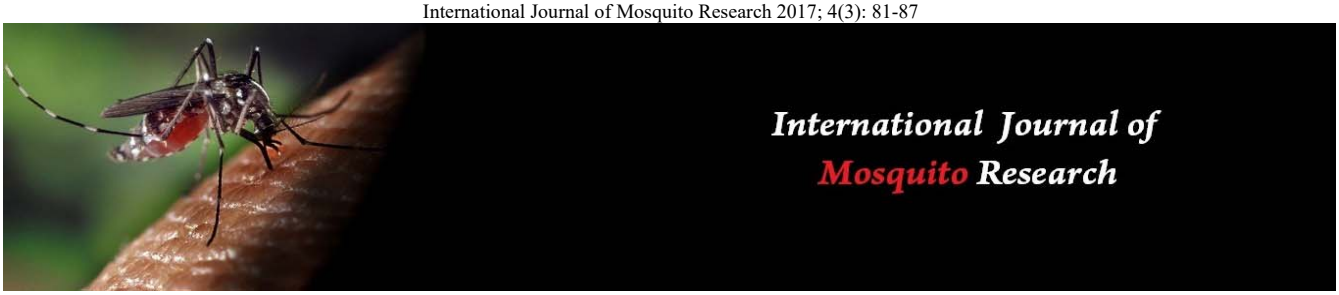

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2017; 4(3): 81-87

Received: 01-03-2017
Accepted: 02-04-2017

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Larvicidal efficacy of ethanolic leaf extracts of four selected local plants from hail region, northern Saudi Arabia, against the dengue fever vector, *Aedes aegypti* (L.) Under laboratory conditions

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Abstract
This study was conducted to evaluate the efficacy of ethanolic leaf extracts from four selected local plants, *Citrullus colocynthis* (bitter apple), *Artemisia annua* (sweet wormwood), *Pergularia tomentosa* (Fattaka), and *Rhanterium epapposum* (Arfaj), from Hail region, northern Saudi Arabia, against the larval stages of the Dengue fever vector, *Aedes aegypti*. Data analyses revealed that ethanolic extracts of both *R. epapposum* and *A. annua* were more toxic to the 4th instar larvae of *Ae. aegypti* compared to the other two plants. The lethal effect of the ethanolic extract of *A. annua* on the larvae was more effective with time, as the mortality rate increased with spent time, with the highest mortality rate recorded, 95%, was after 72 hours. Moreover, the study revealed that the ethanolic extract of *A. annua* showed more larvicidal efficacy against *Ae. aegypti* larvae under lower concentrations compared to the other three plants.

Keywords: *Aedes aegypti*, *Rhanterium epapposum*, *Artemisia annua*, *Pergularia tomentosa*, ethanolic extract, larvicidal activity.

Introduction
Mosquitoes are responsible for the transmission of many diseases to both humans and animals worldwide. There are more than 300 different species of mosquitoes, but only a few are of major concern to human health [1]. Mosquitoes are well-known disease vectors, transmitting the causative agents of some of the most serious diseases such as malaria, dengue fever, West Nile fever, and lymphatic filariasis. In addition to being disease vectors, mosquitoes are also known as notorious nuisance causers, through persistent biting, to occupational, recreational and social activities [1, 2].

Mosquitoes belong to the Dipteran family of Culicidae, which consists of approximately 3500 recognized species, grouped into two subfamilies: Anophelinae and Culicinae [3]. The subfamily Culicinae is the largest of the two mosquitoes' subfamilies, containing around 3,060 species in 109 genera [4], whereas subfamily Anophelinae has three genera, with 481 recognized species [5]. There are three important species of mosquitoes worldwide, which include the *Anopheles gambiae* complex, the *Culex pipiens* complex, and the *Aedes* subgenus *Stegomyia. Aedes* species, including *Aedes aegypti* and *Ae. albopictus*, are medically important as they are vectors for some serious diseases such as the yellow fever and dengue fever [6, 7, 8].

