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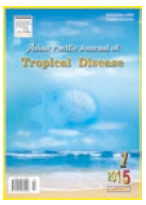

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Chemical properties of essential oil from *Rhizophora mucronata* mangrove leaf against malarial mosquito *Anopheles stephensi* and filarial mosquito *Culex quinquefasciatus*

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ABSTRACT

Objective: To identity and evaluate mosquito larvicidal and repellent activity against the malarial mosquito *Anopheles stephensi* (*A. stephensi*) and filarial mosquito *Culex quinquefasciatus* (*C. quinquefasciatus*).

Methods: The eggs and egg rafts of *A. stephensi* and *C. quinquefasciatus* were procured from National Centre for Communicable Diseases, Mettupalayam, Tamilnadu, India. Filter paper attached with eggs was dipped into a plastic tray containing 500 mL of dechlorinated water for 30-40 min, long enough to allow the eggs to hatch into larvae.

Results: The larval mortality was observed for late fourth instar after 24-h treatment. The maximum activity of LC_{50} value was 0.051 mg/mL and 0.0514 mg/mL for *A. stephensi* and *C. quinquefasciatus* respectively in essential oil treated at minimum concentration. Skin repellent test at 1.0, 2.0, 3.0 and 4.0 mg/cm^2 concentration of *Rhizophora mucronata* (*R. mucronata*) gave 100% protection up to 11 h. These results clearly revealed the potent larvicidal and repellent activity of *R. mucronata* essential oil. The present study was conducted to identify the chemical constituents with the GC-MS analysis in the most effective volatile oil of *R. mucronata*.

Conclusions: The results have clearly demonstrated the possibility of transforming the potential natural products into commercial larval and repellent agent after being tested in different geo-climates and mosquito species.

1. Introduction

Mosquito borne diseases have an economic impact, including loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates; however, no part of the world is free from vector-borne diseases[1]. Mosquitoes are the most important single group of insects in terms of public health importance, which transmit a number of diseases, such as malaria, filariasis, dengue, Japanese encephalitis, *etc*. causing millions of deaths every year. *Aedes aegypti*, a vector of dengue, is widely distributed in the tropical and subtropical zones. Dengue fever incidence has increased fourfold since 1970 and nearly half the world's population is now at risk. In 1990, almost 30% of the world population, 1.5 billion people, lived in regions where the estimated risk of dengue transmission was greater than 50%[2]. *A. stephensi* is the major malaria vectors in India. With an annual incidence of 300-500 million clinically manifest cases and a death toll of 1.1–2.7 million, malaria is still one of the most important communicable diseases. Currently, about

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40% of the world's population remains in region where malaria is endemic[3]. *C. quinquefasciatus*, a vector of lymphatic filariasis and widely distributed tropical diseases with around 120 million people infected worldwide and 44 million people have common chronic manifestation[4]. Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations. It has also resulted in the development of resistance[5], undesirable effects on non-target organisms, and fostered environmental and human health concern. Chemical control methods using synthetic insecticides are in practice due to their speedy action and ease of application. Use of chemical agents, however, results in environmental degradation in addition to accumulation of toxicants as residual deposits in non-target species.

Researchers are now looking for natural insecticides which do not have any ill effects on non-target population and are easily degradable. The search is underway to find out newer insecticides and repellent which will be effective and safe, and also easily available at lower cost. Due to spiraling costs of insecticides and labour, paucity of funds and resistance developed by plasmodia or anophelines to chemicals, diseases carried by mosquitoes have reappeared since 1980s. Natural products are generally preferred due to their less harmful nature to non-target organisms and because their innate biodegradability essential oils are natural volatile substances found in a variety of plants. When isolated from plants, essential oils are not usually extracted as chemically pure substances, but consist of mixtures of many compounds[6]. It is well known that plant-derived natural products are extensively used as biologically active compounds. Among them, essential oils were the first preservatives used by man, originally in their natural state within plant tissues and then as oils obtained by water distillation. Essential oils composed of isoprenoid compounds, mainly mono and sesquiterpenes are the carriers of the smell found in the aromatic plants[7]. Marine halophytes are salt tolerant plants having enormous diversity. Most of the plants have special adaptation potential to accumulate salts and some to excrete through the leaves. Previously mangrove plants have displayed various levels of biological activities including antibacterial, antifungal[8], antimicrobial[9], antiplasmodial[10], hepatoprotective[11], larvicidal[9-11] and antifertility[12]. The most common representatives are *Rhizophora mucronata*, *R. mangle* and *R. apiculata*. *R. mucronata* is an astringent, a folk remedy for angina and hemorrhage; its old leaves or roots are used for childbirth. Although information has been available for hundreds

