ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 15:6 (2021) 476-486

records of natural products

Study of *Anacyclus pyrethrum* Lag. Root Extract against *Aedes aegypti* Linn. Larvae: Potential Vector Control for Dengue Viral Fever

Atia-tul-Wahab [1*], Muniza Shaikh [1,3], Uzma Naqeeb [2], Shakil Ahmed [4], M. Iqbal Choudhary [1,2,5*] and Atta-ur-Rahman [1,2]

[1]*Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[2]*H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[3] *Third World Centre for Science and Technology, H. E. J. Research Institute of Chemistry International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[4]*Industrail Analytical Center, H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[5]*Department of Biochemistry, Faculty of Sciences, King Abdul Aziz University, Jeddah-21589, Saudi Arabia*

(Received February 19, 2021; Revised April 22, 2021; Accepted April 23, 2021)

Abstract: *Anacyclus pyrethrum* Lag. is a medicinal plant. In this study we evaluated the susceptibility of larvae of *Aedes aegypti* Linn. against potential larvicidal effects of root extract of *A. pyrethrum*. Bioassays were performed using World Health Organization methodologies. GC-MS analysis was employed to determine the contents of the extract. The underlying mechanisms of larvicidal activity were analyzed by using morphological, histological, and Reactive Oxygen species (ROS) studies. The GC-MS of *A. pyrethrum* root extract showed nine compounds. During initial screening, the root extract killed 90% of 3rd instar larvae of *A. aegypti* at 0.2 mg/mL concentration as compared to standard permethrin. Different larval stages exhibited 100% larvicidal activity, especially more naive 1st and 2nd instar larvae. The dose dependent larvicidal activity against 3rd instar larvae, showed reduced effect with decreased concentration. Disruption in gut line of 3rd instar larvae is proposed to be mediated from the ROS production, evident in fluorescent oxidant sensitive assay. The extract exhibited non-cytotoxic profile in brine shrimp lethality assay with IC_{50} of 126.93 ± 0.3 µg/mL as compared to standard etoposide *i.e.*, IC_{50} = 7.46 ± 0.05 µg/mL. The study inferred that the root extract of *A. pyrethrum* can serve as promising biopesticide for the control of *A. aegypti*, which is a vector of many viral diseases.

Keywords: *Anacyclus pyrethrum* Lag.; *Aedes aegypti* Linn.; larvicidal activity; ovicidal activity; dengue hemorrhagic fever; biopesticides. © 2021 ACG Publications. All rights reserved

1. Introduction

Mosquitoes are among the vectors of many vector-borne diseases. The species *Aedes aegypti* is the most common mosquito in the world that is responsible for the transmission of vector borne diseases and its impact on human health is very significant. *Aedes aegypti*, commonly known as yellow fever mosquito, has been known to transmit many tropical fevers causing viruses, including dengue, Zika, urban yellow fever, and chikungunya viruses. These are potentially deadly tropical infections in Asia, South America, and Africa [1]. Its elimination by biopesticide is highly desirable for the control of these

* Corresponding author: E-Mail: atia.tulwahab@iccs.edu (Atia-tul-Wahab); iqbal.choudhary@iccs.edu (M. Iqbal Choudhary)

The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products November-December 2021 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.243.21.02.1979

tropical diseases. The vector is responsible to affect 50-100 million people every year worldwide [2], with 250,000 suffering from dengue hemorrhagic fever. A large proportion of deaths *i.e.*, 25,000 per year have been reported worldwide [3].

The use of various commercially available synthetic pesticides, comprising of persistent toxic substances, endocrine signaling disruptors, and persistent organic pollutants has serious environmental and health impacts. This requires the development of ecofriendly, biodegradable, indigenous, and economical methods of vector control. One of these approaches is to use commonly available herbal products as bio-insecticides.

Anacyclus pyrethrum Lag. is a species, belongs to family Asteraceae. Its herb reaches 30 to 50 cm in height. The roots of *A. pyrethrum* commonly known as 'pellitory root' which possess interesting pharmacological properties including, anti-convulsive, anticancer, aphrodisiac, androgenic, antiparasitic, bio-insecticide, antidiabetic, anti-amnesic, antifungal, and immunostimulant effects. In addition, this plant is also recommended for the treatment of partial paralysis of lips and tongue, gout, and sciatica [4]. It has been reported that *A. pyrethrum* has antimicrobial effects on *Candida albicans*, *Staphylococcus aureus* and strong larvicidal activity against malaria causing mosquitos [5].

We have investigated the larvicidal activity of the root extract of *A. pyrethrum* Lag. against *A. aegypti* larvae. The study also includes the effects of the root extract on different stages of developing larvae. Ovicidal effects were also evaluated, followed by the determination of cytotoxicity through brine shrimp lethality assay. The mechanistic investigation includes morphological and histopathological studies, and measurement of the formation of free radicals as a means of larvicidal activity.

2. Materials and Methods

2.1 Materials

Glass Teflon homogenizer (Thomas scientific), 96-well flat bottom plate (Iwaki, Japan), spectrophotometer (Tecan, Austria), 90i microscope, water bath, rotary microtome (Leica RM 2245, Leica, Mannheim, Germany).

2.2 Chemicals and Drugs

HPLC grade absolute ethanol, PBS tablets (MP Biomedicals, Inc., France), sodium chloride (Sigma Aldrich, Germany), 5% permethrin (GlaxoSmithKline, UK), etoposide (Tokyo Chemical Industry, Japan), xylene HPLC grade. Hematoxylin and eosin staining (Sigma Aldrich, Germany) formaldehyde (Sigma Aldrich, Germany), glacial acetic acid (Sigma Aldrich, Germany).

