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Research Article

INSECTICIDAL AND GENOTOXIC POTENTIAL OF *ACORUS CALAMUS* RHIZOME EXTRACT AGAINST *DROSOPHILA MELANOGASTER*

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ABSTRACT

Objectives: Insect pest problems in agriculture have a considerable shift during first decade of 21st century due to global climate changes. Various synthetic insecticides were used from last 50 years to overcome the pest problem, but major concerns in using these pesticides were: Public health and pesticide resistance in pests. To abolish the catastrophic effect of synthetic insecticides, urgent need to develop new drugs for pest control. In this study we evaluate the insecticidal and genotoxic activity of ethanolic extract of *Acorus calamus* rhizome against *Drosophila melanogaster.*

Methods: Different concentration of ethanolic extracts of *A. calamus*, fed to different developmental stages of *D. melanogaster* to determine insecticidal activity and genotoxicity; genotoxicity was determined by the comet assay in male flies.

Results: Lethal concentration 50 (LC_{50}) values of ethanolic extract against larvae, adult males and females of *D. melanogaster* were 109.54, 52.51 and 41.11 mg/L respectively. Genotoxicity of adult's flies were determined at 30 and 55 mg/L ethanolic extract of *A. calamus*, The mean comet tail length was 4.24±0.653 µm and 6.13±0.721 µm and the respective DNA damage was 5.1% and 7.3% with reference to controls.

Conclusion: The present finding suggests that ethanolic extract of *A. calamus* rhizome showed higher insecticidal potential in adults than larvae of *D. melanogaster*. Results from genotoxicity studies further support the insecticidal activity against adult stages of *D. melanogaster*. The study reflects ethanolic extract of *A. calamus* could be used as an alternative pest control negotiator for minimizing the noxious effects of pesticides in the environment.

Keywords: Insecticidal activity, Genotoxicity, Ethanolic extract, Botanical pesticides, *Acorus calamus*, *Drosophila melanogaster.*

INTRODUCTION

Synthetic insecticides have been used extensively over the past 50 years globally to the routine control of insect and plant pests. The emergence of detrimental side-effects of synthetic insecticides was occurred to human beings and pet animals due to extensive use in past decades [1,2]. The use of approximately 3 billion kg of pesticides throughout the world [3], results in more than 26 million cases of non-fatal pesticide poisonings in humans [4]. Among these, about 3 million cases are hospitalized and approximately 220,000 people cases lead to death [4]. These major types of chronic illness caused by pesticides include neurological effects, respiratory problem, reproductive problem, and cancer [5]. Moreover due to various reasons such as development of genetic resistance [6,7], toxicity to non-target organisms [1], residual toxic effect, high cost, environmental pollutant [8] and handling hazards [2] has generated worldwide interest in the development of alternative strategies. These include the use of new types of insecticides derived from traditional botanical pest control agents, which are less expensive [9] and comparatively safer to mammals and higher animals [10]. The identification of botanical derivatives and their effects on insect physiology and behavior is essential for the development of an alternative bioenvironmental control strategy. *Acorus calamus* Linn. commonly known as Sweet Flag and Vach (in India), was being used as ant diabetic [11], immune suppressive [12], antioxidant [13], antifungal [14], antibacterial [15], insecticidal agent [16] and to cure central nervous system abnormalities [17]; The reported bioactivity of *A. calamus*, develop the interest of researcher in this plant. Therefore, present investigation demonstrate the insecticidal activity and genotoxicity of ethanolic extract of *A. calamus* (rhizome) against *Drosophila melanogaster* (earlier described as best model for the insecticidal activity and genetic study [18,19]) in order to develop an environment-friendly control measure against insects.

METHODS

Plant collection and identification

Plant with rhizomes of *A. calamus* Linn. (Family: Araceae) collected from Dehradun region and further identified by Botanical Survey of India, Dehradun, India. Voucher specimen of the plant was stored in the institute herbarium (voucher specimen number MITSGWL-131AC) for future reference.

Preparation of plant extract

The rhizomes were washed with distilled water, dried under shade and grind to fine powder. Rhizome powder (100 g) was submerged with ethanol (400 ml) and agitated for 8 hrs at room temperature and filtered with Whatman No.1 filter paper. The filtrate was dried in rotary evaporator and stored at 4°C until used.