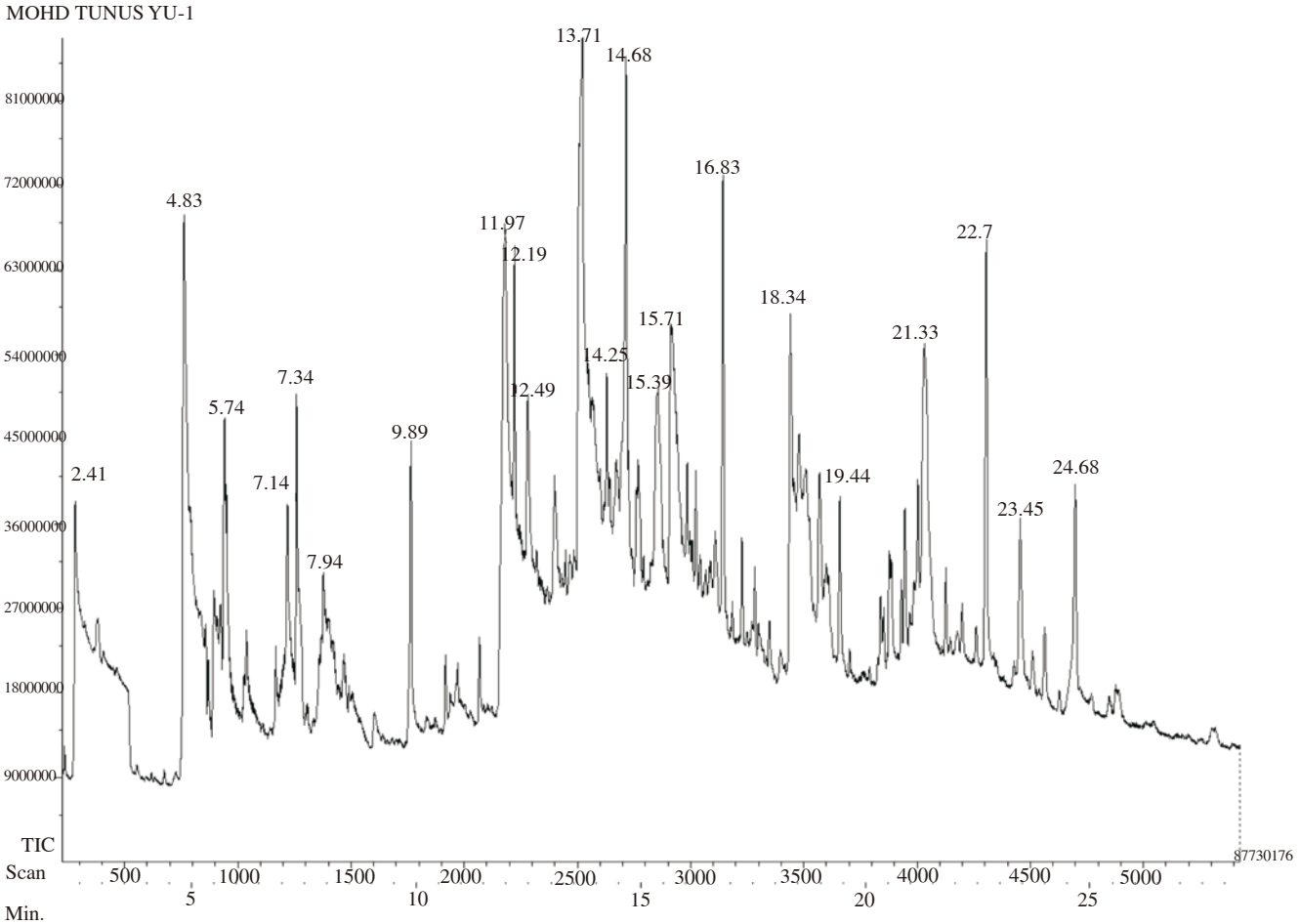

Figure 1. GC analysis of *R. mucronata* essential oil.