2.3. Artificial Rearing of Aedes aegypti *Linn.*

The egg strips of identified *A. aegypti* Linn. mosquito were obtained from the insectary of the Department of Zoology, University of Karachi, established in 1994, and maintained by Dr. R. M. Tariq. The larvae have been deposited (specimen voucher no. NHMZUK-2(5): Dip. Ae. 37, 1992) by Prof. Dr. S.N.H. Naqvi. Larvae were reared in plastic bowls after immersing the egg strips for 2-3 days in 800 mL of water. Each bowl containing approximately 400 larvae with temperature controlled at 27 ± 2 °C, and relative humidity of 75% ± 5. The bowls were covered with net covers. Dried ground prawns were provided as food for the survival of larvae until they were converted into pupae. The pupae were collected in beaker containing 80 mL tap water and were transferred into the mosquito cages (60 × 60 × 60 cm) for adult emergence. The pupae shed their exoskeleton and converted into adult mosquitos in 3-4 days. About 200 adult mosquitoes were kept in each cage, and fed with 10% sugar solution, and soaked in cotton circles in petri plates. After two days of emergence, blood meal from chicken was provided to the female mosquitos in every two days while male mosquitos were fed with 10% sugar solution. The blood meal helped in the survival and oviposition. In each cage cylindrical plastic bowls were placed for oviposition, containing 500 to 600 mL of tap water, and lined with 3 cm broad, and 8 inches long filter paper strips. After 2-3 days when the oviposition occurred, the bowls were kept in above mentioned temperature for

hatching or the filter paper was removed, dried under laboratory conditions, and stored in sealed plastic bags [6].

2.4. Plant Material

The roots of *A. pyrethrum* Lag. were purchased from local herbal market, and deposited in National Herbarium Islamabad. The taxonomic identification was made by Dr. Amir Sultan from National Herbarium Bio-Resource Conservation Institute (BRC), National Agriculture Research Center (NARC). The voucher specimen was given as HEJ-1, and reserved for future reference.

2.5 Preparation of Ethanol Extract of the Root A. pyrethrum *Lag.*

Initially, plant material was soaked in 80% ethanol-water for 72 hrs. After that the extract was obtained by filtration and, the filtrate was dried in rotary evaporator at 60 °C. The resulting material was left for some time. Extracts were used for screening purposes.

2.6. GC-MS of Plant Extract

GC/MS of *A. pyrethrum* root extract was performed on a 7890-gas chromatography equipped with an Agilent auto sampler and coupled to an Agilent 5975 single Quad system (Agilent Technologies, USA). An HP-5MS 30 m-250 mm (i.d.), fused-silica capillary column (Agilent J&W Scientific, CA, USA) was used. The column was chemically bonded with a cross-linked stationary phase of 0.25 mm thickness comprising of 5% diphenyl 95% dimethylpolysiloxane. Helium was used as the carrier gas at 1.0 mL/min and the sample was injected in split-less mode. The injector, and source temperatures were 280 °C. The oven temperature was initially maintained at 60 °C and was then increased at 5 °C/min to 180 °C for 15 min. In post run, temperature was further increased to 300 °C, for remaining 1 min. Electron ionization (EI) was used as an ionization source for the GC/MS analysis at 70 eV. Data was acquired in the full scan mode from *m*/*z* 50 to 700 with a scan time of 0.5 sec.

2.7. Identification of Constituents Present in Plant Extract

Data processing was performed by using the Agilent Mass Hunter Qualitative Analysis (version B.04.00). Identification of individual constituents of the extract was achieved based on their retention indices (determined with a reference to homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns (NIST database/ChemStation data system) [7].

2.8. Bioassays

2.8.1. Larvicidal Activity of Root Extract against 3rd Instar Larvae of Aedes aegypti *Linn.*

A stock solution of 20 mg /mL was prepared. From the stock solution, 0.5 mL was dispersed in 49.5 mL of distilled water for test solution of 0.2 mg/mL. After that, ten larvae were transferred in the flask containing test solution. For positive and negative controls, permethrin and ethanol were used, respectively [8]. Mortality rate was determined after 24 hrs by using the following formula:

$$Percent\ Mortality = \frac{\text{Percent survival in the untreated control} - \text{Percent survival in the treated sample}}{\text{Percent survival in the untreated control}} x100 \quad (1)$$

2.8.2. Ovicidal Activity of Root Extract against Eggs of Aedes aegypti *Linn.*

From the stock solution of 20 mg/mL, 0.5 mL was mixed with 49.5 mL tap water for 0.2 mg/mL test solution. Then, 20 recently laid eggs were introduced into 0.2 mg/mL test solution. Egg mortality was scored 120 hrs post-treatment. Eggs that were not hatched after this period were considered as dead. For

positive control, permethrin was used, and for negative control ethanol was used [9]. Formula for ovicidal activity is as follows:

$$Percent\ Egg\ Mortality\ = \frac{\text{Number of unhatched eggs}}{\text{Total number of eggs introduced}} x100 \quad (2)$$

2

2.8.3. Dose Dependent Larvicidal Activity

Stock solutions of plant extracts were made in ethanol. Various concentrations were used to characterize the activity of plant root extracts. Results were noted after 24 hrs. Ethanol was used as negative control, and permethrin as positive control. Both controls were also checked to the relevant concentration of 0.4, 0.1, and 0.05 mg/mL same as that of plant extract i.e., 0.2 mg/mL (200 ppm).

