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Research Article

Larvicidal effect of *Artemisia vulgaris* leaves, flower and leaves essential oil extracts against *Aedes aegypti* larvae

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Abstract: Laboratory reared *Aedes aegypti* mosquito larvae of the Hlaing Tharyar Township Yangon, Myanmar strain were used for testing larvicidal properties of ethanolic leaf extracts, flower extracts and essential oil from leaves of *Artemisia vulgaris. Artemisia vulgaris* leaves and flowers were collected from Taunggyi Township in Shan State. 100 gram of dried leaf and flower powder was extracted using 95% ethanol 1:5 wt./v by refluxing in a soxhlet extractor at 70ºC for 6 hours. 100 g of leaf powder was mixed with 1000ml of distilled water and subjected to hydro distillation in Clevenger-type distilling apparatus for 2 hours for essential oil extraction. Different concentrations of ethanolic leaf and flower extracts as well as essential oil of *Artemisia vulgaris* leaves were prepared freshly in 100 ml each of distilled water in 150 ml plastic cups. Detail testing was done according to the WHO standard method. Each replicate of *Aedes* larvae (70x4 replicates) were exposed for 24 hrs. in different concentrations in the laboratory. Acute toxicity and allergenicity tests were done according to OECD 2008 Guidelines for the testing of Chemicals 425. Results revealed that the highest dose 1500ppm of leaf and 4000-ppm flower crude extracts produced 98.57% mortality followed by 88.93% and 86.79% mortality of 3rd and 4th instars *Aedes* larvae. A 96.07% mortality of *Aedes* larvae in was recorded at very low amount of doses (100ppm) than the crude extracts of leaves and flowers within 24 hrs. exposure period. The effective lethal concentrations for 50% mortality (LC_{50}) and for 90% mortality (LC_{90}) against *Aedes* larvae were found to be 792.40ppm and 1240.07ppm respectively for leaf extract, 735.89ppm and 2152.92ppm for flower extract and 16.30ppm and 70.82ppm for essential oil of leaves of *Artemisia vulgaris.* We did not find any acute toxicity in mice or allergenicity in guinea pigs in laboratory tests. Repellent activity of *Artemisia vulgaris* oil provided 100% protection against *Aedes aegypti* adult females landing to probe human skin at a dose of 0.08g/ml or 0.0002g/cm^2, with protection declining to 97.62% after 30 minutes, 92.86% after 60 minutes, and 85.71% after 120 minutes *Artemisia vulgaris* essential oil leaf extracts did not cause dermal irritation when applied to human skin. The leaf and flower crude extracts as well as essential oil of *Artemisia vulgaris* exhibited strong larvicidal activity against 3rd and 4th stages of *Aedes* larvae, while leaf essential oil provided a high level or protection against bites of *Aedes* mosquitoes.

INTRODUCTION

The mosquito species *Aedes aegypti* L. is a major vector of Dengue fever (DF), Dengue Haemorrhagic Fever (DHF), Chikungunya, Yellow fever and Zika fever. Dengue has been known for more than a century in the tropical areas of Southeast Asia and the Western Pacific region. However, dengue haemorrhagic fever outbreaks were recognized as a new disease, first in the Philippines in 1953 caused by dengue type one (DENV1) [1] and Myanmar in 1970 caused by dengue type one (DENV1) [2]. The main vector of dengue virus in Myanmar is *Aedes aegypti, largely* because of its anthropophilic feeding behavior, its resting behavior inside houses and its capability to exploit most water holding containers for breeding [3]. In Southeast Asia DHF has been observed mainly in children, with peak prevalence in the age group 4-7. . In Myanmar DF and DHF are increasingly serious public health problems especially among young children [4,5]. Each year, an estimated 50 to 100 million new cases of dengue occur around the world. Of these, 500, 000 cases correspond to dengue haemorrhagic fever with a mortality of 5% (25000 cases) [6,7]. Due to the lack of a vaccine or an antiviral treatment, disease prevention is based on vector control [8]. Dengue virus transmission (Serotypes 1-4) by *Aedes* (*Stegomyia*) mosquitoes is a public health problem that principally affects tropical countries. Almost half of the global population lives in high risk areas and currently more than 100 countries experience dengue fever and dengue haemorrhagic fever [8,9] .

