RESEARCH ARTICLE

Larvicidal activity and Biochemical Effects of Apigenin against Filarial Vector *Culex quinquefasciatus*

Abhay Deep Johnson[1], Ajay Singh[2*]

[1]Research scholar, Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur, (U.P.) India

[2]Professor, Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur, (U.P.) India

[*]**Address for Correspondence:** Dr. Ajay Singh, Professor, Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur, (U.P.) India

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ABSTRACT- Mosquito-borne diseases have intruded the globe since immemorial time. The present scenario for commanding the mosquitoes is aimed at application of target and stage-specific, cost-effective and biodegradable phytoproducts. Plant extracts are safer for non-target organisms including man. Plant based formulations would be more feasible environmental products with proven potential as insecticide. Therefore, in the present study of larvicidal activity of biologically active compound Apigenin extracted from the leaf of *Jatropha gossypifolia* against the filarial vector, *Culex quinquefasciatus* was studied. Standard WHO protocols with minor modifications was adopted for the larvicidal bioassay. The active compound Apigenin extracted through ethyl alcohol solvent from the leaf of *J. gossypifolia* plant of family Euphorbiaceae was administered for 24 h or 96 h to the larvae of *C. quinquefasciatus*. Exposure of larvae over 24 h to sub-lethal doses (40% and 80% of LC_{50}) of apigenin, significantly ($P<0.05$) altered the level of total protein, total free amino acid, glycogen and activity of enzyme acetyl cholinesterase, acid and alkaline phosphatase activity in the whole body tissue of *C. quinquefasciatus* larvae. The alterations in all these biochemical parameters were significant ($P<0.05$) time and dose dependent.

Key-words- *Jatropha gossypifolia*, Euphorbiaceae, *Culex quinquefasciatus*, biochemical effects, *Wuchereria bancrofti*

INTRODUCTION

Mosquitoes transmit several public health problems, such as malaria, filariasis, and dengue causing millions of deaths every year [1]. Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water and, thus, are easy to deal with them in this habitat. The use of herbal products is one of the best alternatives for mosquito control [2]. Mosquitoes are the major vectors for the transmission of malaria, dengue fever, chikungunya, filariasis and Japanese encephalitis affecting humans and domestic animals worldwide, causing millions of deaths every year [3]. *C. quinquefasciatus* Say (Diptera: Culicidae) is a predominant house-resting mosquito in many tropical countries [4] breeding in polluted waters such as blocked drains, damaged septic tanks, or soak age pools close to human habitations. It is a pan tropical pest and urban vector of *Wuchereria bancrofti*, which causes filarial fever [5].

Synthetic pesticides are generally used for public health sprays in most parts of the world [6,7]. It's unlimited, un-interrupted and indiscriminate use as the principal agent, results in development of insecticide resistance in mosquitoes and also poses a threat to life and our environment [8-12]. Plants are rich source of alternative agents for control of mosquitoes, because they possess bioactive chemicals, which act against a number of species including specific target-insects and are eco-friendly. Plant based pesticides are less toxic, delay the development of resistance and are easily biodegradable [13]. Plant based products do not have any hazardous effect on the ecosystem. Plant's secondary metabolites and their synthetic derivatives provide an alternative source in the control of mosquitoes biodegradable. The crude extracts can be effectively used in the control of mosquitoes by replacing the chemical pesticides, which cause environmental pollutions and other burdens [14].

In the present study, the larvicidal activity of Apigenin extracted from *J. gossypifolia* leaf as well as its biochemical effects on larvae of *C. quinquefasciatus* were investigated, these extracts cannot be applied to commercial use without a study of these aspects as well and it can replace the chemical pesticides which cause environmental pollutions and other health problems [14].