Test organisms and culture

Culture of *D. melanogaster* obtained from the Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, India, was used as test organisms. The culture was maintained on an artificial diet in glass culture tubes at a temperature 22-25°C, 60-95% relative humidity and a photoperiod of 16:8 (light:dark) hrs. Ingredients used for the preparation of diet were: 450 ml distilled water, 2.5 g agar, 17 g corn flour, 15 g sugar, 3 g yeast powder, 1 g nepagin (methyl-4-hydroxybenzoate), 1 ml propionic acid and 1 ml 70% ethanol.

Lethal concentration 50 (LC_{50}) value

For determination of LC_{50} value; 30 larvae (3rd instar) and 2 days old 30 adult flies (both males and females taken separately) of *D. melanogaster* ware feed to various concentrations of ethanolic extract of *A. calamus* rhizomes along with diet. After 24 hrs feeding, mortality was recorded and evaluates the LC_{50} by log-probit method.

Genotoxicity determination by comet assay

The DNA damage studies were carried out using single cell gel electrophoresis, commonly called as comet assay. The procedure (Alkali method) was followed as described by Singh *et al.* 1988 [18] with minor modifications as described below. Comet slide were prepared by using 30 male flies fed with sublethal (30 mg/L) and lethal (55 mg/L) concentration of ethanolic extract of *A. calmus* as well as control flies for 24 hrs in culture tube at standard condition (temperature 22-25°C, 60-95% relative humidity). After 24 hrs flies were homogenized in homogenizing buffer (0.075 M NaCl and 0.024 M ethylenediaminetetraacetic acid [EDTA]). Homogenate was centrifuged at 1000 rpm for 10 minutes and pellet was gently resuspended in 1 ml of chilled homogenizing buffer to nuclei preparation. Then frozen microscopic slide was placed horizontally and casted a homogenous thin layer of 1% normal melting agarose, isolated nuclei and 1% low melting agarose ((1:4), 100 µl) was mixed and casted on the pre-coated slides and kept at 4°C for 20 minutes. The slides were immersed into the freshly prepared chilled lysis buffer (2.5M NaCl, 100 mM EDTA pH 10, 5% dimethylformamide, 1% and triton - ×100) for 1 hr in dark at 4°C. After complete lysis the slides were placed for 20 minutes in ice cold electrophoresis chamber containing alkaline electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH>13) for unwinding of DNA strands, the process was subsequently conducted for 20 minutes at 25 V/300 mA. The slides were washed thrice with neutralizing buffer (0.4 M Tris pH 7.5) for 5 minutes and just before visualization. Slides were then stained with ethidium bromide (10 µg/ml, 40 µl/slide) for 10 minutes in the dark. They were dipped once in chilled distilled water to remove excess ethidium bromide and subsequently coverslips were placed over them. The slides were stored in a dark, humidified chamber and analyzed within 3-4 hrs.

Comet capture and analysis

A total of 100 cells from each slide were analyzed by image analysis using fluorescence microscope (Leica DM4000B) with an excitation filter of 515-560 nm and a barrier filter of 590 nm using ×40 objectives. The photographs of the individual cells were taken by Leica Digital DFC 320R-II camera. Comet tail length and percentage of fragmented DNA in tail were measured with an Image Analysis system (Leica Qwin) and comet score software version 1.5 (TriTek Corporation, Sumerduck, VA).

Data analysis

The results were recorded on standard excel spreadsheets. LC_{50} values were analyzed by log-probit method [19]. Statistical analysis was done by comparing means t-test and one-way analysis of variance using Statplus 2009 software.

RESULTS

Insecticidal activity

Dose-response relationship of ethanolic extract of *A. calamus* rhizome was evaluated in both larval (3rd instar) and adult (male and female) stages of *D. melanogaster*.

In the larvae of *D. melanogaster*, tested dose range for ethanolic extract of *A. calamus* rhizome were 50-125 mg/L and the recorded mortality ranges 10-62.22% (Fig. 1) whereas the calculated LC_{50} value was 109.54 mg/L. In the different dose ranges, ethanolic extract of *A. calamus* showed significant mortality of larvae of *D. melanogaster* compared to the control (overall analysis of variance significance, F=81.05; df=10,4; p<0.001) (Table 1).

