leaves are presented in Table 1. The FTIR spectrum of *C. sinensis* showed the presence of alcohol, alkenes, aromatic amines, phenyl, ether, methylene, 1° and 2° amines and aliphatic chloro compound. The major band was observed at 3308cm⁻¹ due to O-H stretching vibrations of alcohol group (Figure 1). *M. koenigii*

Table 1 Phytochemical composition of *Citrus sinensis* and *Murraya koenigii* leaves

Plant species	Phytocompounds								
	Alkaloids	Carbohydrates	Flavonoids	Glycosides	Phenols	Proteins	Saponins	Steroids	Tannins
<i>Citrus sinensis</i>	+++++	+++	++	++++	++++	++++	+++++	++++	+++++
<i>Murraya koenigii</i>	+++++	++++	+++++	+++	+++	++++	+++++	-	+++

+++++ Above 80%; ++++ 50-75%; +++ Below 50%; ++ Below 25%; and – Nil

Figure 1 FTIR analysis of *Citrus sinensis* leaf Figure 2 FTIR analysis of *Murrayakoenigii* leaf

indicated the presence of alcohol, alkenes, aromatic amines, phenyl, ether, methylene, cyclic ether and vinyl groups. The major band was observed at 3398.2cm⁻¹ due to O-H stretching vibrations of alcohol group (Figure 2).

No larval mortality was observed in control. The percentage mortality of *A. aegypti* and *C. quinquefasciatus* larva treated with the various concentrations of *C. sinensis* after 24, 48, 72 and 96 hours are presented in Figure 3 and 4 and its LC₅₀ values were reported 0.69, 0.54, 0.48 and 0.36%; and 0.61, 0.53, 0.44 and 0.34% respectively. One Way ANOVA, comparing treated and control group, with a significance level established at $P < 0.001$ showed that *C. sinensis* concentrations significantly influenced the mortality of larvae; F value 82.52 for *A. aegypti* and 59.46 for *C. quinquefasciatus*. In the case of *M. koenigii*, the percentage mortality of *A. aegypti* and *C. quinquefasciatus* after 24, 48, 72 and 96 hours are presented in Figure 5 and 6 and its LC₅₀ values are 1.05, 0.73, 0.38 and 0.24%; and 0.54, 0.50, 0.32 and 0.22% respectively. One Way ANOVA, comparing treated and control group, with a significance level established at $P < 0.001$ showed that *M. koenigii* concentrations significantly influenced the mortality of larvae, F value 30.27 for *A. aegypti* and 21.81 for *C. quinquefasciatus*. *C. sinensis* was found to be more effective against third instar larvae of *C. quinquefasciatus*. Hence, these mosquito larvae treated with *C. sinensis* leaf powder were further investigated to reveal the impact of phytochemicals on their proteins. SDS-PAGE analysis revealed that the phytochemicals from *C. sinensis* suppressed the expression of certain proteins present in *C. quinquefasciatus* (Figure 7).

Figure 3 Per cent larval mortality of *Aedes aegypti* by *Citrus sinensis*

Figure 4 Per cent larval mortality of *Culex quinquefasciatus* by *Citrus sinensis*

Figure 5 Per cent larval mortality of *Aedes aegypti* by *Murraya koenigii*

Figure 6 Per cent larval mortality of *Culex quinquefasciatus* by *Murraya koenigii*

Figure 7 SDS PAGE analysis of proteins from *Culex quinquefasciatus*
Lane 1: Molecular weight marker; Lane 2: Control larva; and
Lane 3: Treated larva

4 Discussion

Mosquito control is vital and is still in a state of evolution, dependent upon synthetic organic insecticides, many of which have been removed from the arsenal of weapons (Das et al., 2007) and botanicals replaced as the new weapons. Vector control is preferably performed at the larval stage, due to its larger vulnerability (Zoubiri & Baaliouamer, 2014). The activities of botanicals are often attributed to the complex mixture of their phytochemical active compounds. Plant families, viz., Asteraceae, Boraginaceae, Fabaceae, Piperaceae and Rutaceae (Garcez et al., 2013) are highlighted as producers of compounds with larvicidal activity. Results of current study revealed that both plants are rich sources of bioactive compounds and have great potential of eradicating mosquito larvae (Ghosh et al., 2012; Samuel & William, 2014; Pathak et al., 2018; Kaushik et al., 2019; Nathan, 2020). Among these two, *C. sinensis* was found more efficient against *C. quinquefasciatus* in dose and time dependent manner. *C. sinensis* also has an intense larvicidal activity against *Anopheles. labranchiae* (El-Akhal et al., 2015) and *A. aegypti* (Galvao et al., 2015) besides acting as a potent fumigant against mosquitoes (Ezeonu et al., 2001). Similarly, Sattar et al. (2016) also reported the larvicidal property of *C. sinensis* leaf powder against *C. quinquefasciatus*. Also, the results of the present study are in accordance with Bilal et al. (2012) who have reported that the leaf extract of *C. sinensis* exhibited 97% mortality against *A. albopictus* larva. Similarly, Murugan et al. (2012) studied the effect of ethanol peel extract of *C. sinensis* against *Anopheles stephensi*, *A. aegypti* and *C. quinquefasciatus* larvae and reported their LC₅₀ values