2.9. Morphological Studies

Morphological studies of 3rd instar larvae of control and treated larvae was carried out at the concentration of 0.2 mg/mL root extract incubated for 24 hrs. Slides containing treated and control larvae were mounted with DPX and the results were observed *via* 90i microscope at 10X DIC.

2.10. Histological Analysis

2.10.1. Paraffin Embedding

3rd Instar larvae, after treatment with plant root extracts, were fixed in a mixture of solution (60% ethanol, 30% formaldehyde, 10% and 37% glacial acetic acid), and preserved for five days at 4 °C. For histological procedure, the larvae were exposed to serial washes with 70, 80 and 90% ethanol for 10 min, respectively. It was then followed by two washes for 10 min each with 100% ethanol and were further immersed in 100% ethanol for 20 min. afterward, larvae were exposed to xylene for 10 min, and were embedded in paraffin at 56°C for 24 hrs. Larvae were fixed in aluminum boats and subjected to serial microtome sectioning at 7 µm thickness.

2.10.2. Hematoxylin and Eosin Staining

Slides containing specimen were washed in a series of solutions, xylene (2 washes of 5 min), 100% ethanol (2 washes for 3 min), 95% ethanol (3 min), 70% ethanol (3 min) and distilled water (3 min). Then the slide was stained with hematoxylin for 45 sec, rinsed with tap water followed by water solution containing (2 g $NaHCO_3$, 10 g $MgSO_4$, in 1 L dist. H_2O) for 2 min, distilled water for 1 min, 70% ethanol for 1 min, counter stained with eosin dye for 2 min, 95% ethanol (30 sec), 100% ethanol and xylene for 30 sec, each. Later the slides were fixed with cover slips using DPX (mounting media), then examined under bright field microscope [10].

2.11. Reactive Oxygen Species (ROS) Studies

Larvae were treated with the plant root extract of concentration 0.2 mg/mL for 24 hrs. After treatment, larvae were perforated by pricking with needle so that the media may enter the haemocoel. 20 min incubation was done in PBS with 2 µM of CM-DCFDA (5-(and-6)-chloromethyl-2′,7′-dichloro-dihydrofluorescein diacetate, acetyl ester), an oxidant-sensitive probe, at room temperature in the dark. 0.1 M probe free PBS was used for washing larvae. Microscopic analysis was carried out in a dye/probe free medium, and detection was carried out on green filter [11].

2.12. Cytotoxicity (Brine Shrimp Lethality Assay)

Root extract of *A. pyrethrum* (20 mg) was dissolved in 2 mL of respective solvent at concentrations of 10, 100 and 1000 µg/mL. The solution was kept for evaporation overnight. The volume of each vial was made up with 5 mL seawater and 10 brine shrimps (*Artemia salina*) were added in each vial. The vials were incubated at 25-27 °C for 24 hrs. Respective solvent was used as negative control and cytotoxic drug (etoposide) was used as a positive control. Afterwards, the mortality of brine shrimps in each vial was observed. Data analysis was carried out by Finney software to determine LD_{50} [12].

3. Results and Discussion

3.1. GC-MS of Ethanolic Root Extract of A. pyrethrum *Lag.*

GC-MS was used for identification of compounds. The results of the GC-MS of roots extract of *A. pyrethrum* showed nine components listed in Table 1. *γ*-Sitosterol, *n*-hexadecanoic acid, and 9-12-octadecadienoic acid were identified as the main components by GC-MS.

Table 1. Identified compounds from the ethanolic extract of the root of *Anacyclus pyrethrum* by GC-MS analysis.

Compound	Relative Retention Indices (RRI)	Percentage Area (%)
Benzofuran 4-7 dimethyl	1244	18.4
n-Hexadecanoic acid	1968	20.1
Hexadecanoic acid ethyl ester	1978	8.1
2-Methyl-*Z*,*Z*-3,13 octadecadienol	2104	8.9
Octadecanoic acid ethyl ester	2177	0.9
9-12- Octadecadienoic acid	2183	11.3
Ethyl oleate	2185	4.4
Linoleic acid ethyl ester	2193	6.0
γ-Sitosterol	2731	21.8
Total		**99.9**

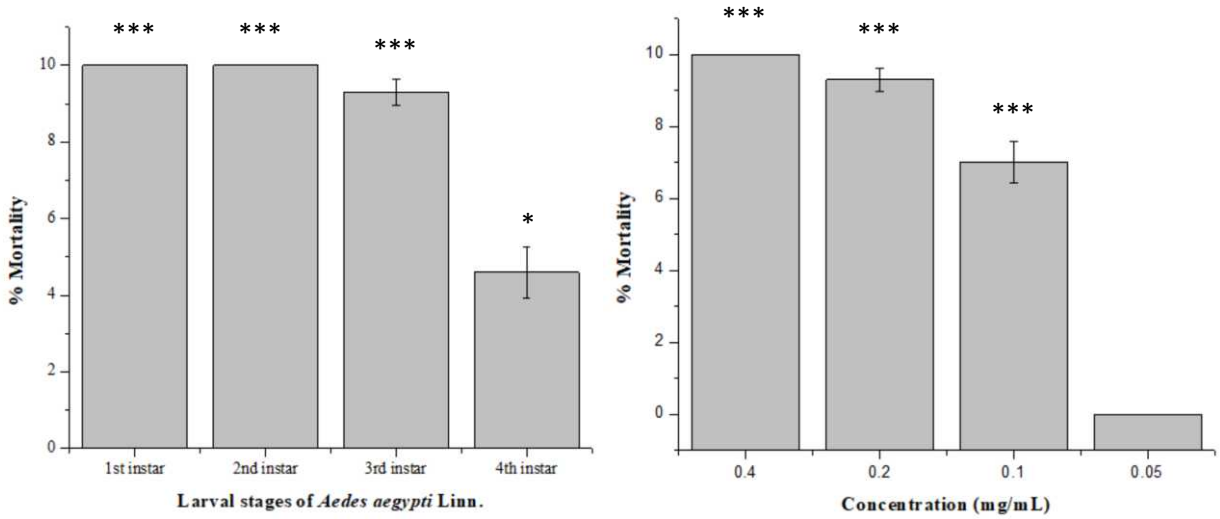
3.2. Larvicidal Activity of A. pyrethrum *Lag. against 3rd Instar larvae of* Aedes aegypti *Linn.*