Dengue fever and dengue hemorrhagic fever are cosmopolitan vector-borne diseases currently present in more than 100 countries in tropical, subtropical, and temperate regions of the world and poses a public health threat to more than 2.5 billion people worldwide, with around 80 million people being reported infected annually at an attack rate of 4% [9-17]. In the western parts of Saudi Arabia, Dengue fever has a major effect on human populations' wellbeing and the country's economy, specifically in Jeddah city, which is one of the main entry points to Saudi Arabia [18, 19].

Recent mosquito-control research has been focusing on the interruption of disease transmission either by killing, preventing the disease-vectors, mosquitoes, from biting humans or by killing the larvae at their breeding sites. The wide use of conventional chemical insecticides, such as malathion and DDT, against adult mosquitoes have shown promising results in combating the spread of mosquitoes. However, several mosquito strains developed resistance to those chemical pesticides [20-23], in addition to their apparent side-effects as they have found to be toxic (such as leaving toxic residues on treated crops) and have adverse effects on the environment (by contaminating air, water, and soil), humans, and animals [24-28].

This has turned the focus of researchers to develop environmentally safe, biodegradable insecticides from natural sources. Insecticides of botanical origins are promising as they are effective, easily biodegradable, environment-friendly, and moreover, inexpensive [29-35, 8]. Extracts from several different plant families have been used and evaluated in many areas around the world, and have shown new and promising larvicides, including extracts from leaves, flowers and roots of plants [31-43].

The current study is an attempt to evaluate the efficacy of ethanolic leaf extracts from four selected local plants: *Citrullus colocynthis* (bitter apple), *Artemisia annua* (sweet wormwood), *Pergularia tomentosa* (Fattaka), and *Rhanterium epapposum* (Arfaj), from Hail region, northern Saudi Arabia, against the larval stages of the Dengue fever vector, *Ae. aegypti* under the laboratory conditions. Several studies were carried on the bio efficacy of the four plant extracts against mosquitoes in other countries. For example, [44-46] worked on *C. colocynthis* in India and Sudan; [47] studied *A. annua* in Vietnam. Regarding *R. epapposum*, no previous studies were done on its larvicidal efficacy against mosquitoes except for the study done by [48] on the use of the essential oils of *R. epapposum*, collected north of Riyadh, Saudi Arabia, as an insect-repellent. Similarly no previous records were found for studies on larvicidal efficacy of *P. tomentosa*, except for the study done by [49] on the effects of different extracts three different extracts (water, ethanol and acetone) of *P. tomentosa* against the fourth instar larvae of the Dengue Fever mosquito vector *Ae. aegypti*, which showed that the ethanolic extract had a higher larvicidal success against *Ae. aegypti* larvae compared to the other solvents.

Materials and Methods

Plant materials

Four different plants (*Citrullus colocynthis* (bitter apple), *Artemisia annua* (sweet wormwood), *Pergularia tomentosa* (Fattaka), and *Rhanterium epapposum* (Arfaj)) were chosen for their medicinal uses in traditional folk medicine. Fully developed leaves of these plants were collected from different localities in Al-Nafud Desert, which is located in northern of Hail region, Saudi Arabia. The collected plants were carefully isolated from combined impurities such as weeds, soil particles and other inessential matters. The leaves were then washed with tap water, dried in the shade (for three weeks) and then finely ground, and sieved to obtain fine powder that was used to prepare the extracts.

Ethanol extracts of each of these plants were obtained by taking 50 g of dried leaves powder in a container and homogenized with 100 ml of absolute ethanol, which was added to the powder and kept for 24 hour with periodic shaking. The crude preparations were left for 24 hour in the shaker at room temperature and were then centrifuged at 4000 rpm for 20 minutes. The supernatant containing the plant extracts was transferred to a beaker and concentrated by evaporating the solvent sing a rotary vacuum evaporator at 60 °C, following the procedure of [50]. The solid substance was weighed and dissolved in a known volume of distilled water to obtain the final concentrations: 5.00%, 0.50%, 0.05%, and 0.005%, and later stored in a refrigerator at 4ºC in air-tight bottles.