In case of male *D. melanogaster*, tested dose range for *A. calamus* rhizome ethanolic extract was 25-100 mg/L with 17.78-91.11% mortality (Fig. 2), whereas the calculated LC_{50} value was 52.51 mg/L. In the different dose ranges, ethanolic extract of *A. calamus* recorded significant mortality of males *D. melanogaster* compared to control (overall analysis of variance significance, F=152.67; df=10,4; p<0.001) (Table 2).

In female *D. melanogaster*, tested dose range for *A. calamus* rhizome ethanolic extract was 25-100 mg/L with 26.67-100% mortality (Fig. 3), whereas the LC_{50} value was 41.11 mg/L. In the different dose ranges, ethanolic extract of *A. calamus* showed significant mortality of males *D. melanogaster* compared to the control (overall analysis of variance significance, F=517.23; df=10,4; p<0.001) (Table 3).

Genotoxicity

The effect of ethanolic extract *A. calamus* rhizome on DNA damage in individual cells of adult male *D. melanogaster* was assessed by two distinct types of DNA damage measurements: The length of DNA comet tail and the percentage of fragmented DNA present in the tail after electrophoresis. The DNA damage was assumed by using the comet assay method in adult *D. melanogaster* fed with sublethal (30 mg/L) and lethal (55 mg/L) as well as control concentrations of ethanolic extract of A. calmus for 24 hrs. The observation showed that DNA damage was significant i.e., 5.1% and 7.3% at 30 mg/L and 55 mg/L concentrations of ethanolic extract in comparison to controls (Fig. 4). The comet tail length increases with increase in concentration of ethanolic extract *A. calamus* rhizome, the tail length was 4.24±0.653 µm and 6.13±0.721 µm at 30 mg/L and 55 mg/L concentration respectively with reference to control (Fig. 4). The relative information produced by comet assay is suggested that the ethanolic extract *A. calamus* rhizome inducing the targeted genotoxicity in *D. melanogaster*.

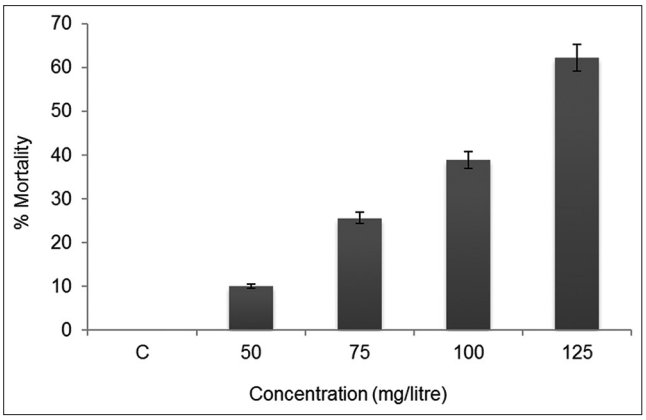

Fig. 1: Mortality rate of *Drosophila melanogaster* larvae feed at different concentration of ethanolic extract of *Acorus calamus* rhizome

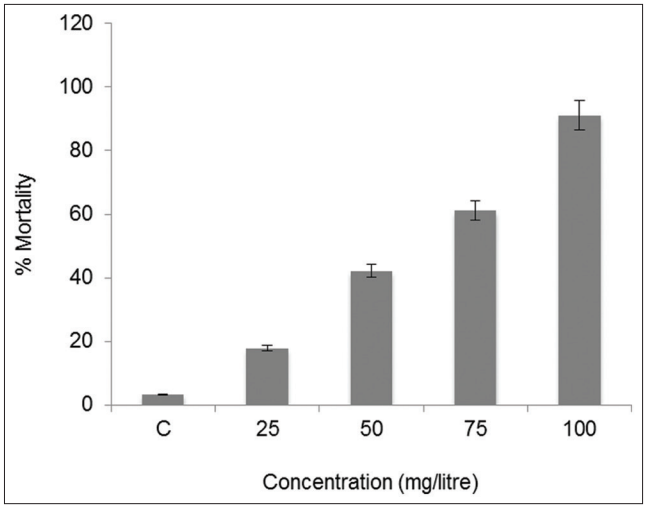

Fig. 2: Mortality rate of male *Drosophila melanogaster* feed at different concentration of ethanolic extract of *Acorus calamus* rhizome