Rapid, poorly planned urbanization in association with weak regulatory policies for discharge of solid waste has resulted in the accumulation of solid waste which in turn results in the accumulation of discarded containers in most developing countries. These accumulations have favored the establishment and geographic spread of this *Aedes aegypti*. The strategies to control dengue transmission used by the public vector control programs have not been adequate in most countries. The emergence of insecticide resistance, the difficulty of eliminating larval populations through environmental sanitation and lack of efficacy of ultra-low volume insecticide spraying to control adults are all factors which have limited the effectiveness of vector control programmes [10]. The indiscriminate use of synthetic insecticides is creating multifarious problems like environmental pollution, insecticide resistance, and toxic hazards to humans. Globally, there have been conscientious efforts to overcome these problems, and great emphasis has been placed recently on environmentally friendly and economically viable methodologies for pest control. Therefore it is need to test toxicity and allergenicity evaluation of transgenic seeds plants and plant parts for safety of life [11]. Phytochemicals obtained from the huge diversity of plant species are an important source for safe and biodegradable chemicals, which can be screened for mosquito repellent, larvicidal and insecticidal activities [12,13]. Large numbers of plant products have been reported to have mosquito larvicidal and repellent activity against adult mosquitoes [14]. In recent years, essential oils have received much attention as potent bioactive compounds against various mosquito species [15]. *Artemisia vulgaris* L. is a member of the Asteraceae family. It is a tall (1-1.5 meter), aromatic, threatened perennial herb distributed throughout the northern temperate regions of Africa, Asia, Europe, India, Myanmar and North America. In Myanmar *Artemisia vulgaris* L. plants are abundantly present in Chin, Kachin and Shan State. In traditional medicine, this plant is widely used for the treatment of diabetes and extracts of the whole plant are used for epilepsy, psychoneurosis, depression, irritability, insomnia and anxiety states [16].

Synthetic insecticides are toxic and adversely affect the environment by contaminating soil, water and air [17]. Therefore, there is a need to find alternative ways for environmental safety, biodegradable, cost effective and indigenous methods for vector control. In view of the recently increased interest in developing natural insecticides of plant origin as an alternative to synthetic chemical insecticides, this study was undertaken to assess the larvicidal, ovicidal, and repellent potential of different solvent crude extracts from the medicinal plant *Artemisia vulgaris* L. The present study was conducted to determine the effects of ethanol leaf and flower extracts and essential oil from leaves of *Artemisia vulgaris* on *Aedes aegypti* mosquitoes.

MATERIALS AND METHODS

Study area

Present study was done in Medical Entomology Research Division, Department of Medical Research(DMR) Yangon from 2014-2015, to evaluate the larvicidal and repellency tests and toxicity and allergenicity evaluation of transgenic leaf and flower extracts and leaves essential oil of *Artemisia vulgaris* were done in Laboratory Animal Services (DMR). All the works were done by the approval of Department of Medical Research ethical review community ERC005814, Approval Number: Ethics/DMR/2014/066.

Mosquito larvae

Aedes aegypti mosquitoes of the Hlaing Tharyar Township, Yangon, Myanmar strain were reared in the laboratories of the Medical Entomology Research Division, Department of Medical Research, Yangon. Larvae were fed on DMR larval food, comprising a mix of dry fish meal, maize, peanut cake, sesame cake, wheat, gram power, rice bran, rice power and yeast in the ration of 15:15:12.5:2.5:15:12.5:15:12.5 Adults were provided with 10% sucrose solution and 8 week old mice for blood meal. Mosquitoes were held at 26±2ºC, 65-75 % relative humidity with a photoperiod of 12-h light and 12-h dark. Laboratory reared larvae and adult mosquitoes were used for testing insecticidal properties of ethanolic leaves, flowers and watery essential oil extracts of *Artemisia vulgaris,* the plants collected from Taunggyi Township in Shan State.

Mosquito species identification

Larval and adult mosquitoes were identified by morphological methods [18].

Collection and preparation of extracts of *Artemisia vulgaris* leaves, flowers and essential oil of leaves

Artemisia vulgaris (Myanmar name Shanmanaing) leaves and flowers were collected from Taunggyi Township, Shan State. A total of 10 kilograms of *Artemisia vulgaris* leaves and flowers were cleaned and dried at room temperature for 30 days. Dried leaves and flowers were separately ground into powder by a grinding machine and 100 gram each of finely ground dried leaves and flowers were separately refluxed using 95% ethanol 1:5 wt./v in a Soxhlet extractor at 70ºC for 6 hours. Complete removal of the solvent from the extract was accomplished in a glass rotary evaporator. This process yielded 8 gm. of viscous material from 100 gm. of dried leave powder and 7gm of viscous material from 100gm dried flower powder. The viscous material was stored at 4ºC for three days. . For extraction of essential oils, 100 gm. of *Artemisia vulgaris* leaves powder was mixed with 1000ml distilled water and subjected to hydro distillation in a Clevenger-type distilling apparatus for two hours. The resulting oil was dried over anhydrous sodium sulphate and stored in airtight fuscous glassware in a refrigerator at 4ºC until use. The **e**xtraction was done in the laboratories of the Department of Botany, Yangon University.