of years, there is no scientific investigation on the mode of action of the active substances to determine the plant part of the tree which has biological activity against insects.

The present investigation is aimed at understanding the biting behaviour of mosquito larvae and adult female mosquito testing the repellency of essential oils derived from *R. mucronata* for the development of suitable biodegradable larvicidal and repellent against *A. stephensi* and *C. quinquefasciatus*.

2. Materials and methods

2.1. Collection and extraction of R. mucronata

R. mucronata mangrove plants were collected from Pichavaram mangrove forest (latitude 11°27′ N, longitude 79°47′ E) in south east coast of India. The plant samples were shade dried and subjected to percolation by soaking in ethanol and water mixture (3:1, v/v). After 21 days of dark incubation, the filtrate was concentrated separately by Soxhlet apparatus evaporation (> 45 ℃) and then freeze-dried at -80 ℃ to obtain solid residue.

2.2. Mosquito larval culture

To satisfy the enormous number of mosquitoes needed for the day to day bioassays, a colony is essential. The eggs and egg rafts of *A. stephensi* and *C. quinquefasciatus* were procured from National Centre for Communicable Diseases (NCCD), Mettupalayam, Tamilnadu, India. Filter paper attached with eggs was dipped into a plastic tray containing 500 mL of dechlorinated water for 30-40 min, time enough to allow for eggs to hatch into larvae. They were reared indoors at (28 ± 2) ℃ and 14:10 light and dark period cycle. The larvae were fed with powdered mixture of dog biscuits and yeast powder in the ratio of 3:1. After five days emergence, female mosquitoes were moved into a mosquito cage where the emergent adults were fed with a 100 g/L sucrose solution and allowed for blood feed using white albino rats for 2-3 h. A few days after having a blood meal, the gravid mosquito laid their eggs.

2.3. Isolation of essential oil components from R. mucronata

A volume of 1 mL concentrated extract was dissolved in 20 mL petroleum ether and 2 mL 2 mol/L methanolic KOH was added. The mixture was shaken for 2 min and allowed to stand for 10 min. The upper layer was removed and washed with water and formed double layers. The upper layer was collected. This oil (as the methyl esters of the fatty acids) was analyzed by using GC-MS.

2.4. Larvicidal activity

Larvicidal activity of the essential oil of *R. mucronata* against *A. stephensi* and *C. quinquefasciatus* was assessed by using the WHO standard method[13]. For experimental treatment, 1 mL of essential oil was dissolved in 100 mL distilled water using acetone. A series of concentrations ranging from 0.0125 to 0.1 mg/mL of the extract were prepared in distilled water. After that, 25 larvae of late fourth instar stage were taken on a strainer with fine mesh and transferred gently to the test medium by topping. The control experiments also run parallel with each replicate. The larval mortality was calculated after 24 h of the exposure period. The corrected percent of mortality was calculated by applying Abbott's formula[14]. The data were subjected to probit analysis in order to estimate the LC_{50} and LC_{90} values[15].