The 3rd instar larvae of *Aedes aegypti* was incubated with 0.2 mg/mL of ethanolic root extract of *A. pyrethrum* for preliminary screening. A substantial larvicidal activity of 90% was witnessed against 3rd instar larvae.

3.3. Larvicidal Activity of A. pyrethrum *Lag. against all Instar Larvae of* Aedes aegypti *Linn.*

Root extract of *A. pyrethrum* Lag. was screened at 0.2 mg/mL concentration against 1st, 2nd, 3rd, and 4th instar larvae of *Aedes aegypti*. Replicates of ten larvae from each larval stage were transferred in the flask containing root extract of *A. pyrethrum* at 0.2 mg/mL. 100% mortality was observed for 1st and 2nd instar, while 90%, and 46% mortality was observed in 3rd and 4th instar, respectively (Figure 1A).

Different concentrations of roots of *A. pyrethrum* extract were used to determine the sensitivity of plant extract. *A. pyrethrum* root extract showed a reduction in dose dependent manner in larvicidal activity against 3rd instar larvae of *Aedes aegypti* (Figure 1B).



A **B**

Figure 1. (A) % Mortality of *A. pyrethrum* against all instar larvae of *Aedes aegypti* Linn at 0.2 mg/mL. (B) Effect of different concentrations of *A. pyrethrum* on 3rd instar larvae of *Aedes aegypti* Linn.
*** = P value <0.05 when compared with control
** = P value = 0.05 when compared with control
* = P value > 0.05 when compared with control

3.4. Ovicidal Assay

Root extract of *A. pyrethrum* was subjected to ovicidal activity against the eggs of *A. aegypti*. Results were found to be non-significant with % egg mortality of 21.6.

3.5. Effects of Root Extract of A. pyrethrum *Lag. on Morphology of Larvae*

Morphology of the larvae treated with 0.2 mg/mL root extract was compared with untreated larvae (Figure 2) *via* observing under compound microscope at 4x. This showed a remarkable difference in the morphology of the treated larvae by root extracts of *A. pyrethrum* that affected the gut line. Internal disruption of the gut line was manifested as a black line in the abdominal region (Figure 3).

3.6. Histological Analysis

The histopathological study of *Aedes aegypti* incubated with the ethanolic root extract of *A. pyrethrum* revealed collapsed and broken epithelial layers and displayed a total damage in the midgut region and cecum. The extract treatment disorganized the epithelial layer in the treated larvae and affected the tissues of whole gut line. In the midgut of abdominal region, a complete disintegration was observed (Figure 4).

3.7. Reactive Oxygen Species (ROS) Studies

By using 90i Nikon Eclipse fluorescent microscope and (5-(and-6)-chloromethyl-2′, 7′-dichloro-dihydrofluorescein diacetate, acetyl ester) or CM-DCFDA, an oxidant sensitive probe, ROS production was detected. Three individual 3rd instar larvae, pre-incubated with 0.2 mg/mL *A. pyrethrum* ethanolic root extract, were observed for ROS production.