Mosquito cultures

Eggs of *Ae. aegypti* were obtained from the Laboratory of Public Health Pests, Jeddah Governorate, Saudi Arabia. The colonies of *Ae. aegypti* were cultured and maintained in the laboratory at the College of Science, University of Hail, at 27 ± 2°C and 75-85% relative humidity under a 14:10 light and dark cycles. The larvae were reared in plastic trays containing tap water, and were fed on a diet of aquarium fish food that contained macro nutrients, trace elements and necessary vitamins. The Pupae were transferred daily from the trays to a cup containing tap water and were kept inside chiffon mosquitoes breeding cages (measuring 50×50×50 cm) covered with a fine mesh for trapping adults after their emergence. Adults were provided with 10% sugar solution for males feeding. The females were given a blood meal by placing a pigeon on top of the breeding cages overnight. Plastic Petri dishes filled with 50 ml of tap water were lined with filter paper and kept inside the cages for oviposition.

Larvicidal bioassay

Four different test concentrations from each of the four different plants were prepared by adding different ranges of stock solution to 250 ml of water. The effects of the tested extracts were determined by following the WHO standard procedure [51], where, 25 fourth instar larvae of *Ae. aegypti* were exposed to the prepared 250 ml of test concentrations. Each concentration was replicated four times. The control experiments were also run parallel to each replicate [52]. The larval mortality was observed and counted after 24, 48 and 72 hours, respectively, of the exposure period.

Statistical analysis

Before performing analysis, tested concentrations were transferred to log [53]. The mortality percentage of *Ae. aegypti* larvae was determined by the following formula:

$$Mortality\ \% = (no.of\ dead\ larvae/no.of\ introduced\ larvae) \times 100$$

Calculated percentages of the mortality of *Ae. aegypti* larvae were plotted with dependable concentrations on logarithmic probability paper for completing the corresponding log-concentration Probit lines. In order to detect the lethal concentrations of 50% (LC_{50}) on *Ae. aegypti* larvae, regression lines were formulated. The data were then tested to create the Probit and toxicity index (LC_{50}) according to [54-56]. Statistical analysis were carried out using the statistical package SPSS® 14.0 for Windows.

Results

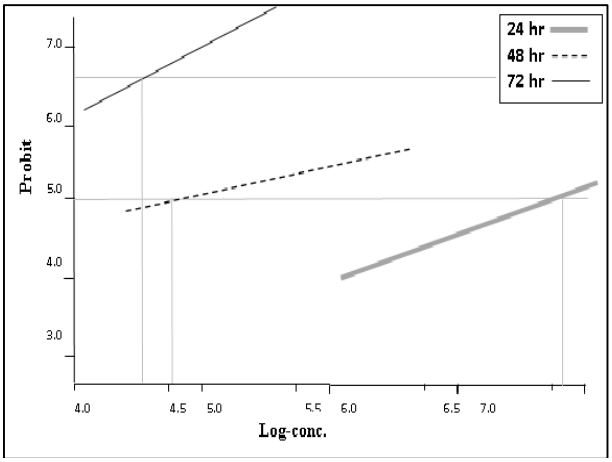
The obtained results revealed that, under the laboratory

conditions, and at the lower concentration (50 ppm), the ethanolic extracts resulted in percentage mortality ranged between 10% (*P. tomentosa*) to 50% (*C. colocynthis*) in the 4th instar larvae of *Ae. aegypti*. Mortality percentages of *Ae. aegypti* larvae ranged between 15% (*P. tomentosa*) to 61% (*A. annua*) at the concentration of 500 ppm. At the concentration of 5000 ppm the percentage mortalities ranged between 18% (*P. tomentosa*) to 80% (*A. annua*). The tested ethanolic extracts at 50000 ppm resulted in percentage mortality ranged between 25% (*P. tomentosa*) to 99% (*R. epapposum*) in the 4th instar larvae of *Ae. aegypti* larvae (Table 1).