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MATERIALS AND METHODS

Collection and maintenance of experimental insects: Fully fed adult females of Culicines were collected from the different residential areas of

Gorakhpur district, India. Collections were made from human dwellings with the help of an aspirator supplied by W.H.O. and kept in 30x30x30 cm cages with cotton pads soaked in 10% glucose solution and water containing enamel bowl for egg laying. Experimental conditions of water determined by the method of APHA/AWWA/WEF [15] were atmospheric temperature 30.4°±1.7°C, water temperature 27.5°±1.2°C, pH 7.4–7.6, dissolved oxygen 7.6–8.3 mg/L, free CO_2 4.2–5.2 mg/L, bicarbonate alkalinity 104.5–105.8 mg/L.

Collection of plant material: Plant *J. gossypifolia* (family: Euphorbiaceae) was collected locally from Botanical garden of Deen Dayal Upadhyay Gorakhpur University, Gorakhpur, and identified by Prof. S. K. Singh, Plant taxonomist, Department of Botany, Deen Dayal Upadhyay Gorakhpur University, Gorakhpur, Uttar Pradesh, India, where a voucher specimen was deposited (Fig. 1).

Fig. 1: *J. gossypifolia* plant

Extraction of active compounds: The Apigenin was isolated from the leaves of *J. gossypifolia* by the method of Subramanian [16]. The leaves of these plants were washed properly in tap water and the leaves were cut by scissors, then dried in shady places and finally dried in an incubator at about 35^0C temperature; dried leaves were powdered by electric Grinder. About 50 g powder of the leaves was subjected in Soxhlet extraction unit with about 250–300 mL ethyl alcohol for about 72h at 30–40^0C. In case of compound Apigenin after extraction, the aqueous layer was collected and left to stand in a cold place for 72 hours; a yellow precipitate separated out from the solution. The precipitate was filtered and washed with a mixture of chloroform: ethyl acetate: ethanol (2:1:1). The un-dissolved part of the precipitate was dissolved in hot methanol and filtered, the filtrate was evaporated to dryness to give 280 mg yellow powder of Apigenin. Confirmation of the compound (Fig 2) was made through IR and R_f value data of Dordevice [17] and was also confirmed by comparing it with the authentic sample obtained from Sigma Chemical Company, USA.

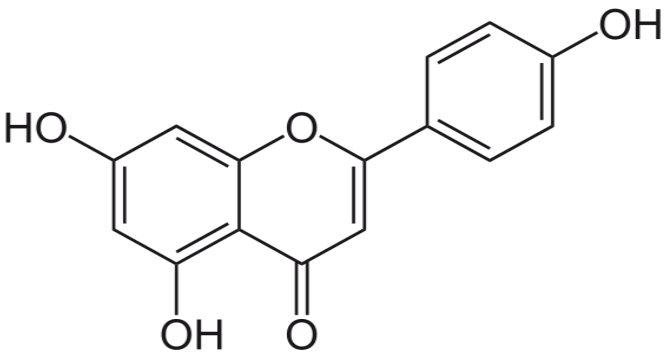

Fig. 2: Chemical structure of Apigenin (Source: https://en.wikipedia.org/wiki/Apigenin)

Toxicological experiment: Toxicity experiment was performed by using the method of W.H.O. [18]. Twenty late, third instar larvae of *C. quinquefasciatus* mosquito was exposed to four different concentrations of apigenin. Doses were maintained in 500 ml of de-chlorinated tap water in glass beakers (15 cm in diameter and 7.5 cm in height) containing twenty mosquito larvae in each test concentration. Six replicates were maintained for each concentration. Control larvae were kept in similar conditions without treatment. *Culex* larvae were exposed for 24 h to 96 h at four different concentrations (70 mg, 80 mg, 90 mg and, 100 mg) of apigenin. Mortality was recorded after every 24 h up to 96 h exposure periods. LC values, upper and lower confidence limits, slope value, t-ratio and heterogeneity were calculated by the POLO computer programme of Robertson [19].