Table 1: The effect of *A. calamus* ethanol extract against larvae of *D. melanogaster* at different concentrations in the diet

Group	Concentration (mg/L)	% Mortality	LC_{50} (mg/L)	t-test	df[b]	ANOVA	df[b]
1	C[a]	0	109.54	-	4	81.05*	10,4
2	50	10		5.19*			
3	75	25.6		8.69*			
4	100	38.89		13.22*			
5	125	62.22		15.53*			

*Significant values with respect to control at p<0.001; [a]Control; [b]Degree of freedom (10-vertical, 4-horizontal) in t-table, *A. calamus: Acorus calamus, D. melanogaster: Drosophila melanogaster*, LC_{50}: Lethal concentration 50

Table 2: The effect of *A. calamus* ethanol extract against adult males of *D. melanogaster* at different concentrations in the diet

Group	Conccentration (mg/L)	% Mortality	LC_{50} (mg/L)	t-test	df[b]	ANOVA	df[b]
1	C[a]	0	52.51	-	4	152.67*	10,4
2	25	17.78		16.00*			
3	50	42.2		10.53*			
4	75	61.11		20.78*			
5	100	91.11		22.74*			

*Significant values with respect to control at p<0.001. [a]Control, [b]Degree of freedom (10-vertical, 4-horizontal) in t-table. *A. calamus: Acorus calamus, D. melanogaster: Drosophila melanogaster*, LC_{50}: Lethal concentration 50

Table 3: The effect of *A. calamus* extract against adult females of *D. melanogaster* at different concentrations in the diet

Group	Concentration (mg/L)	% Mortality	LC_{50}	t-test	df[b]	ANOVA	df[b]
1	C[a]	0	41.11	-	4	517.23*	10,4
2	25	26.67		8.00*			
3	50	48.89		44.00*			
4	75	83.33		43.30*			
5	100	100		ND[c]			

*Significant values with respect to control at p<0.001. [a]Control, [b]Degree of freedom (10-vertical, 4-horizontal) in t-table, [c]Not determined. *A. calamus: Acorus calamus, D. melanogaster: Drosophila melanogaster*, LC_{50}: Lethal concentration 50

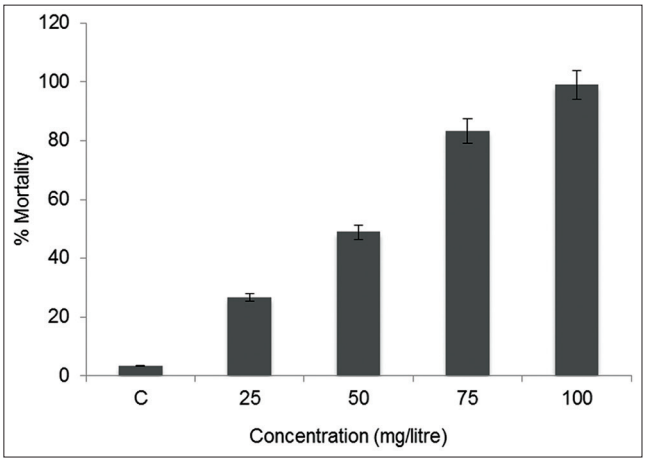

Fig. 3: Mortality rate of female *Drosophila melanogaster* feed at different concentration of ethanolic extract of *Acorus calamus* rhizome

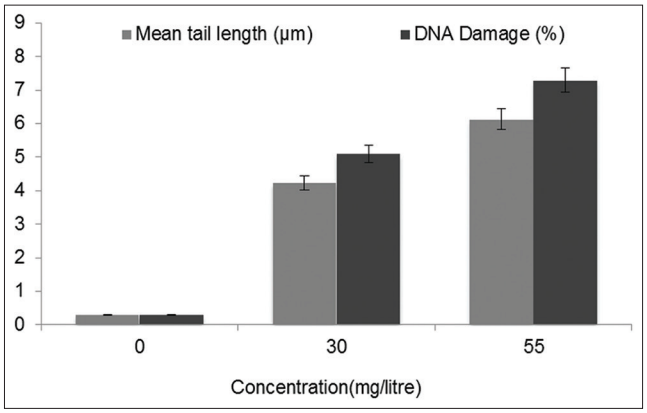

Fig. 4: Effect of ethanolic extract of *Acorus calamus* rhizome on comet tail length and percent DNA damage against male *Drosophila melanogaster*