2.5. Repellent activity

The repellency of the essential oil was evaluated by using the human-bait technique to stimulate the condition of human skin to which repellents will be eventually applied[13,16]. Evaluation was carried out in a net cage (45 × 30 × 25 cm^2) containing 100 blood-starved female *A. stephensi* and *C. quinquefasciatus* of 3–4 days old at (28 ± 2) ℃ and relative humidity of 65%–80%. The volunteer had no contact with lotions, perfumes, oils or perfumed soaps on the day of the assay. As to the arms of the volunteer, only 25 cm^2 dorsal side of the skin on each arm was exposed and the remaining area was covered by rubber gloves. The essential oil of *R. mucronata* was applied at 1.0, 2.0, 3.0 and 4.0 mg/cm^2 separately in the exposed area of the fore arm. Ethanol alone served as control. Each bioassay was conducted between 08.00 and 19.00 h. The control and treated arms were introduced simultaneously into the mosquito cage, and by gently tapping the sides on the experimental cages, the mosquitoes were activated. Each test concentration was repeated for six times. The volunteer conducted their test of each concentration by inserting the treated and control arm into the same cage for one full minute for every 5 min[1]. The mosquitoes that landed on the hand were recorded and then shaken off before imbibing any blood, making out a 5-min protection. The percentage of repellence was calculated by the following:

$$\frac{\text{No. of bites received by control} - \text{No. of bites received by treated}}{\text{No. of bites received by control}} \times 100$$

2.6. GC-MS analysis

The fatty acid composition was determined. Briefly 100 μL oil plus 1 mL 10% potassium hydroxide in methanol were heated for 45 min at 85 ℃. Fatty acid was methylated with 1 mL boron trifluride-methanol-complex (20% solution in methanol) plus 1 mL methanol for 45 min at 60 ℃ and then extracted from the methonolic phase with petroleum ether. A volume of 1 μL of the analyte was injected in the column equipped with column oven temperature of 60 ℃ and column flow of 0.99 mL/min. The compounds were identified by the GC-Mass spectra, intensity of retention time and by comparison with the NIST library of compounds present in the SAIF, Indian Institute of Technology, Chennai. The result was expressed as the relative percentage of each individual fatty acid present in each sample given by the corresponding retention time.

3. Results

The toxicities of oils from *R. mucronata* to late fourth instar *A. stephensi* and *C. quinquefasciatus* larvae were noted, and the LC_{50}, LC_{90}, 95% confidence limits of LC_{50}, regression equation, and *chi*-square were also calculated (Tables 1 and 2). The LC_{50} and LC_{90} values of *R. mucronata* were 0.500 and 0.092 mg/mL of *A. stephensi* and 0.0514 and 0.0943 mg/mL of *C. quinquefasciatus* respectively. The regression equation of *R. mucronata* for 4th instar larvae of *A. stephensi* and *C. quinquefasciatus* were $Y = 0.995 + 0.56x$ ($R^2 = 0.991$), $Y = 1.102 + 0.62x$ ($R^2 = 0.983$) and analysis of variation was significant at $P < 0.05$ level respectively. The essential oil of *R. mucronata* showed significant repellency against *A. stephensi* and *C. quinquefasciatus* (Tables 3 and 4). It showed that repellency depended on the strength of the essential oil concentration. A higher concentration of 4.0 mg/cm^2 provided average range 97% protection up to 9 h. The present study also identified the chemical composition with the GC-MS analysis in the most effective volatile oil of *R. mucronata* extract. The result suggested that a total of 14 different compounds were identified with varied retention times (5 to 25 min) and varied peaks of 2.411-24.68 (Figure 1 and Table 5). The major compounds and high intensity peak were those of oleic acid (33.87%) andαα-pinene (35.87%) among the other fatty acids.

Table 1

Larvicidal activity of essential oil derived from *R. mucronata* mangrove plant against *A. stephensi.*

Concentration (mg/mL)	Percentage of mortality ±SE	LC_{50} (mg/mL)	LC_{90} (mg/mL)	Regression equation	95% confidence		R^2	*P* value
					LCL	UCL		
0.0125	10.0±1.0							
0.025	39.0±1.2							
0.05	51.0±2.8	0.051	0.092	$Y = 0.995 + 0.56x$	42.54	58.65	0.991	0.045
0.75	68.0±1.3							
0.1	91.0±0.8							
Control	2.00±0.79							

Percentage mortality values are mean of six replications ± SE.