Biochemical experiment: The late third instar larvae were treated with 40% and 80% of 24 h LC_{50} of Apigenin obtained from *J. gossypifolia* leaf for 24 h. Six beakers were set up for each dose and each beaker contained 50 larvae in 1L de-chlorinated tap water. The LC_{50} value of Apigenin was 93.11 mg/L for 24 h against *C. quinquefasciatus* larvae. 40% and 80% of 24 h, LC_{50} of ethyl alcohol extract was selected as sub-lethal dose to analyze its time and dose dependent effects in this present study and at that dose there was no mortality were observed in the treated larvae. After the stipulated time (24h), the dead larvae were removed from the beaker and washed with water and the whole body tissue stored in deep freezer for biochemical analysis. Control larvae were held in the same condition without any treatment. Each experiment was replicated six times and the values are expressed as mean±SE of six replicates. Student's 't' test was applied to locate significant changes with controls[20,21].

Total protein: Total protein level was estimated by the method of Lowry [22]. Homogenates (10 mg/mL) was prepared in 10% tri-chloroacetic acid (TCA). Bovine serum albumin was used as a standard.

Total free amino acids: Total free amino acid level was estimated by the method of spies [23]. Homogenates (10 mg/mL) were prepared in 95% ethanol. Glycine was used as a standard.

Glycogen: Glycogen level was estimated by the method of Van der Vies [24]. Homogenate (10 mg/mL) was prepared in 5% TCA. Glucose was used as a standard.

Acetylcholinesterase activity: Acetylcholinesterase activity was measured by the method of Ellman [25]. Homogenate (50 mg/ml, w/v) was prepared in 0.1 M-phosphate buffer, pH 8.0 for 5 min in an ice bath. The change in optical density at 412 nm caused by the enzymatic reaction was monitored for 3 min at 25°C.

Acid and alkaline phosphatase activity: Acid and alkaline phosphatase activity was determined by the method of Andersch and Szcypinski [26]. Homogenates (2% w/v) were prepared in ice-cold 0.9% NaCl solution and centrifuged at 5000 xg at 0°C for 15 min.

Statistical analysis: Each experiment was replicated at least six times and data has expressed as mean ±SE. Student's t-test as applied for locating significant differences Sokal and Rohlf [20].

RESULTS AND DISCUSSION

In the present study exposure to the apigenin extracted from *J. gossypifolia* leaf caused significant behavioural changes in the larvae of mosquito *C. quinquefasciatus.* Behavioural changes appear after 4–5 hours of exposure. Larvae were incapable of rising to the surface shown restlessness, loss of equilibrium, lethargic and finally death. No such behavioural symptoms and mortality occurred in the control groups, indicating that the plant moieties were actual factors responsible for altered behavior and larval mortality.

Percent mortality produced by apigenin for the periods ranging from 24 to 96 h is shown in Table 1. The toxicity of ethyl alcohol extract was time and dose dependent for *C. quinquefasciatus* larvae. The LC_{50} values of apigenin are shows in Table 1. There was a significant negative correlation between LC values and exposure periods. i.e. LC_{50} values of ethyl alcohol extract of *J. gossypifolia* leaf decreased from 93.11 mg/L (24 h)> 86.26 mg/L (48 h)> 77.81 mg/L (72 h)> 71.92 mg/L (96 h) in case of *C. quinquefasciatus* larvae (Table 1).