Larvicidal testing procedure

Based on preliminary tests, dilutions of ethanolic *Artemisia vulgaris* leaf extract concentrate were prepared using distilled water, as indicated in Table 1, for *Artemisia vulgaris* flower extracts as indicated in Table 2, and for *Artemisia vulgaris* essential oil extract as indicated in Table 3. Dilutions were prepared freshly in 100 ml each of distilled water in 150 ml

plastic cups. Seventy (70) 3rd and 4th size instar larvae were put into each of different concentrations and a negative control test was also done simultaneously by way of 100ml of pure distilled water. Detail testing was done according to the WHO standard method [19]. Larvae were exposed for 24 hrs. in each in each concentration in the laboratory at 27-29ºC and 70 to 80% relative humidity. Four replicates were carried out and knockdown was checked and recorded after 60 minute exposure period and mortality was checked and recorded after 24 hrs. of exposure period. Knockdown and dead larvae were identified when the larvae failed to move after probing with a needle in the thoracic region of the body. Lethal concentration LC_{50} and LC_{90} values for 95% confidential limits were calculated by the formula of Finney [20].

Table: 1 Knockdown and mortality effect of different dilutions of *Artemisia vulgaris* leaves crude extracts against 3rd and 4th instars of *Aedes aegypti* larvae

Concentration (ppm)	Total larvae	Knockdown	% Knockdown	Mortality	% Mortality
1500	280	277	98.93	276	98.57
1250	280	227	81.07	249	88.93
1000	280	172	61.43	193	68.93
750	280	103	36.79	129	46.07
500	280	34	12.14	45	16.07

Table: 2 Knockdown and mortality effect of different dilutions of *Artemisia vulgaris* flower crude extracts against 3rd and 4th instars of *Aedes aegypti* larvae

Concentration (ppm)	Total larvae	Knockdown	% Knockdown	Mortality	% Mortality
4000	280	267	95.36	276	98.57
2000	280	192	68.57	243	86.79
1000	280	79	28.21	153	54.64
500	280	31	11.07	76	27.14
250	280	5	1.79	22	7.86

Table: 3 Knockdown and mortality effect of different dilutions of *Artemisia vulgaris* essential oil against 3rd and 4th instars of *Aedes aegypti* larvae

Concentration ppm	Total larvae	Knockdown	% Knockdown	Mortality	% Mortality
100	280	267	95.36	269	96.07
50	280	225	80.36	233	83.21
25	280	152	54.29	174	62.14
12.5	280	80	28.57	98	35.00
6.25	280	25	8.93%	42	15.00

Toxicity and Allergenicity tests

Toxicity test: Acute toxicity test of the samples on albino mice model

***Theory*:** To determine the symptomatology consequent to application of the plant extracts and to determine the nature and degree of toxicity produced by these extracts and also to find out the medium lethal doses (LD_{50}), acute toxicity tests were done according to OECD 2008 [12]. Usually the acute lethality of a compound is determined on the basis of deaths occurring in 24 hrs. but the survivors should be observed for at least seven days in order to detect delayed effects [21]. In this study, the acute toxicity effect of *Artemisia vulgaris* leaves essential oil (two dose) was determined on albino mice, at the Laboratory Animal Services Division, Department of Medical Research (DMR), Yangon.

***Procedure*:** The acute toxicity of different doses of *Artemisia vulgaris* leaves essential oil extracts were evaluated by the methods of OECD Guidelines for the Testing of Chemicals 425 [12]. In accordance with that test description, we used a total number of 18 adult female albino mice weighing between 25to 30g and divided into three groups. Each group contained six animals. All were fasted for 18 h before giving the extracts. Group (1) mice were orally administrated with *Artemisia vulgaris* leaves essential oil extract at a 2000 mg/kg dose. Group (2) mice were similarly administered orally with *Artemisia vulgaris* leaves essential oil extract but at a dose of 5000 mg/kg. Group (3) mice performed as a control group and they were treated with clean water and normal animal food. The mice were kept in three appropriately labelled mouse cages at a room temperature of 26 ± 1º C. After administration of extracts on each group of animals, they were observed continuously for the first six hours for

mortality and behavior changes. Following that the animals were checked each 24 h for fourteen days. The mortality during this period was noted (Nil or percent death). The results obtained were promptly recorded after each observation.

Irritation Test

Primary skin irritation is the production of reversible inflammatory changes in the skin following the application of a test substance as it involves the interaction of chemicals with the sensory receptors in the skin at the site of application. The skin irritation test was done according to 'DBT, Guidelines for toxicity and allergenicity [11]. In the present study three young adult guinea pigs of the Myanmar white strain were taken from our animal husbandry facility, two samples for test and one sample for control. All animals were housed in metal cages fitted with perforated floors. Water and standard guinea pig feed were given. The room temperature was maintained at 22 ± 3 ° C with 30 - 70 % relative humidity. The light conditions were controlled to give 12 hours artificial light (8 a.m. - 8 p.m.) each day. A minimum of 7 days acclimatization was allowed before the commencement of the study. Each guinea pig cage was attached with a tag marked with the animal number, the test and the product name.