DISCUSSION

Synthetic insecticides have become a major health problem due to their persistent in the environment while plant-based insecticides were biodegradable and act as natural insecticide with less or no side-effect. There has recently been an interest increasing in the insecticidal activity of plant extracts. Rajkumar and Jebanesan [20] demonstrated that ethanolic extract of *Cassia obtusifolia* (leaf) have larvicidal activity against late third instar larvae of *Anopheles stephensi*, with LC_{50} and LC_{90} values were 52.2 and 108.7 mg/L, respectively. Kumar *et al.* 2010 [21] reported the significant larvicidal activity of ethanol extract of dried fruits of three species of pepper corns: Long pepper (*Piper longum* L.), black pepper (*Piper nigrum*), and white pepper (*P. nigrum*) against *Aedes aegypti*. The LC_{50} values of *P. longum*, white *P. nigrum* and black *P. nigrum* against early fourth instar larvae have 0.248, 0.356, and 0.405 mg/L, respectively, and the LC_{90} values are 0.605, 0.758, and 0.801 mg/L, respectively. Whereas, LC_{50} values against third instar larvae have 0.022, 0.015, and 0.016 mg/L and the LC_{90} values are 0.054, 0.034, and 0.046 mg/L, respectively.

In this study, LC_{50} values of ethanolic extract of *A. calamus* rhizome against *D. melanogaster* larvae and adults (male and female) were 109.54, 52.51 and 41.11 mg/L respectively. The insecticidal activity of naturally occurring compound nootkatone extracted from methanolic extract of *Alpinia oxyphylla*, have LC_{50} value of 11.5 mMol/L of diet against larvae of *D. melanogaster* and LD_{50} value 96 µg/adult against adults *D. melanogaster* [22]. The insecticidal activity of methanolic

extract of rhizome of four alkaloids of *Nuphar joponicum* DC. against *D. melanogaster* larva in which two alkaloids showed potent activity with their LC_{50} values 1.0 mg/L and 4.33 mg/L respectively. For adult one compound with its LD_{50} of 0.86 µg/adult showed potent activity [23].

The study revealed that the ethanolic extract of *A. calmus* rhizome showed noxious effect in both the developmental stages (larva and adult) of *D. melanogaster*. The observed insecticidal activity was more in adult than the larvae of *D. melanogaster*. It was also observed that the adult females were more susceptible than males to an ethanolic extract of *A. calamus*. The results can be compared with other studies on *A. calamus*, which showed significant insecticidal activity against various insects [24-27].

The study on genotoxicity, DNA damage was observed in adult flies with comet tail length 4.24±0.653 µm, 6.13±0.721 µm and DNA damage 5.1%, 7.3% in comparison to controls at 30 mg/L and 55 mg/L, respectively. The comet assay outcomes revealed that mortality in the insect model caused by DNA damage. A similar study was carried out by Oberholster *et al.* 2009 [28]; DNA fragmentation was observed by well-known cyanobacterial secondary metabolite (microcystin-LR) against insects of *Periplaneta americana* (American cockroach), *Tenebrio molitar* (yellow mealworm), *Gryllusbi maculatus* (common cricket) and crustacean zooplankton (*Artemia salina*) within the first 48 hrs after exposure. Dua *et al.* 2013 [29] also reported the insecticidal and genotoxic activity of essential oil of *Psoralea corylifolia* Linn. against *Culex quinquefasciatus* with LC_{50} and LC_{90} values (for larvae) of 63.38±6.30 and 99.02±16.63 ppm and LD_{50} and LD_{90} values (for adult) of 0.057±0.007 and 0.109±0.014 mg/cm^2 respectively. The mean comet tail length was 6.2548±0.754 µm and 8.47±0.931 µm and the respective DNA damage have 6.713% and 8.864% in comparison to controls at 0.034 and 0.069 mg/cm^2 of essential oil of *P. corylifolia* Linn.

The results of this study indicate that the ethanolic extract of *A. calamus* could be used as environmentally safe and eco-friendly pest control agent. Ethanolic extract of *A. calamus* can be used to replace the use of synthetic insecticide and minimizing the noxious effects of insecticides in the environment. Furthermore the study could draw the attention of researchers for confirmation of insecticidal screening using genotoxicity assays.

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