Table 1: Toxicity (LC values) of different concentrations of Apigenin extracted from leaf of *J. gossypifolia* plant against *C. quinquefasciatus* larvae at 24 h to 96 h exposure period

Exposure Period (hours)	Effective dose (mg/L)	Limits (mg/L)		Slope value	't' ratio	Heterogeneity
		LCL	UCL			
24	LC_{10}= 67.06	37.75	75.83	8.99±6.13	2.84	0.02
	LC_{50}=93.11	85.33	117.43			
	LC_{90}=129.29	108.01	324.75			
48	LC_{10}=64.02	40.26	72.49	9.90±5.98	3.20	0.09
	LC_{50}=86.26	78.80	96.33			
	LC_{90}=116.21	101.52	194.46			
72	LC_{10}=57.83	31.76	67.28	9.95±6.03	3.16	0.01
	LC_{50}=77.81	66.41	84.29			
	LC_{90}=104.69	93.76	156.36			
96	LC_{10}=52.92	21.15	63.79	9.62±6.35	2.90	0.07
	LC_{50}=71.92	53.24	78.63			
	LC_{90}=97.74	88.32	147.03			

Batches of twenty mosquito larvae were exposed to four different concentrations of the extract.
Concentrations given are the final concentration (w/v) in the glass beaker containing de-chlorinated tap water. Each set of experiment was replicated six times.
Mortality was recorded after every 24h.
Regression coefficient showed that there was significant (P<0.05) negative correlation between exposure time and different LC values.
LCL: Lower confidence limit; UCL: Upper confidence limit.
There was no mortality recorded in the control group.

After exposure to sub-lethal doses of 40% and 80% of LC_{50} of apigenin extracted from *J. gossypifolia* leaf for 24h or 96h caused significant (P<0.05) alterations in total protein, total free amino acids and glycogen metabolism in the whole body tissue of the larvae of *C. quinquefasciatus* (Table 2). Total protein and glycogen levels were significantly reduced, while free amino acid level was significantly enhanced after the exposure to sub-lethal doses. Total protein levels were reduced to 92% of control after exposure to (24h) of apigenin extracted from *J. gossypifolia* leaf. The maximum decrease in protein level (86% of control) was observed in larvae treated with 80% of LC_{50} (24h). Total free amino acid levels were induced to 104% of controls after treatment with 40% of LC_{50} (24h) and maximum increase in total free amino acid level (110% of control) was observed in larvae treated with 80% of LC_{50} (24 h) of the ethyl alcohol extract of *J. gossypifolia* leaf and the

glycogen level was reduced up to 83% and 76% respectively (Table 2).

Table 2: Changes in total protein, glycogen and total free amino acid activity in whole body tissue of *C. quinquefasciatus* larvae after 24 h exposure to sub-lethal doses (40% and 80% of LC_{50} of 24 h) of active compound Apigenin extracted from leaf of *J. gossypifolia* plant

Parameters	Control	40% of LC_{50} (+, £) (37.24 mg/L, 24h LC_{50})	80% of LC_{50} (+, £) (74.49 mg/L, 24h LC_{50})
		24h	
Protein	1.90±0.003 (100)	1.75±0.003 (92)	1.64±0.004 (86)
		24h	
Glycogen	1.20±0.003 (100)	1.00±0.004 (83)	0.91±0.006 (76)
		24h	
Amino acid	0.50±0.005 (100)	0.52±0.004 (104)	0.55±0.004 (110)

Values are mean ±SE of six replicates.
Values in brackets indicate percent biochemical activity with control taken as 100%.
Doses are 40% and 80% of LC_{50} for period for which animals were exposed.
+, significant (P<0.05) when two way variance was applied to see whether protein, glycogen and amino acid alterations was time and dose dependent.
£, significant (P<0.05) when Student 't' test was applied between control and treated groups.

Table 3 was clearly shown that sub-lethal exposure of apigenin at 40% and 80% of LC_{50} the AChE activity decreases 82% , 71% at 24h with respect to control but at 96h exposure the AChE activity also decreases as 78%, 64% at 40% and 80% of LC_{50} respectively with respect to control.

According to Table 3 at sub-lethal treatment of apigenin of 40% and 80% of LC_{50} (24 h), acid phosphatase activity decreases by 88% to 82% respectively with respect to control. At longer duration (96h) exposure, 40% and 80% of LC_{50} of apigenin also decreases the activity of acid phosphatase by 73%, 71% respectively with respect to control. In the case of enzyme alkaline phosphatase, exposure of 40%, 80% LC_{50} of apigenin also decreases the enzyme activity by 90% to 80% and 71% to 60% at 24h or 96h respectively with respect to control (Table 3).