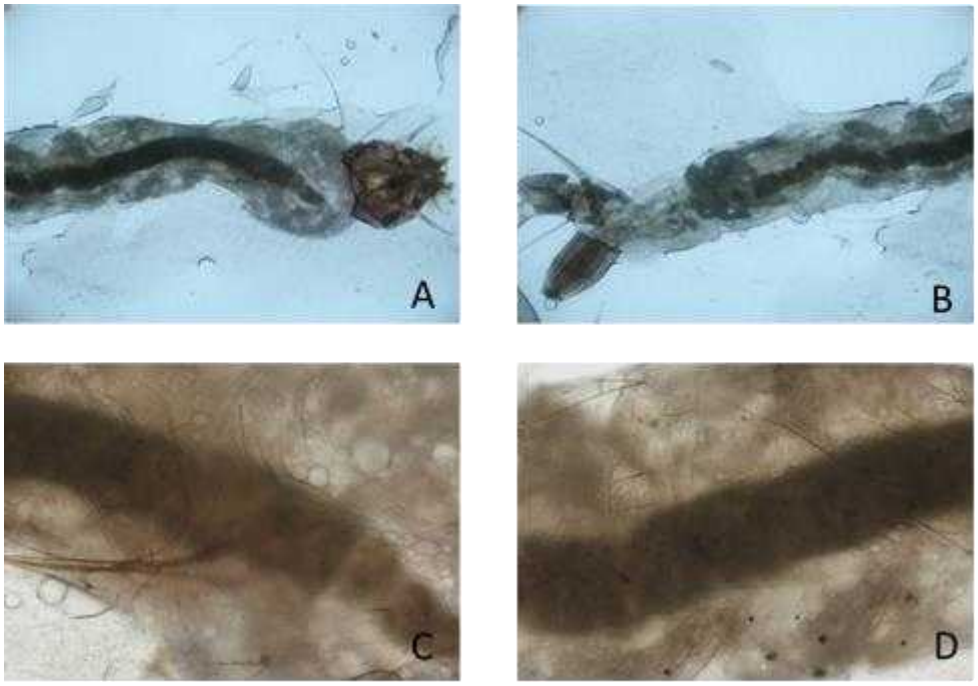


Figure 2. Morphological study of 3rd instar larvae of *Aedes aegypti*. Control (untreated) larvae of *Aedes aegypti* showing intact (A) head, thorax, midgut, and (B) tail region at 2X. While (C) and (D) are showing intact head, thorax, and tail at 10X, respectively.

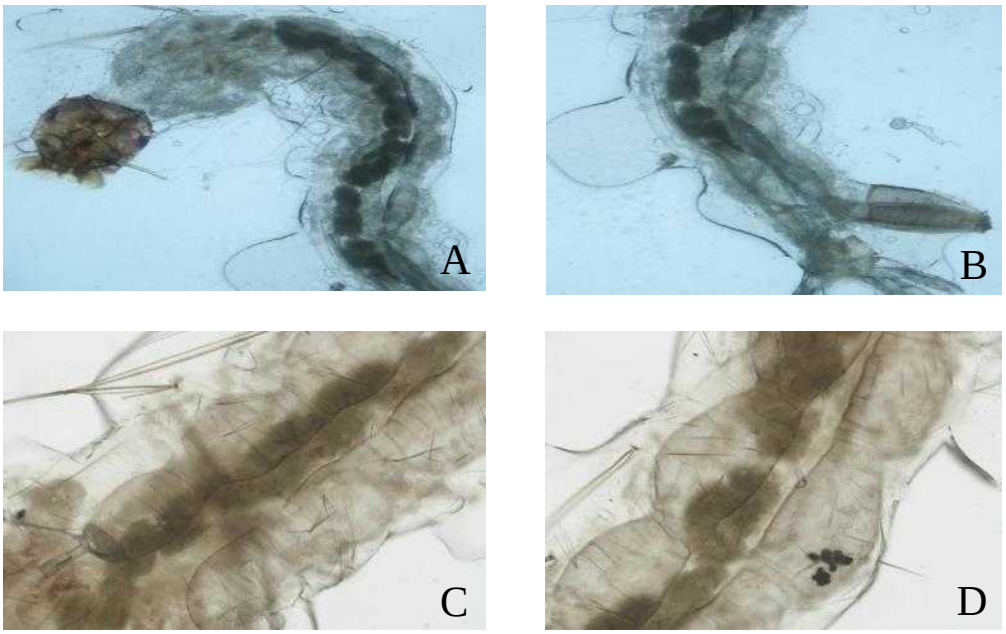


Figure 3. Morphological study of *Aedes aegypti* 3rd instar larvae, treated with root extract of *A. pyrethrum* Lag. At 0.2 mg/mL the treated larvae showing fragmented and disorganized midgut in (A) head thorax, and abdomen and (B) tail region at 2X. (C) and (D) are showing the high-resolution images of midgut at 10X.

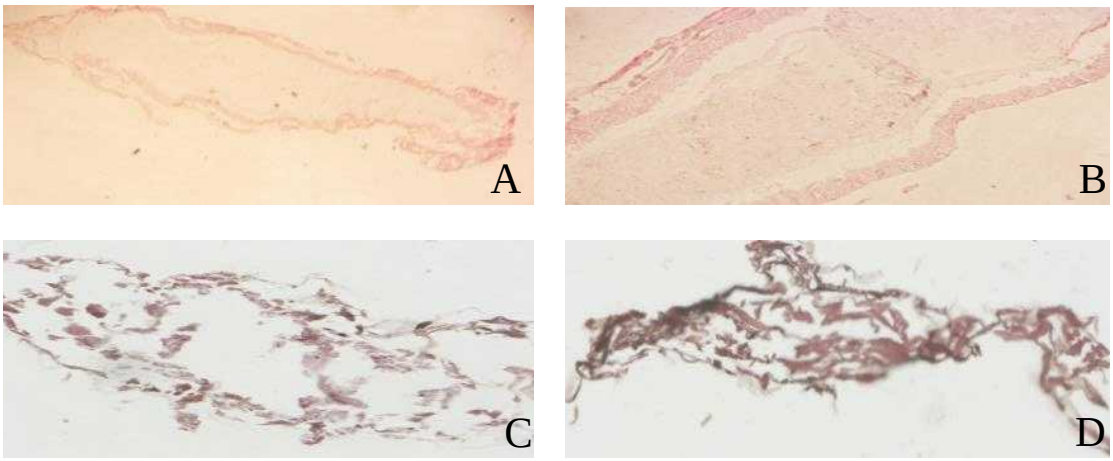


Figure 4. Histopathological study on control and treated third instar larvae of *Aedes aegypti*. Control (A, and B) showed an organized epithelial layer and intact mid gut. Larvae treated with *A. pyrethrum*. Lag. *in* (C) and, (D) are showing the disorganized and broken epithelial layer, complete breakup of mid gut and completely collapsed larval structure.