Table 2

Larvicidal activity of essential oil derived from *R. mucronata* mangrove plant against *C. quinquefasciatus.*

Concentration (mg/mL)	Percentage of mortality ±SE	LC_{50} (mg/mL)	LC_{90} (mg/mL)	Regression equation	95% confidence		R^2	*P* value
					LCL	UCL		
12.5	31.00±0.76							
25.0	42.0±1.0							
50.0	55.00±1.64	0.0514	0.0943	$Y = 1.102 + 0.62x$	50.51	58.91	0.983	0.005
75.0	77.00±1.54							
100.0	89.0±2.7							
Control	1.00±0.02							

Percentage mortality values are mean of six replications ± SE.

Table 3

In vitro repellent activity of essential oil from *R. mucronata* against *A. stephensi.*

Concentration (mg/cm^2)	Time post application of repellent (h)																						% of Protection	Protection time (h)
	8-9		9-10		10-11		11-12		12-13		13-14		14-15		15-16		16-17		17-18		18-19		Total			
	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C		
1.0	0	0	0	0	0	5	0	5	0	6	0	7	1	8	2	10	4	10	6	10	6	10	19	71	73.2	7.2
2.0	0	0	0	0	0	6	0	6	0	6	0	7	3	9	3	9	2	10	1	10	1	12	10	75	86.6	7.8
3.0	0	0	0	0	0	5	0	8	0	6	0	8	0	9	2	9	2	10	1	11	1	13	6	79	92.4	8.5
4.0	0	0	0	0	0	6	0	8	0	7	0	8	0	9	0	9	1	10	1	11	0	13	2	81	97.5	9.1

T: Treatment; C: Control.

Table 4

In vitro repellent activity of essential oil from *R. mucronata* against *C. quinquefasciatus.*

Concentration (mg/cm^2)	Treatment period (h)																						% of Protection	Protection time (h)
	8-9		9-10		10-11		11-12		12-13		13-14		14-15		15-16		16-17		17-18		18-19		Total			
	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C		
1.0	0	0	0	5	0	6	0	5	0	6	2	7	1	8	1	10	3	10	3	10	6	10	16	77	79.2	6.5
2.0	0	0	0	3	0	6	0	6	0	8	3	7	3	8	3	8	2	11	2	12	2	10	12	79	84.8	6.8
3.0	0	0	0	5	0	5	0	7	0	6	0	8	0	9	1	9	0	10	2	10	3	11	6	80	92.5	8.2
4.0	0	0	0	0	0	6	0	7	0	8	0	9	0	9	0	10	1	10	1	12	0	8	2	87	97.7	9.3

T: Treatment; C: Control.

Table 5
List of compounds of *R. mucronata* essential oil identified by GC-MS.

Peak	Retention time	Compounds	Relative content (%)
1	2.14	Cholro 4-methoxy 3-methylquinoline	0.05
2	5.74	Methyl-3-acetyl,2,4,6-triethyl	0.59
3	9.92	Malonic acid, Mononitrite	1.33
4	13.91	2.15- Heptadecadiene 9(ethoxy methyl)	2.39
5	14.71	Oleicacid	33.87
6	19.49	α-pinene	35.87
7	21.38	Oxiraneoctanic acid 3-octyscis	1.98
8	7.24	Trichloroactic acid	0.87
9	22.74	Heptadecane, 9-hexyl	2.98
10	12.52	Galatonic phenylhydrazide	0.76
11	16.87	Acrylophenon 3-(diethyl amino) 2-methoxy	1.86
12	12.08	N-acetylmannosamine	0.53
13	16.03	Heptadecanenitrate	1.97
14	12.53	Phenol 2.5bis	0.54