Table 3: Changes in acetylcholinesterase, acid and alkaline phosphatase activity in whole body tissue of *C. quinquefasciatus* larvae after 24 h or 96 h exposure to sub-lethal doses (40% and 80% of LC_{50} of 24 h) of active compound Apigenin extracted from leaf of *J. gossypifolia* plant

Parameters	Control	40% of LC_{50} (+, £) (37.24 mg/L, 24 h LC_{50})	80% of LC_{50} (+, £) (74.49 mg/L, 24 h LC_{50})
	AChE activity (µm SH hydrolyzed/min/mg protein		
		24h	
AChE	0.072±0.006 (100)	0.059±0.004 (82)	0.051±0.004 (71)
		96h	
	0.072±0.006 (100)	0.056±0.004 (78)	0.046±0.004 (64)
	µm *p*-nitrophenol formed/30 min/mg protein		
		24h	
	0.170±0.003 (100)	0.150±0.003 (88)	0.140±0.003 (82)
Acid phosphatase		**96h**	
	0.180±0.003 (100)	0.132±0.004 (73)	0.127±0.003 (71)
	µm *p*-nitrophenol formed/30 min/mg protein		
Alkaline phosphatase		**24h**	
	0.400±0.004 (100)	0.360±0.003 (90)	0.320±0.004 (80)
		96h	
	0.380±0.004 (100)	0.270±0.004 (71)	0.228±0.004 (60)

Values are mean ±SE of six replicates.
Values in brackets indicate percent biochemical activity with control taken as 100%.
Doses are 40% and 80% of LC_{50} for period for which animals were exposed.
+, significant (P<0.05) when two way variance was applied to see whether enzyme inhibition was time and dose dependent.
£, significant (P<0.05) when Student 't' test was applied between control and treated groups.

Statistical analysis of the data on toxicity brings out several important points. The X^2 test for goodness of fit (heterogeneity) demonstrated that the mortality counts were not found to be significantly heterogeneous and other variables, e.g. resistance etc. do not significantly affect the LC_{50} values, as these were found to lie within the 95% confidence limits. The dose mortality graphs exhibit steep values. The steepness of the slope line indicates that there is a large increase in the mortality of the larvae of *C. quinquefasciatus* with relatively small increase in the concentration of the toxicant. The slope is, thus an index of the susceptibility of the target animal to the plant origin pesticides used.

The mosquito larval control using larvicidal agents is a major component in the control of vector borne diseases. Plant as potential larvicides is considered as viable and preferred alternative in the control of the mosquito

species at the community level. A large number of plant extracts have been reported to have mosquitocidal or repellent activities against mosquito vectors, but few plant products have shown practical utility for mosquito control [27].

In the present study the apigenin extracted with ethyl alcohol from *J. gossypifolia* leaf has potent larvicidal activity of *C. quinquefasciatus* mosquitoes. Exposure to sub-lethal doses of compound apigenin of *J. gossypifolia* leaf against larvae of *C. quinquefasciatus* significantly altered the level of total protein, total free amino acid, glycogen and enzyme activity of acetyl cholinesterase, acid and alkaline phosphatase activity. Significant exceptional changes as given in result section of *C. quinquefasciatus* larvae like ecdysial failure, abnormalities during intermediate stages, prolongation of the life span of treated instars, emergence of adultoids after treatment with ethyl alcohol extract of *J. gossypifolia* leaf may be due to the effect of active moiety present in the plant extract. The effect of compound depends on the synthesis or release of ecdysone and in the absence of it, the insect lapses into a state of developmental stand still [28]. It resulted in to ecdysial failure. The male and female emerged from treated groups were unable to feed on sugar solution as well as on mammal blood ultimately they died sooner. Laboratory observations revealed that, their mouth parts were undeveloped, legs were paralysed and the females were incapable of egg laying after treatment, eventually they died sooner.