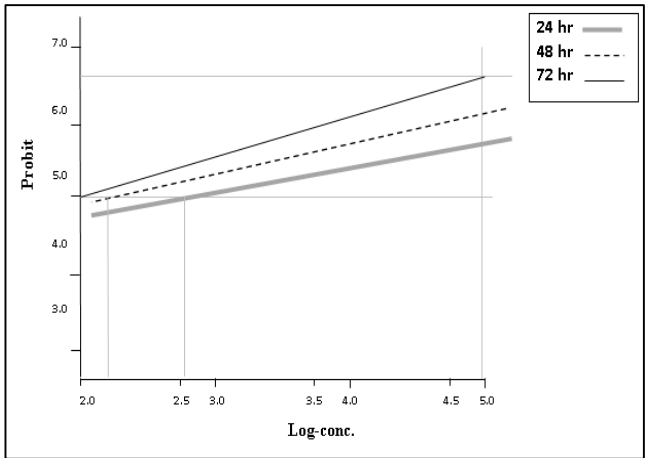
Out of 12 treatments (4 plant products × 3 tested periods] only five treatments resulted in mortality of 50% of *Aedes aegypti* larval population (LC_{50}) using concentrations ranged between the lower two tested concentrations (50 – 500 ppm) of which the LC_{50} of *A. annua* (72 hr) at 100 ppm, *A. annua* (48 hr) at 130.7 ppm, *C. colocynthis* (72 hr) at 177.8 ppm, *R. epapposum* (72 hr) at 365.7 ppm, *A. annua* (24 hr) at 379.3 ppm. Four plant products has LC_{50} ranged between 5000 – 50000 ppm of which *R. epapposum* (48 hr) at 695.2 ppm, *R. epapposum* (72 hr) at 1399.8 ppm, *P. tomentosa* (72 hr) at 15615.2 ppm and *C. colocynthis* (72 hr) at 35111.2 ppm. Three plant products required highest concentration than the higher tested one (50000 ppm) to produced 50% mortality, of which, *P. tomentosa* (48 hr) at 17752.8 ppm and *C. colocynthis* (24 hr) at 464×10^5 ppm and *P. tomentosa* (24 hr) at 100×10^6 ppm (Table, 2 and Appendix, 1).

It was clear that, the lethal effect of each of the tested ethanolic extracts (under the laboratory conditions) on *Ae. aegypti* larvae was more effective with raising concentration (from 50 to 50000 ppm) and submission time from 24 to 72 hours (and this observation can be attributed to the time required for the toxic components to dissolve and accumulate in higher concentrations). Moreover, the data showed that the ethanolic extracts of *C. colocynthis* and *A. annua* were more effective to control *Ae. aegypti* larvae under the lower concentration (after 72 hours) compared to the other plants, whereas, *R. epapposum* and *A. annua* were more effective to control *Ae. aegypti* larvae (mortality reached 95% and more) under the higher concentration (after 72 hours) compared to the others.

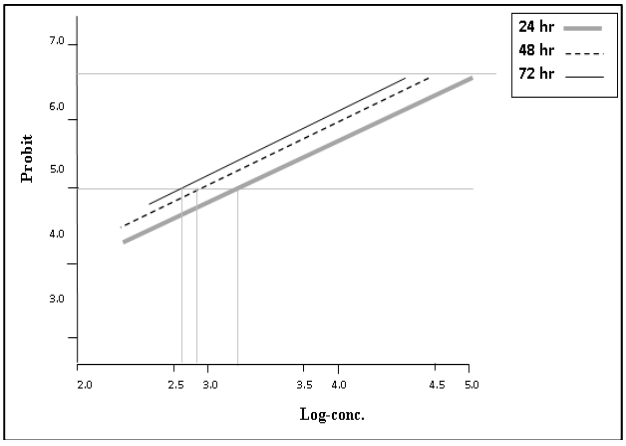
Appendix (1): Log- Probity curves of the four ethanolic leaf extracts that affected the mortality of *Aedes aegypti* larvae after 24, 48 and 72 hours. A) *Citrullus colocynthis*; B) *Artemisia annua*; C) *Rhanterium epapposum*; D) *Pergularia tomentosa*. (x-axis: Logarithm concentration (ppm); y-axis: Probit mortality).