4. Discussion

The bioactivity of phytochemicals against mosquito larvae can vary significantly depending on plant species, plant parts, age of plant parts, solvent used in extraction and mosquito species[10]. Most studies on phytochemicals focus on herbs and other medicinal plants. This is because historical experiential knowledge and some scientific studies have shown them to be particularly active against certain organisms. A wide selection of trees and shrubs has been found to contain phytochemicals that may be of use in the control of mosquitoes. Some trees and shrubs have also been tested more frequently due to observations indicating a degree of resistance to pests such as termites or herbivorous insects[6]. Plants and plant parts have been provided as a good source of novel drug compounds, as plant derived drugs have made large contribution to human health. The use of plant extracts, as well as other alternative forms of medical treatment, is enjoying great popularity in the late 1990s. The plant based repellents may play a good role in reducing the biting nuisance and infections by preventing the man-fly contact[1,17,18] and precluding any adverse effect that could emerge from the use of synthetic repellents[19,20]. Many plants derived oils and preparations have been tested as potential insect repellents[21-23] and are available in the market either in single or in combination with others. Mangroves are thus widespread in tropical and subtropical regions and grow in the saline intertidal zones of sheltered coast lines. The results of the present study showed that topical application of essential oils extracted from the chosen mangrove plant *R. mucronata* significantly reduced the biting rate and larvicidal activity. The activity of the extracted essential oil is often attributed to the diverse mixture of active compounds.

Preliminary screening is a good mean to evaluate the potential larvicidal and repellent activity of essential oil. The essential oil of *R. mucronata* maximum larvicidal activity with minimum concentration of essential oil showed LC_{50} = 0.051 and LC_{90} = 0.092 mg/mL and LC_{50} = 0.0514 and LC_{90} = 0.0943 mg/mL when compared with other concentrations of essential oil against mosquito larvae of *A. stephensi* and *C. quinquefasciatus* respectively. Moreover, repellent activity of essential oil against *A. stephensi* showed maximum percentage of protection (97.5%) for protection time of 9.1 h at the 4 mg/cm^2. Similarly, *C. quinquefasciatus* showed maximum percentage of protection (97.7%) and protection time (9.3 h) at the 4 mg/cm^2. The present study also aimed to identify the fatty acid chemical classes of the active essential oil of *R. mucronata*. The results provided a total of 14 peaks for fatty acid compounds in essential oil of *R. mucronata* with different retention time intervals with unique peak area. Fatty acid profile revealed the presence of oleic acid and α-pinene which could be further used as a chemical marker for authentication and checking adulteration in the finished products of insecticides of marine origin. The results of this study clearly showed that the oil extract of *R. mucronata* that contains these fatty acid constituents demonstrated a high larval and repellent activity. This result is also comparable to earlier reports in which the observed larvicidal activity of *Ocimum canum* oil against vector mosquitoes, namely, *A. aegypti* and *C. quinquefasciatus* (LC_{50} = 0.301 mg/mL) and *A. stephensi* (LC_{50} = 0.234 mg/mL)[23]. Traboulsi *et al.* (2005) reported that the larvicidal activity of essential oils of *Citrus sinensis*, *Eucalyptus* spp., *Ferrula hermonis*, *Laurus nobilis*, and *Pinus pinea* against *C. pipiens* showed LC_{50} values of 0.060, 0.120, 0.044, 0.117 and 0.075 mg/mL respectively[23]. This is supported by many researches, which have demonstrated the improved repellency of plant-derived topical repellent products after formulation with vanillin[24]. The essential oil extracts of *R. mucronata* have potential to be developed as natural larvicidal agent. In this context, the highly bioactive compounds of *R. mucronata,* which is grown widely in most areas of India, offer an opportunity for developing alternatives to replace rather expensive and environmentally hazardous organic insecticides. Furthermore, the findings of the high correlation between the phytocompounds and larval and repellent activity would also open the door for using fatty acids like oleic acid and α-pinene as natural larvicidal agents. It is inferred from the present study that the extracts from mangrove plant of *R. mucronata* can be explored as a potential source of safe and efficacious mosquito control agents for the management of malarial and filarial diseases.

The essential oils appeared to have no toxic effects in this study and a history of herbal medicine use indicates safety, which is further confirmed here by the dermatological test carried on the mice model. The present results have clearly demonstrated the possibility of transforming the potential natural products into

commercial larval and repellent after testing in different geo-climates and mosquito species.

Conflict of interest statement

We declare that we have no conflict of interest.

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