Twenty-four hours before the test (dose application), hair on the back of each guinea pig was shaved over an area covering approximately 9 cm^2 of skin. A dose of 0.0018gm of essential oil was desorbed in 2ml of ethanol and this evenly applied to a small area (approximately 9 cm square) of the shaved skin of each of three guinea pigs. The site of application was not covered with a cotton gauze patch or any other material. Similarly, a control guinea pig was treated with only 2ml of ethanol. Skin reaction at the site of application was subjectively assessed and scored once daily at 1, 24, 48, 72 hours, 7 and 14 days after treatment of skin (post-test observation period). The reaction at the site of application was assessed and scored according to the following classification system : Skin reaction: (A) erythema and eschar formation (B) Edema formation. Irritation was followed by evaluation of a primary skin irritation index: Non Irritant 0.0, Negligible Irritant 0.1- 0.4, Slight Irritant 0.41-1.9, Moderate Irritant 2.0 - 4.9, Severe Irritant 5.0 - 8.0.

Repellent activity testing

The repellent study followed the method of World Health Organization [13]. Five day old blood-starved female *Aedes aegypti* mosquitoes were kept in two steel net cages (59 x 59 x 59 cm), with 50 female mosquitoes per cage; one cage was designated for male human test volunteers and the other for female volunteers. *Aedes aegypti* is a day biter, therefore the tests were done between 08:00 hour and 16:00 hour. Evaluation tests were carried out in a (12x15x15ft) room at 24-28ºC and relative humidity of 70-85%. The volunteers had no contact with lotions, perfumes, or perfumed soaps on the day of the assay or for 12 hours before commencement of tests. On the arms of volunteers, one ml of ethyl alcohol (95%) diluent used in the preparation of the test repellent was applied evenly across the forearm skin between the wrist and elbow to cover an area of average 380 cm2 (two volunteers =one male + one female) of , then allowed to dry 1 minute. Before insertion of the control arm into the cage containing 50 *Aedes* female mosquitoes, the hands were covered with plastic gloves to protect against mosquito bites. In this first step, the ethyl alcohol treated forearm was inserted into the cage and the number of mosquitoes that landed on the skin during the next 30-second period counted. The control forearm was carefully withdrawn and this arm then treated with one ml of 0.1g/10ml of *Artemisia vulgaris* oil solution and allowed to dry. The treated arm was placed in the cage for another 30 second period and observed for mosquito landing, the number of landing mosquitoes recorded on a data-sheet.. This procedure was repeated for each additional incremental dose of *Artemisia vulgaris* oil. The tests were carried out one after the other without delay and *Artemisia vulgaris* oil dose at each test was calculated as the sum of the doses applied to that particular arm to arrive at the cumulative dose for each test. The repellency test was continued for a subsequent dose if the mosquito landing rate on the exposed forearm was less than 10 female in a 30 second exposure period. Two trained technicians were observers who recorded the number of landings. At the conclusion of the dose response experiment, 1 ml of ethyl alcohol was applied on the other forearm and allowed to dry. This forearm was inserted in the cage for 30 seconds to verify that the number of landings was more than 10 per 30 seconds as was observed at the beginning of the experiment. Protection (P) was expressed as a proportion of the number of mosquito landing on a treated arm (T) in relation to the number of landings on the control arm (C) of the same individual; C is the average number of landing mosquitoes on two untreated arms. The formula used to derive P is

$$P=1-(T/C) =(C-T)/C$$

Estimation of complete protection time

The protection test followed the same procedure described as above for repellent activity. Two mosquito cages (size 59x59x59cm), each containing 50 non-blood fed 5-day old *Aedes aegypti* female mosquitoes, were used. One cage was used for testing by female volunteers and another cage for testing male volunteers. Before testing the arms of volunteers, one ml of ethyl alcohol (95%) diluent used in the preparation of the test of *Artemisia vulgaris* oil extract repellent was applied evenly using a pipette to average 342 cm^2 of forearm skin between the wrist and elbow and allowed to dry 1 minute. Before insertion of the arm into the cage containing 50 *Aedes* female mosquitoes, the hands were protected by plastic gloves to protect against mosquito bites. As a first step, ethyl alcohol was applied on the forearm and inserted into the cage, the number of mosquitoes that landed on the skin during a subsequent 3 minute period then counted. The control forearm was carefully withdrawn from the cage. Then 0.08g of *Artemisia vulgaris* oil extract was prepared in one ml of ethyl alcohol solution and applied evenly on an average 342 cm^2 of another forearm skin between the wrist and elbow. The treated arm was placed in the cage for 3 minute period and observed for mosquito landing.