Hydrogen peroxide treatment group was included as positive control in the study. The treatment with hydrogen peroxide, at a concentration of 200 µM, showed a significant fluorescence which was observed through fluorescence microscopy. Compared with the fluorescence produced by hydrogen peroxide, *A. pyrethrum* showed a moderate fluorescence indicating that their larvicidal activity might be partially due to the ROS production (Figure 5).

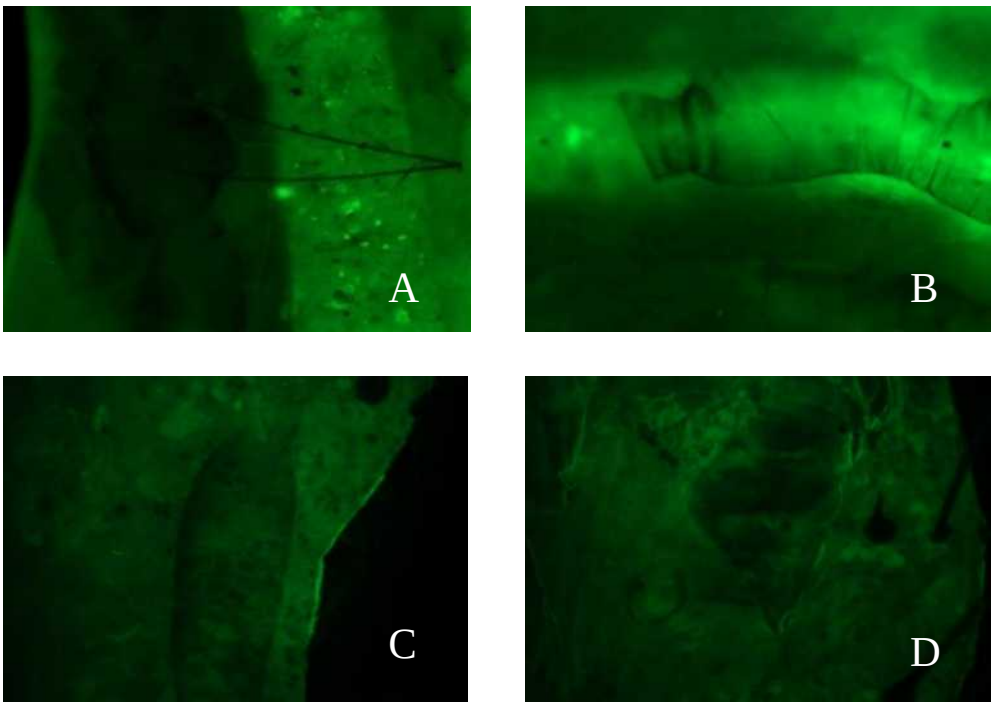


Figure 5. ROS studies of *Aedes aegypti* 3rd instar larvae *via* CM-DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester). An oxidant-sensitive probe was used for fluorescence observation of 3rd instar larvae of *Aedes aegypti* after 24 hrs treatment with *A. pyrethrum* at 0.2 mg/mL. The green color in figures is depicting fluorescence and ultimately ROS production. (A), and (B) are showing different parts of control larvae abdomen, while (C), and (D) are showing head region along with abdomen of treated larvae.

3.8. Cytotoxicity Assay

Toxicity of the *A. pyrethrum* root extract was examined by brine shrimp lethality assay at 10, 100, and 1,000 µg/mL concentrations. LC_{50} of the tested extracts was determined by Finney software (prob. analysis). The root extract was inferred to be having non-cytotoxic behavior towards brine shrimps with LC_{50} of 126.93 ± 0.3 µg/mL, as compared to standard etoposide *i.e*., LC_{50} = 7.46 ± 0.05 µg/mL.

A. pyrethrum Lag. (Aqer Qerha), is a perennial herb that belongs to family Asteraceae. It is commonly found in Arab countries, North Africa, India, and Pakistan. It is native to North India, Himalayas, and Jammu and Kashmir (Pakistan). It has been used as an effective remedy for the treatment of various diseases in Unani System of Medicine. Based on the reported activities, the plant roots are designated as muqawwi-e-bah (anabolic and aphrodisiac), muhallil-e-awram (anti-inflammatory), Moqawwi and moharrik-e-aasab (tonic and stimulant of nerves) herb. It is used for the treatment of facial paralysis, hemiplegia, and epilepsy, loosening of teeth, flaccidity of uvula, and stammering. It is available as single as well as in its compound formulations, such as decoction gargle, majun, oil, and liniment, etc. [13].

Currently used synthetic insecticides are significantly toxic to humans, and other life forms, and cause environmental pollution. The adverse effects of these synthetic insecticides have created an urgent need for development of phytochemicals as insecticidal agents which may serve as eco-friendly, safe, and easily available biopesticides in many regions of the world. In future, synthetic insecticides might be replaced with phytochemicals as biopesticides [14].

According to Bowers *et al*. [15], screening of locally available medicinal plants can play an important role in the protection of public health against vector borne diseases by an easy, and cost-effective way. In the current study, root extract of *A. pyrethrum* Lag., a medicinal plant, was systematically screened through WHO recommended bioassay protocols for its potential to control the vector of dengue fever, *Aedes aegypti* Linn. Our findings highlighted the importance of medicinal plants in the control of insect populations, particularly against *A. aegypti*.