Carbohydrates are the primary and immediate source while the protein acts as the next alternative source of energy to meet the increase energy demand. The depletion of the protein fraction in treated mosquito larvae of *C. quinquefasciatus* may have been due to their degradation and the possible utilization for metabolic purposes. The protein content depends on the rate of protein synthesis and its depletion might have been due to their degradation and possible utilization for metabolic purposes. The quantity of protein may also be affected due to impaired incorporation of amino acids into polypeptide chains [29]. The decreased protein content attributed to the destruction or necrosis of cells and consequent impairment in protein synthesis machinery [30]. The total free amino acids content showed a significant increase in whole body tissue of mosquito larvae exposed to sub-lethal doses of ethyl alcohol extract of *J. gossypifolia* leaf. The augmentation in total free amino acids level in the whole body tissue suggests high proteolytic activity. The accumulation of free amino acids can also be attributed to lesser use of amino acids [31] and their involvement in the maintenance of an acid base balance [32]. Another possibility for enhancement of free amino acid level might be due to transamination and amination to keto acids. Stress conditions induce elevation in the transamination pathway [33]. The transamination reaction is probably the most important pathway in the metabolism of many amino acids [34]. In stress condition, carbohydrate reserve depleted to meet energy demand. In the present study, the diminished glycogen content in body tissues of *Culex* larvae indicates its rapid utilization for energy generation; a demand caused by rutin extracted from *J. gossypifolia* leaf as a consequence toxic stress during the experiment.

Finally, glycogenolysis seems to be the result of increased secretion of catecholamine due to stress of plant extract treatment [35]. Larvae also secrete catecholamine in excess amount, during stress, which depletes glycogen reserves [36]. Anaerobic and aerobic segments are two important components of carbohydrate metabolism. In first case, the breakdown of glucose or glycogen through the Embden-Meyerhof pathway (glycolysis) takes place while the next one consists oxidation of pyruvate to acetyl co-A to be utilized through the citric acid cycle [37].

The effect of toxicants on enzymatic activity is one of the most important biochemical parameters, which Affect physiology of the body. When an organ is diseased due to the effect of a toxicant, enzyme activity appears to be increased or it may be inhibited due to the active site being either denature or destroyed. Acetylcholinesterase, or acetyl-hydrolase, is a serine protease that hydrolyses the neurotransmitter acetylcholine. AChE found mainly at neuromuscular junctions and brain synapse, where its activity serves to terminate synaptic transmission. It belongs to the carboxyl esterase family of enzymes.

Enzyme alkaline phosphatase plays an important role in animal metabolism. Vorbrodt [38] has reported that the role of this enzyme is the transport of metabolites across the membrane. The enzyme has been shown to be intimately associated with protein synthesis and is thus involved in the synthesis of certain enzymes [39]. Acid phosphatase is the lysosomal enzyme and plays an important role in catabolism, pathological necrosis, autolysis and phagocytosis [40].

CONCLUSIONS

In conclusion, the larvicidal activity of the apigenin extracted through ethyl alcohol from *J. gossypifolia* leaf is highly toxic to larvae of *C. quinquefasciatus* mosquito. This extract significantly suppresses the population build up of the mosquito by morphogenetic action on insects. Sub-lethal doses of ethyl alcohol extract significantly alter the protein, amino acids, glycogen, enzyme activity like acetylecholinesterase, acid, and alkaline phosphatase activity of *Culex* larvae. We therefore believe that the plant extracts may eventually be of great value for the control of *C. quinquefasciatus* mosquitoes on aquatic stage.

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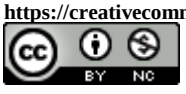

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