After 30 minutes, the *Artemisia vulgaris* oil repellent treated arm was inserted again into the cage and exposed for 3 minutes to determine landing activity. This procedure was repeated at 30-minute intervals for 180 minutes and the procedure was used consistently throughout the experiment. Tests were repeated two times using two volunteers. The mosquitoes that landed on the hand were recorded and then shaken off before imbibing any blood. Complete protection time was estimated after experiment.

Data analysis

Data entry and processing was made using Microsoft Excel software. The average larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90} values and other statistics at 95% confidence limits of upper confidence limit and lower confidence limit. Chi-square values were calculated using the dose-effect probit analysis [20]. Results with $p<0.05$ were considered to be statistically significant.

RESULTS

With regard to *Artemisia vulgaris* leaf extract, Table (1) shows that 98.93% effective knockdown of *Aedes aegypti* larvae was achieved at 1500ppm followed by 81.07% knockdown at 1250ppm dilution of extract. The lowest knockdown effect of 12.14% was found at 500-ppm dilution of leaf extract. Highest mortality of *Aedes aegypti* larvae was recorded as 98.57% at 1500ppm and 88.93% at 1250ppm of *Artemisia vulgaris* leaf extract, followed by 68.93% at 1000-ppm dilution of extract. Lowest mortality effect was 16.07% of larvae at 500-ppm dilution of leaf extract.

With regard to *Artemisia vulgaris* flower extract, Table (2) shows that knockdown of *Aedes aegypti* larvae was found to be 95.36% at 4000ppm followed by 68.57% knockdown at 2000-ppm dilution of extract. Lowest knockdown was recorded as 1.79% at 250-ppm dilution of flower extract. Highest mortality of *Aedes aegypti* larvae was found to be 98.57% at 4000ppm and 86.79% at 2000ppm of *Artemisia vulgaris* flower extract, followed by 54.64% at 1000-ppm dilution of extract. Lowest mortality effect was 7.86% of larvae at 250-ppm dilution of flower extract.

Using *Artemisia vulgaris* essential oil, Table (3) shows the knockdown of *Aedes aegypti* larvae to be 95.36% at 100ppm, 80.36% knockdown at 50-ppm dilution of extract, and lowest knockdown 8.93% at 6.25-ppm dilution of essential oil. Effective mortality of *Aedes aegypti* larvae was found to be 96.07% at 100ppm, 83.21% at 50ppm of dilution of *Artemisia vulgaris* essential oil, and 62.14% at 25-ppm dilution of extract. Lowest mortality effect was found to be 15.00% of larvae at 6.25-ppm dilution of essential oil.

Table (4) shows the LC_{50} and LC_{90} values of *Artemisia vulgaris* leaf and flower ethanolic crude extracts and leaf essential oil against 3rd and 4th instars *Aedes aegypti* larvae. Leaf essential oil was found to have much higher effective larvicidal activity than ethanol leaf and flower extracts of *Artemisia vulgaris.*

Toxicity test

Table (5) shows the acute toxicity screening of *Artemisia vulgaris* leaf essential oil extract at dosages of 2000 mg/kg and 5000 mg/kg body weight in albino mice, as recorded fourteen days after administration. The results show no lethality of the mice was observed up to fourteen days administration. None of the animals showed any visible symptoms of toxicity such restlessness, respiratory disorders, convulsion, aggressive activities, coma or death. Even with high doses up to 5000 mg/kg body weight administration, there is no lethality at day fourteen.

Table: 4 LC_{50} and LC_{90} values of *Artemisia vulgaris* leaves and flower ethanolic extracts and leaves essential oil on 3rd and 4th instars of *Aedes aegypti* larvae

Treatment	Hours	Extract	X^2	Df	LC_{50} corrected limits and 95 % confidence interval (upper and lower limit (ppm)	LC_{90} corrected limits and 95 % confidence interval (upper and lower limit) (ppm)
Artemisia vulgaris leaves	24	Ethanolic Leaves	0.0749	4	792.40ppm 826.50 to 758.31 ppm	1240.07ppm 1309.91 to 1170.23 ppm
Artemisia vulgaris flower	24	Ethanolic Flower	0.1113	4	735.89 ppm 797.14 to 674.64ppm	2152.92ppm 2442.66 to 1863.17ppm
Artemisia vulgaris leaves oil	24	Watery Essential oil	0.1105	4	16.30 ppm 17.6046 to 14.9996 ppm	70.82 ppm 81.5455 to 60.0891 ppm

Table: 5 Acute Toxicity effect of *Artemisia vulgaris* leaves essential oil on albino mice model after two weeks administration

No	Groups	Extract Administration	Dosage	No. of death	% of death
1	Group 1	*Artemisia vulgaris* leaves essential oil	2000 mg/kg	Nil	0 %
2	Group 2	*Artemisia vulgaris* leaves essential oil	5000 mg/kg	Nil	0 %
3	Group 3	No administration	Nil (clean water)	Nil	0 %