Initial screening *via* incubation for 24 hrs with 3rd instar larvae at 0.2 mg/mL concentration of root extract showed a 90% mortality. This significant larvicidal activity developed our interest in *A. pyrethrum* to further investigate the underlying mechanism for *A. aegypti* mortality. To check the effectiveness of root extract, different stages of larvae were incubated with 0.2 mg/mL concentration of *A. pyrethrum.* A gradual decrease in mortality was observed at higher larval stages, suggesting the naïve and immature larvae of 1st and 2nd instar are prone to *A. pyrethrum* root extract *i.e.,* 100% mortality (Figure 1A). As the larval stage got advanced, the larvae managed to survive the larvicidal effects, reflecting from 46% mortality of 4th instar larvae (Figure 1A). Increasing the concentration of root extract of *A. pyrethrum* showed a positive correlation with the % mortality, hence, confirming the larvicidal effect of *A. pyrethrum*. The extract exhibited a dose dependent mortality effect with descending pattern from 100% at 0.4 mg/mL to no mortality at 0.05 mg/mL (Figure 1B).

GC-MS technique was used for the identification of compounds. The results of the GC-MS of root of *A. pyrethrum* showed nine components including benzofuran 4-7 dimethyl, *n*-hexadecanoic acid, hexadecanoic acid ethyl ester, 9-12-octadecadienoic acid, 9-12- octadecadienoyl chloride, linoleic acid ethyl ester, 9-1-octadecadienoyl chloride, octadecanoic acid ethyl ester, and γ-sitosterol, listed in Table 1. Chemical composition of root of *A. pyrethrum* showed presence of *n*-hexadecanoic acid (palmitic acid) which is reported to have larvicidal activity against *Aedes aegypti* [16]. 9-12-Octadecadienoic acid (Linoleic acid) is also reported for its larvicidal activity [17].

The morphological changes in 3rd instar larvae brought about by the incubation with root extract and control larvae were also studied (Figures 2, and 3). Black pigmentation was observed in the abdominal region of treated larvae that could possibly be arising from abnormalities in the cuticular melanization, resulting from inhibition of chitin synthesis [18]. The similar morphological deformities have also been reported by Saxena *et al.* [19] in *Culex quinquefasciatus,* caused by *Ageratum conyzoides* extract.

The digestive system of *Aedes aegypti* larvae is divided into three parts (foregut, midgut, and hindgut). The midgut can be separately observed in the anterior middle and posterior regions in the control larvae. An intact foregut, midgut, and hindgut with brush borders and epithelial layer can be observed in Figure 4 A and B.

The histopathological study of *Aedes aegypti* incubated with root extract of *A. pyrethrum* revealed collapsed and broken epithelial layers and showed an entire damage in the midgut and cecum areas. The root extract treatment disorganized the epithelial layer in the treated larvae and affected the tissues of whole gut line. A complete disintegration of abdominal region was observed, particularly in the midgut (Figure 4 C, and D).

Generation of reactive oxygen species (ROS) can be one of the various mechanisms of mortality in 3rd instar larvae [20]. *A. pyrethrum* has been shown to cause the production of ROS up to a medium extent that is visible as green fluorescence under fluorescent microscopy (Figure 5). This suggested the partial role of ROS in mediating the larvicidal activity [21].

The study was followed by cytotoxic evaluation in brine shrimp lethality assay exhibiting non-cytotoxic activity, with IC_{50} of 126.93 ± 0.3 µg/mL, as compared to etoposide (standard cytotoxic drug, IC_{50} = 7.46 ± 0.05). The ovicidal activity of the root extract of *A. pyrethrum* against the eggs of *A. aegypti* exhibited 21.6% activity which is found to be non-significant as compared to standard permethrin.

The results suggest that some bioactive compounds of *A. pyrethrum* root extract caused their larvicidal activity *via* metamorphosis disrupting effects with accompanying disturbance in the regulation of molting. Therefore, the inference could be drawn that the larval structure was fully collapsed.

Acknowledgments

ATW and MIC acknowledge the enabling role of the Higher Education Commission, Islamabad, Pakistan, through a financial support under the project entitled, “Studies of Control of Vector *Aedes ageypti* by Natural Products- An Approach towards Controlling Dengue Fever Epidemics” (Project # 2490) under National Research Program for Universities (NRPU).

ORCID
Atia-tul-Wahab: 0000-0002-2730-5547
Muniza Shaikh: 0000-0002-4956-9877
Uzma Naqeeb: 0000-0002-5016-7584
Shakil Ahmed: 0000-0002-0905-1299
M. Iqbal Choudhary: 0000-0001-5356-3585
Atta-ur-Rahman: 0000-0002-3462-5143

References

[1] A.M. Marques, L.S. Velozo, M.A. Carvalho, M.T. Serdeiro, N. Honório, M.A. Kaplan and M. Maleck (2017). Larvicidal activity of *Ottonia anisum* metabolites against *Aedes aegypti*: A potential natural alternative source for mosquito vector control in Brazil, *J. Vector Borne Dis*. **54**(**1**), 61-68

[2] J.W. Tang, M.R. Khanani, A.M. Zubairi, W.Y. Lam, F. Lai, K. Hashmi and P.K. Chan (2008). A wide spectrum of dengue IgM and PCR positivity post-onset of illness found in a large dengue outbreak in Pakistan, *J. Med. Virol.* **80** (**12**), 2113-2121.

[3] S.T. Hakim, S.M.H. Tayyab, S.G. and Nadeem (2011). An Experience with dengue in Pakistan: An expanding problem, *Ibnosina J. Med. Biomed*. *Sci*. **3**(**1**), 3-8.