291.69, 342.45, and 385.32ppm respectively. Warikoo et al. (2012) also testified the activity of hexane extract of *C. sinensis* leaf extract against *A. aegypti* larvae and reported 446.84ppm LC₅₀ value. George (2019) stated that the aqueous peel extract of *C. sinensis* caused 100% larval mortality in *Anopheles* species at 2.0g/mL for 24 hours exposure. The results of the present study also validates with the report of Arivoli & Samuel (2011) who investigated the larvicidal effect of different solvent extracts of *M. koenigii* leaves against *A. aegypti*, *An. stephensi* and *C. quinquefasciatus* larvae and confirmed the presence of various active substances. Arivoli et al. (2015) reported that the hexane fraction of *M. koenigii* leaves produced 100, 99.2 and 97.6% larval mortality rate at 100ppm after 24 hours against *A. aegypti*, *C. quinquefasciatus* and *An. stephensi* respectively.

Mortality of larvae is related to the phytochemical constituents present in the leaves of *C. sinensis* and *M. koenigii*. Active ingredients in botanical derivatives owning mosquitocidal properties due to the presence of alkaloids, flavonoids, steroids, tannins, terpenes and terpenoids (Scherer et al., 2010; Farooq et al., 2014) directly spasm the nervous system, disturb the mid-gut epithelium and secondarily distress the gastric caeca and malpighian tubules in mosquito larvae (Rey et al., 1999). Further, they act as mitochondrial poison (Mann & Kaufman, 2012), and work by means of networking and intermingling with the larvae cuticle membrane, and ultimately disarranging the membrane which would be the utmost probable reason for larval death (Hostettmann & Marston, 1995). The larvicidal activity of *C. sinensis* might be due to the high quantity of alkaloids, saponins and tannins. Kumar et al. (2012) reported the presence of alkaloids, flavonoids and terpenoids in the petroleum ether leaf extracts of *C. sinensis* which caused mortality in *A. aegypti* larvae. Musau et al. (2016) suggested that alkaloids act as anticholinesterases that bind to acetylcholine enzymes and disrupts the membrane integrity, impair microtubules functioning and could cause impairment in digestive system by inhibiting hydrolytic enzyme. Further, these alkaloids slow down larval movement by interfering nerve impulse transmission (Mansour et al., 1998; Armadhani, 2014). Phytochemical saponins are toxic as they disrupt the oxygen supply to larvae and disrupts the larvae before it could pupate (Bagavan et al., 2008; Chapagain et al., 2008). Tannins work as a stomach poison and can interfere with the larvae digestion process by binding with proteins in the digestive system (Boudko et al., 2011). On the other hand, *M. koenigii* extract is also a rich source of alkaloids, saponins and flavonoids and larvicidal properties shown in current study might be due to the presence of these chemicals. Results of current study are in agreement with the findings of Sukari et al. (2013) who reported the larvicidal property of *M. koenigii* phytocompounds against *A. aegypti*. According to Palanikumar et al. (2017) flavonoids available in *M. koenigii* inhibits the larval respiration and disrupts the transport of electrons as they enter through the respiratory siphon and are forwarded to trachea

throughout the body and attacks the central nerve ganglion which gets disturbed, which leads to paralysis of nerve cells and eventually death of mosquito larvae (Adnyani & Sudarmadja, 2016). Further, this plant is rich in coumarins, acridine alkaloids and carbazole alkaloids (Ito, 2000). Apart from the specific role of certain compound, synergistic effect of closely related compounds in plants could also contribute to death of mosquito larvae (Isman, 1997).

SDS-PAGE analysis of proteins from third instar of *C. quinquefasciatus* revealed the absence of some of the proteins when treated with *C. sinensis*. In the control sample, 13 protein bands were clearly visible after CBB staining; however in the treated mosquito sample only nine proteins were visualized. This is mainly due to suppression or inhibition of protein synthesis by phytochemicals. These phytochemicals enter into the body of mosquito larvae through digestive tract and by diffusion. The reduction in protein level in the treated mosquito larvae has been reported previously in *Culex* larvae by the action of *Artemisia annua* extract which caused a ruptured and degenerated body wall of larval tissues (Sharma et al., 2006). Senthilkumar et al. (2009) reported that protein levels in *An. stephensi* larvae treated with *Annona squamosa*, *Artemisia annua*, *Centella asiatica*, *Cymbopogon citratus*, *Eucalyptus globulus*, *Justicia gendarussa* and *Myristica fragrans* extracts were reduced and resumed that it was the result of interference of the phytochemicals with normal protein synthesis mechanism. In general, protein is the essential element of the animal body as well as insect structure. Its higher amount in their body indicates larger body mass, which establishes higher reproductive success in insects, improved competitive ability (Warren et al., 2006), and disease vulnerability and stress resistance (Lee et al., 2008; de Souza Wuillda, 2019).

Conclusion

In conclusion, the present study established the larvicidal activity of the two Rutaceae species against *A. aegypti* and *C. quinquefasciatus*. Botanicals are one of the best alternatives to their synthetic counterpart and this study confirms and recommends that *C. sinensis* and *M. koenigii* are safe and eco-friendly alternative of synthetic pesticides in vector control. However, the mechanism of action and the structure activity relationship for larvicidal activity remain unclear. Therefore, understanding the mechanism of action of secondary metabolites with larvicidal action can help in reducing the resistance of insecticides and aid the production of analogs with more pronounced activity with specific or multiple sites of action.

Conflict Of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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