Allergenicity (Irritation) test

The sample has been tested as per 'DBT, Guidelines for toxicity and allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' for non- clinical laboratory studies and the result of the sample of *Artemisia vulgaris* oil dose 0.0018g/6cm^2 was found to be 'nonirritant' to the skin of guinea pigs when compared with the sample of control guinea pigs (only ethanol alcohol treated guinea pig). Table (6) shows that successive cumulative dose of *Artemisia vulgaris* oil applied on 380 cm^2 area of arm for 100% protection of *Aedes aegypti* mosquito landing to probe the skin was 0.08g/ml or 0.0002g/ cm^2. Table (7) shows that repellency activity of complete protection time of *Artemisia vulgaris* oil dose 0.0002g/cm^2 provided 85.71% protection for 120 min against *Aedes aegypti* and over 90% protection was found for 60 minutes i.e. 97.62% protection for 30min and 92.86% protection for 60min. Leaf essential oil of *Artemisia vulgaris* was found to be more effective than leaf and flower extracts of *Artemisia vulgaris.*

Table: 6 Doses of *Artemisia vulgaris* oil applied showing cumulative dosage rates, associated mosquito landing rates, and percentage protection against *Aedes aegypti* bites

Application sequence 4 replicates	**Repellent solution concentration to be applied in 1ml (g/ml)**	**Cumulative amount of repellent (g/380cm^2 area)**	**Average Mosquito landing to probe the skin**	**%Protection P=(C-T)/C x100**
Left arm control Average area 380cm^2	1ml Ethyl alcohol only	0	18±1.83	Average control (Left + Right) 17.5 (C)
Left arm dose 1	0.01g/ml	0.01g	4 ±0.82	77.14%
Left arm dose 2	0.01g/ml	0.02g	3±0.82	82.86%
Left arm dose 3	0.02g/ml	0.04g	2±0.82	88.57%
Left arm dose 4	0.04g/ml	0.08g	0	100%
Right arm control	0	0	17±1.41	0

Table: 7 Repellency test for complete protection time of *Artemisia vulgaris* oil 0.08g/ml using the arms of four volunteers against *Ae. aegypti* mosquitoes in the laboratory

Tested persons (Repellent area)	**Control 3 min**	**Duration of repellency test (After applying *Artemisia vulgaris* oil 0.08g in one ml of ethyl alcohol)**					
	0 min	30 min	60 min	90 min	120 min	150 min	180 min
Male 1 (362cm^2)	16	1	2	3	5	7	9
Male 2 (359cm^2)	17	0	1	2	2	4	7
Female 1 (325 cm^2)	22	1	2	2	3	6	7
Female 2 (321 cm^2)	29	0	1	2	2	4	7
Total (Average 342 cm^2)	84 ± 5.94	2 ± 0.58	6 ± 0.58	10 ± 0.5	12 ± 1.14	19 ± 1.5	30 ± 1
% protection total no. (4 replicates)	84	2 (97.62%)	6 (92.86%)	10 (88.10%)	12 (85.71%)	19 (77.38%)	30 (64.29%)

DISCUSSION

Mosquitoes are a serious threat to public health and transmit several dangerous diseases to over two billion people in the tropics [1,3,4 ,6]. Insecticide residues in the environment as a result of chemical insecticide usage have attracted increasing research attention towards natural products. Plants synthesize a variety of secondary metabolites which playa vital role in defense of plants against insects, including mosquitoes. Mosquito repellents may be one of the most effective tools for protecting human from vector borne diseases and nuisance caused by mosquitoes. Natural products are safe for humans compared to most synthetic compounds. According to Bowers et al., [22], the screening of locally available medicinal plants for mosquito control could generate local employment, reduce dependence on expensive imported products and stimulate local efforts to enhance public health. Different parts of plants contain a complex of chemicals with unique biological activity [23,24] thought to be due to toxins and secondary metabolites which act as mosquitocidal agent [25]. Smoke is still the most widely used common method for repelling biting insects used throughout the world. Most households in the developing world rely on personal protection measures of limited effectiveness, such as burning mosquito coils or leaves. Bundles of dried *Artemisia vulgaris* L. are burned to repel biting insects since it contains insect repellents that can be released from the plant by combustion [26].