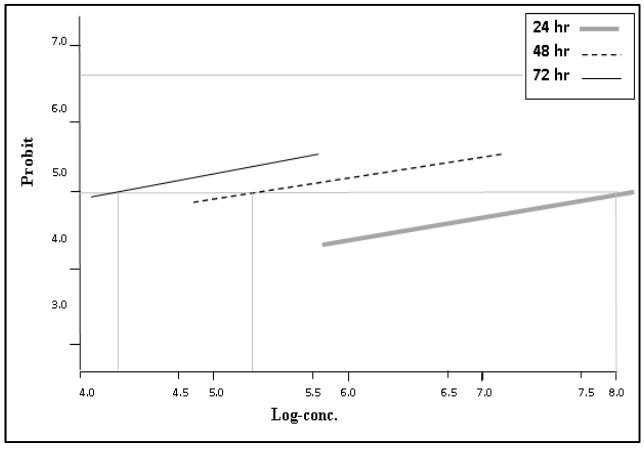
A) *Citrullus colocynthis*



B) *Artemisia annua*



C) *Rhanterium epapposum***.**



D) *Pergularia tomentosa***.**

Table 1: Percentage mortality (%) of *Aedes aegypti* larvae treated with different concentrations of the four ethanolic leaf extracts of (*Citrullus colocynthis*; *Artemisia annua*; *Rhanterium epapposum* and *Pergularia tomentosa*) after 24, 48 and 72 hours.

Conc. (ppm)	*C. colocynthis* (24h)	*C. colocynthis* (48h)	*C. colocynthis* (72h)	*A. annua* (24h)	*A. annua* (48h)	*A. annua* (72h)	*R. epapposum* (24h)	*R. epapposum* (48h)	*R. epapposum* (72h)	*P. tomentosa* (24h)	*P. tomentosa* (48h)	*P. tomentosa* (72h)
50	13	26	50	38	44	48	17	25	34	10	10	22
500	22	36	49	48	58	61	29	34	46	15	25	35
5000	20	40	51	68	73	80	45	54	64	18	29	40
50000	30	52	54	78	87	95	97	99	99	25	41	59
Control	0	0	0	0	0	0	0	0	0	0	0	0

Table 2: The transformed percentage mortality (%) and toxicological parameters of *Aedes aegypti* larvae treated with different concentrations of the four ethanolic leaf extracts of (*Citrullus colocynthis*; *Artemisia annua*; *Rhanterium epapposum* and *Pergularia tomentosa*) after 24, 48 and 72 hours.

Conc (ppm)	Log (conc)	*C. colocynthis* (24h)	*C. colocynthis* (48h)	*C. colocynthis* (72h)	*A. annua* (24h)	*A. annua* (48h)	*A. annua* (72h)	*R. epapposum* (24h)	*R. epapposum* (48h)	*R. epapposum* (72h)	*P. tomentosa* (24h)	*P. tomentosa* (48h)	*P. tomentosa* (72h)
50	1.699	3.87	4.36	5.00	4.69	4.85	4.95	4.05	4.33	4.59	3.72	3.72	4.23
500	2.699	4.23	4.64	4.97	4.95	5.20	5.28	4.42	4.59	4.90	3.96	4.33	4.61
5000	3.699	4.16	4.75	5.03	5.47	5.61	5.84	4.87	5.10	5.36	4.08	4.45	4.75
50000	4.699	4.48	5.05	5.10	5.77	6.13	6.64	6.88	7.33	7.33	4.33	4.77	5.23
Toxicological parameters													
R-square		0.82	0.97	0.70	0.98	0.99	0.97	0.84	0.81	0.83	0.98	0.92	0.96
Intercept (a)		3.62	4.00	4.91	4.02	4.09	3.88	2.20	2.30	2.77	3.40	3.27	3.70
Slope (b)		0.18	0.22	0.04	0.38	0.43	0.56	0.89	0.95	0.87	0.2	0.33	0.31
LC_{50} (ppm)		$464X10^5$	35111.19	177.83	379.27	130.70	100	1399.8	695.19	365.78	$100X10^6$	174752.8	15615.23