[4] F. Z. Jawhari, A. E. Moussaoui, M. Bourhia, H. Imtara, H. Saghrouchni, K. Ammor and A. Bari (2021). *Anacyclus pyrethrum* var. *pyrethrum* (L.) and *Anacyclus pyrethrum* var. *depressus* (Ball) Maire: Correlation between total phenolic and flavonoid contents with antioxidant and antimicrobial activities of chemically characterized extracts, *Plants* **10**(**1**), 149. doi: 10.3390/plants10010149.

[5] V. Pandey, V. Agrawal, K. Raghavendra and A.P. Dash (2007). Strong larvicidal activity of three species of Spilanthes (Akarkara) against malaria (*Anopheles stephensi* Liston, *Anopheles culicifacies*, species C) and filaria vector (*Culex quinquefasciatus* Say), *Parasitol Res*. **102,**171–174.

[6] J. Couret, E. Dotson and M.Q. Benedict (2014). Temperature, larval diet, and density effects on development rate and survival of *Aedes aegypti* (Diptera: Culicidae), *PLoS One*. **9**(**2**), e87468.

[7] R.P. Adams and O.D. Sparkman (2007). Review of identification of essential oil components by gas chromatography/mass spectrometry, *J. Am. Soc. Mass Spectrom*. **18**, 803-806.

[8] I.H. Bassole, W.M. Guelbeogo, R. Nebie, C. Costantini, N. Sagnon, Z.I. Kabore and S.A. Traore (2003). Ovicidal and larvicidal activity against *Aedes aegypti* and *Anopheles gambiae* complex mosquitoes of essential oils extracted from three spontaneous plants of *Burkina Faso, Parassitologia*. **45**(**1**), 23-26.

[9] S. Phasomkusolsil and M. Soonwera (2012). The effects of herbal essential oils on the oviposition-deterrent and ovicidal activities of *Aedes aegypti* (Linn.), *Anopheles dirus* (Peyton and Harrison) and *Culex quinquefasciatus* (Say), *Trop. Biomed*. **29**(**1**), 138-150.

[10] L.C. Farnesi, J.M. Brito, J.G. Linss, Pelajo-Machado, M.Valle and G.L. Rezende (2012). Physiological and morphological aspects of *Aedes aegypti* developing larvae: effects of the chitin synthesis inhibitor novaluron, *PLoS One* **7** (**1**), e30363, (9 pages).

[11] O. Salvador-Neto, S.A. Gomes, A.R. Soares, FLDS Machado, R.I. Samuels, R. Nunes da Fonseca and J.R.Silva (2016). Larvicidal potential of the halogenated sesquiterpene (+)-obtusol, isolated from the alga *Laurencia dendroidea* J. Agardh (Ceramiales: Rhodomelaceae), against the Dengue vector mosquito *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), *Mar. Drugs*.**14**, 2-20.

[12] T.M.D.A. Alves, A.F. Silva, M. Brandão, T.S.M. Grandi, E.D.F.A. Smânia, A.Smânia, Júnior and C.L. Zani (2000). Biological screening of Brazilian medicinal plants, *Mem. Inst. Oswaldo Cruz*. **95**(**3**), 367-373.

[13] M.A. Kalam, M.S. Karim, M.A. Anzar, G. Sofi, G. Ahmad and A. Shahzad (2015). Aqer Qerha (*Anacyclus pyrethrum* DC.) A nobel drug of unani system of medicine-A review, *Int. J. Pharmacogn*. **2**, 116-122

[14] N.T. Chung, L.T. Huong and I. A. Ogunwande (2020). Antimicrobial, larvicidal activities and composition of the leaf essential oil of *Magnolia coco* (Lour.) DC, *Rec. Nat. Prod*. **14**(**5**), 372-377.

[15] W.S. Bowers, B. Sener, P.H. Evans, F. Bingol and I. Erdogan (1995). Activity of Turkish medicinal plants against mosquitoes *Aedes aegypti* and *Anopheles gambiae*, *Int. J. Trop. Insect Sc.* **16**(**3-4**), 339-341.

[16] S.K. Nanyonga, A. Opoku, F.B. Lewu and A.O. Oyedeji (2012). Chemical composition and larvicidal activity of the essential oil of *Tarchonanthus camphoratus* against *Anopheles arabiensis* mosquito larvae, *J. Essent. Oil Bear. Plant.* **15**(**2**), 288-295.

[17] M. Goyal, L. Shinde and R. Bayas (2019). Study of chemical composition and larvicidal efficacy of secondary metabolites from aromatic phytoextracts against dengue vector: *Aedes aegypti* (Linn) (*Diptera: Culicidae*), *Int. J. Mosq. Res*. **6**(**1**), 26-33.

[18] K.E. Kabir, M.I. Choudhary, S. Ahmed and R.M. Tariq (2013). Growth-disrupting, larvicidal and neurobehavioral toxicity effects of seed extract of *Seseli diffusum* against *Aedes aegypti* (L.) (*Diptera: Culicidae*), *Ecotox. Environ, Safe*. **90**, 52-60.

[19] R.C. Saxena, S. Jayashree, S. Padma and O.P. Dixit (1994). Evaluation of growth disrupting activity of *Ageratum-conyzoides* crude extract on culex-quinquefasciatus (Diptera, Culicidae), *J. Environ. Biol*. **15**(**1**), 67-74.

[20] M.A. Hala (2011). Comparative antioxidant activity study of some edible plants used spices in Egypt, *Am. J. Sci.* **7**(**1**), 1118-1122.

[21] Jajic, T. Sarna and K. Strzalka (2015). Senescence, stress, and reactive oxygen species, *Plants (Basel)*. **4**(**3**), 393-411.

A C G
publications

© 2021 ACG Publications