Larvicidal activity

In the present study the larvicidal potential of ethanol extracts of leaves, flowers and essential oil of leaves of the plant *Artemisia vulgaris* (Linn) were evaluated against a Culicine mosquito, *Aedes aegypti*. The extract of each part showed dose

dependent increase in larvicidal activity. At higher doses, 1500ppm of leaves and 4000ppm of flowers extracts caused over 98% mortality in 3rd and 4th instar larvae of *Aedes aegypti.* The knockdown effect of leaf extract was found to be higher than the flower extract. The essential oil of leaves was found very potent against *Ae. aegypti* larvae and required only very low doses such as 100ppm causing over 95% knockdown and mortality against 3rd and 4th instar larvae of *Aedes aegypti.* Documented reports are available about the insecticidal potential of *Artemisia vulgaris.* Lavor et al. [27] studied the larvicidal activities of essential oils from the leaves of *Artemisia vulgaris*, *Cymbopogon flexuosus* (Nees ex Steud.) Wats. and *Piper tuberculatum* Jacq. against *Aedes aegypti with similar* results confirming very effective control of mosquito larvae. The essential oil of *P. tuberculatum* had the lowest LC_{50} value (106.3 +/- 2.2 micro g/mL), followed by *Artemisia vulgaris* (114.1 +/- 1.7 microg/mL) and *C. flexuosus* (121.6 +/- 0.8 micro/mL). In the present study 95% confidence corrected limits of LC_{50} and LC_{90} values for leaves, flowers and essential oil of leaves were 792.40ppm, 7735.89ppm, 16.30ppm and 1240.07pp, 2152.92ppm, 70.82pp respectively. Essential oil of leaves was found to be very effective against *Aedes* larvae, and needed very low doses for 50% and 90% mortality of larvae. Thujone, an active component of essential oils, extracted from *Artemisia vulgaris* and other plants of the genus *Artemisia*, is a cyclic ketone with insecticidal property [28].

Artemisia vulgaris and some other plant extracts contain toxic substances acting after consumption or topical application [29]. The insecticidal activity of *Artemisia vulgaris* extract has also been found to be very effective against *Sarcoptes scabiei* [30]. *Artemisia vulgaris*, *Artemisia absinthum* and some other plants extracts are known to affect the development and physiological state of Lepidopterous insects, in which respiration and transpiration systems of the insects were the vulnerable targets for extracts, causing a loss in discontinuous gas exchange cycles (DGCs) which resulted in water loss and death of the insects [31]. Other plants of the genus *Artemisia* have also been reported as having larvicidal activity [32]. Ansari et al., [33] observed the larvicidal activity of *Pinus longifolia* oil against three vector mosquitoes namely *Aedes aegypti* (LC_{50} 82.1ppm), *Culex quinquefasciatus* (LC_{50} 85.7ppm) and *Anopheles stephensi* (LC_{50} 112.6ppm). Other research revealed larvicidal activity of essential oils of Brazilian plants against *Aedes aegypti* with an LC_{50} range from 60-533ppm [34]. Prajapati and associates reported that the larvicidal activity of different plant essential oils showed varied LC_{95} values against *Cu. quinquefasciatus.* They were *Pimpinella anism* (149 µg/ml), *Z. officinalis* (202 µg/ml), *Cyperus scariosus* 408 µg/ml and *Nigella sativa* 365µg/ml [35]. During the present study, the larvicidal activity of the ethanol extracts of leaves, flowers and leaf essential oil of *Artemisia vulgaris* were studied against the mosquito *Aedes aegypti* and we found essential oil of leaves to be a potent tool against *Aedes* larvae. Essential oil derived from aerial parts of *Artemisia judaica* (Linn) is also known to have insecticidal properties against the cowpea weevil, *Callosobruchus maculatus* (Fab.) [36]. Very low percentage 2.4% of ethanolic leaves extract of *Citrus hystrix* has an active component which produced high casualty to *Aedes* larvae (99.5% mortality) [37].

The larvicidal, pupicidal, adulticidal, and repellent activity of *Artemisia nilagirica* against the mosquitoes of *Anopheles stephensi* and *Aedesa egypti,* and the author suggested the leaf extract has a potent larvicidal properties [38]. *Artemisia* extracts contain secondary metabolites, mainly monoterpenoids such as vulgarole, spathulenol, vulgarin, triterpenoide: α-amyrin, α-amryin acetate and fernenol [39]. The insecticidal properties of *Artemisia vulgaris* and other plants of the genus *Artemisia* have been attributed to the presence of these secondary metabolites. The compounds isolated from *Artemisia vulgaris* were mainly monoterpenoids such as linalool, camphor, isoborneol, borneol, terpinen-4-ol, isobornyl, Nonanone-3, (α +β)-thujone, bornyl acetate, β-Pinene, myrcene, α-terpinene, limonene, and cineote. These compounds were reported as effective repellent compounds against *Aedes aegypti* [40]. Stems analyzed for essential oil of *Artemisia vulgaris,* yielded multiple components such as camphor, camphene, α-thujone, 1,8-cineole, γ-muurolene and β-caryophyllene. Mosquito larvicidal assays against 3rd instar larvae of *Aedes aegypti* showed 100% larval mortality with 500-ppm oil solution exposed for 8 hours. Results suggest a potential source of natural insecticides[41].