Discussion

Disease-vectors control (especially mosquitoes) using conventional chemically synthetic insecticides is showing evidence of losing the battle, as several mosquito species have developed resistance to these insecticides [57-61]. Thus the need has arisen for the generally safe botanical insecticides [62, 63] to serve as suitable alternatives to chemical insecticides, and which could be found in several regions of the world.

Although the ethanolic extract of *A. annua* and *R. epapposum* were more effective in controlling *Ae. aegypti* larvae, the other two plant extracts, *C. colocynthis* and *P. tomentosa* showed larvicidal properties but at a lesser efficacy. Several previous studies suggested that both *C. colocynthis* and *P. tomentosa* could be used as a natural larvicidal products against several vector larval stages [44, 45, 64, 65, 49]. However, the lesser efficacy recorded in the current study could be attributed to the variation in the chemical composition of the effective essential oils found in these local varieties of both plants.

The effectiveness of the ethanolic leaves extract of *R. epapposum* against the 4th instar larvae of *Ae. aegypti* could be attributed to the chemical composition of its essential oils. *Rhanterium epapposum* (Arfaj) is commonly used in folk medicine in rural areas of Saudi Arabia as a remedy for gastrointestinal disturbances, skin infections, and most importantly as an insecticide [66, 67]. In a recent study, [68] recorded the major constituents of essential oils of *R. epapposum* leaves, which included limonene, sabinene, α-pinene, β-myrcene, in addition to other constituents in lesser percentages. Limonene has been used among the first natural pesticides against mosquitoes, ticks, fleas and other insects [69-71]. Sabinene, α-pinene, and β-myrcene have similar insecticidal properties, as reported in several studies [72-75].

The insecticidal ability of ethanolic extract of *A. annua* was found to be effective in lower doses and longer exposure periods, which is similar to the findings of [76], who worked on the essential oil of *Acorus calamus* (sweet flag or calamus) on adults of five stored-product insect species; and [77] who worked on the toxicity of methanol extract of the rhizome of *Acorus gramineus* (Dwarf sedge) on adults of *Sitophilus oryzae* (rice weevil) and *Lasioderma serricorne* (tobacco beetle); and [78] who worked on the insecticidal activity of essential oils obtained from oregano and savoury against *Acanthoscelides obtectus* (bean weevil). This could be attributed to the slow absorbance/penetration of the effective larvicidal essential oils of *A. annua* through the tissues of *Ae. aegypti*, and thus it appears that the period of exposure is more influential manifesting the larvicidal effect than the concentration of the dosage.

The results of the current study conform with several previous studies that highlight the utilization of the plants' bio-active chemicals, especially essential oils, as potential, environment-safe, alternatives to synthetic insecticides. Such chemical compounds could act as repellents, contact insecticides, fumigants, anti-feedants, or could intervene with any of the vital functions of the insects [79-87, 4].

Further studies are needed to determine the exact chemical composition, larvicidal potency and stability of the bioactive chemical ingredients in the studied plants that are found locally in Hail region and other regions within Saudi Arabia.

Acknowledgements

The authors would like to thank Mr. Fahad Al- Zahrani for facilitating the acquisition of mosquitoes' specimens from the Laboratory of Public Health Pests, Al Amana, Jeddah Governorate.

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