Acute toxicity and Allergenicity (Irritation)

Acute toxicity screening of *Artemisia vulgaris* leaves essential oil extract was done at a dosage of 2000 mg/kg and 5000 mg/kg body weight in albino mice. The results indicated no lethality within mice up to fourteen days after administration, and both groups animals showed no visible symptoms of toxicity like restlessness, respiratory disorders, convulsion, aggressive activities, coma or death. The animals had been tested as per 'DBT, Guidelines for toxicity and allergenicity, using *Artemisia vulgaris* oil evaluated on guinea pigs in the laboratory; the result of the sample of *Artemisia vulgaris* oil at a dose of 0.0018g/6cm^2 was found to be 'nonirritant' to the skin of guinea pigs for 14days trial when compared with the sample of control guinea pigs. The same result was found by researchers from Sri Lanka which revealed that the *Artemisia vulgaris* extract was tolerated well by mice over a period of 14 days (assay of sub-chronic toxicity), showing no overt signs of toxicity or stress [42]. Studies have also been conducted on the antimalarial activity of a leaf extract of *Artemisia vulgaris* in a *Plasmodium yoelii* rodent malaria model, results showing inhibition of parasitaemia. Antinociceptive activity was also seen in a hot plate test indicating a central, supra-spinally mediated mechanism for relieving pain. Results showed oral activity, non-toxicity, suggesting a weed with a potential as a cheap source of plant-based antimalarial compounds.[42].

Artemisia vulgaris essential oils are used for their insecticidal, antimicrobial and anti-parasitical properties. Reports from China indicate that *Artemisia vulgaris* essential oils have a significant fumigant and repellent effect on *Musca domestica* [43]. The oil of *Artemisia vulgaris* collected in N. Akmenė district, North Lithuania included 25.4% compounds of pinane skeleton and 14.6% of menthane. More than half of the oil content was formed by monoterpenoids. The main

constituens were the following: chrysanthenyl acetate (23.6%), 1,8-cineole (13%) and germacrene D (10.3%) [44].

Repellency activity

Artemisia vulgaris oil possessed significant repellent activity against *Aedes aegypti*. A dose of 0.0002g/cm^2 provided 85.71% protection for 120 min against *Aedes aegypti* and over 90% protection for 60 minutes i.e. 97.62% protection for 30min and 92.86% protection for 60min respectively. *Artemisia vulgaris* leaves essential oil did not cause dermal irritation when applied to human skin. No adverse effects on human volunteers were observed after application. Although it exerted an effective biting protection time against *Aedes aegypti* it is lower than the protection time of currently used synthetic compounds such as DEET, A13-37220, A35765 and CIC-4 [45,46]. These chemical compounds provide better and longer protection against many biting insect (ED_{50} and ED_{95} level = 0.37-25.37 µg/cm^2, 3-8 hours). Repellent protection time in the laboratory may change depending on the biological characteristics of the mosquito test population. Various researchers have revealed that differences in species and body size, sugar water availability, adult density in test cages, and mosquito age can affect test results [47,48,49]. A mixture of 5% vanillin and essential oil of *Curcuma longa* was found to have effective repellent activity for 8 hours against *Aedes aegypti, Anopheles dirus* and *Culex quinquefasciatu*s [50]. Venketachalam and Jebanesan [51] also observed that the repellent activity of a methanol extract of *Ferronia elephantum* leaves was effective against *Aedes aegypti* activity at 1.0 and 2.5mg/cm^2 concentrations, giving 100% protection up to 2.14 ± 0.16 h and 4.00 ± 0.2.4 h respectively, and the total percentage protection was 45.8% at 1.0mg/cm^2 and 59.0% at 2.5 mg/cm^2 for 10 hours.

Our results revealed that the plant extracts were effective to control *Aedes aegypti* larvae and provide 85.71% protection for 120 min from *Aedes* mosquito bite during day time. From these results, we concluded that the *Artemisia vulgaris* leaves, flowers and leaves essential oil exhibit larvicidal, and repellent activities against dengue vector mosquitoes. Further analysis to isolate active compounds for larval control are under way in the pharmacology research laboratory. More studies are needed to elucidate *Artemisia vulgaris* activity against a wide range of mosquito species. The active compound responsible for repellent activity should be identified, which could be used to control different mosquito species in the future. These results could encourage the search for new active natural compounds offering an alternative to synthetic repellents and insecticides from other medicinal plants.

CONCLUSION

In conclusion, the results demonstrated that the *Artemisia vulgaris* leaf, flower extracts and leaves essential oil are very active larvicidal activity on *Aedes* larvae and leaves essential oil is a very good repellent activity on *Aedes* adult mosquitoes and is non-toxic to men and animals. Therefore *Artemisia vulgaris* leaf, flower extracts and leaves essential oil have the potential to be a cheap source of plant-based larvicidal and repellent in the future.

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COMPETING INTEREST

The authors have declared that no competing interests exist.

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