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Genotoxicity screening of *Catharanthus roseus* L extracts in *Drosophila melanogaster* M (Diptera: Drosophilidae)

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Abstract

Alcoholic extract of the plant *Catharanthus roseus* L. is used in many traditional medicines to treat various diseases in many developing countries. Be that as it may, the symptoms and safety measures are not well studied and assessed. The present study aims to investigate major phytochemical constituents and genotoxic effect of the alcoholic extracts of *C. roseus*. The results showed that the exposure of *C. roseus* extract brought about considerable changes in protein profile of *Drosophila melanogaster* in both the doses such 1.5 & 0.1875 mg/ml. In lower doses (0.1875 mg/ml) five new proteins with apparent molecular weights 109, 45, 36, 28 and 11kDa were expressed. Disappearance many proteins especially higher molecular group and lower molecular group were recorded in lower dose. Hence, the plant extract should be evaluated for its long term human health hazards and wellbeing completely before it could be used for therapeutic medicinal interventions.

Keywords: Phytochemical screening, genotoxicity, alcoholic extract, *Catharanthus roseus*, *Drosophila melanogaster*

Introduction

Treatment of different illnesses utilizing medicinal plants has been followed from ancient times. This dependence on medicinal plants has been extensively expanded among different developing nations in view of its beneficial effects [1, 2]. However, there is very little report of the best possible assessments of the toxicity of these medicinal plants. Subsequently, proper phytochemical screening of the plant is essential since plants can synthesize lethal substances to secure themselves against insects, microbes and other organisms which feed on them. The toxic effects of these plants are due to different classes of bioactive chemical compounds like alkaloids, esters, cardiac glycosides, cyanogenic glycosides and lectins. Past studies had reported the instances of acute poisoning of patients admitted to hospitals and resulted into death chiefly because of ingestion of medicinal plants with toxic substances [3, 4, 5]. Recent studies have likewise uncovered the presence of genotoxic, carcinogenic and mutagenic compounds in many plants used in traditional medicines both *in vitro* and *in vivo* assays [2, 6, 7].

India is famous for its glorious traditional ayurvedic therapeutic practice from BC 572. *Catharanthus roseus* (L) (Madagascar periwinkle; *Vinca rosea*; *Lochnera rosea*) native to the Caribbean basin is a well-known medically important plant especially in cancer treatment and as an anti-diabetic drug. The traditional herbal medical practitioners of India have been depicted the medicinal effects of several indigenous plants for different diseases. The genetic material damage may prompt mutagenesis, carcinogenesis and other toxic effects. Hence assessing the potential genotoxicity of the traditional medicines is the foremost step in drug discovery [8, 9, 10]. *Vinca* alkaloids are cytotoxic and act on target sites of the cell division, thus preventing mitosis. Despite the fact that, for an ordinary individual this activity of *vinca* can be dangerous however they are helpful agents in stopping the division of different cancerous cells. Recent studies showed that, *C. roseus* extracts are effective in the treatment of various kinds of skin cancer leukemia, breast cancer, lymph cancer, vascular dementia, alzheimer's disease and Hodgkin's disease. The two classes of active compounds of *vinca* are alkaloids and tannins. The major alkaloid vincamine and its semi synthetic derivative ethyl-apovincamate are widely used as medical agents for vasodialating, blood thinning, hypoglycemic and memory enhancing actions.

Stress protein has been proposed as sensitive indicators of lethal exposure to contaminants in environment. They are synthesized at higher levels.

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When the cells are challenged with certain environmental stimulus such as temperature and toxic chemicals cause the induction of stress protein genes, the product of which function in new protein folding and in long effect it cause a phenotypic change [11, 12]. Several stress proteins have been shown to be induced by wide variety of stressors like hyperthermia, heavy metals etc. Stress proteins are placed in to groups or families based on their relative in number and DNA sequence; like high molecular weight stress protein(HMWP), stress protein 70(SP-70), chaperones(60), stress protein-42(sp-42) and low molecular weight stress proteins/metallothioneins (MT).

Over the last few decades, issues of animal use in toxicology research and genotoxicity testing have become one of the basic concerns for both science and ethics. The use of alternatives to mammals in research and education was strongly recommended from different corners of by country to reduce, refine or replace the use of laboratory animals. *Drosophila melanogaster* is the widely used model organism due to its well-explored genetics and developmental history. Additionally, the European Centre for the Validation of Alternative Methods (ECVAM) had recommended utilization of *D. melanogaster* as a model organism [13, 14]. Hence *D. melanogaster* has been evolved as a model organism in toxicological studies [15, 16, 17, 18, 19, 20, 21]. Regardless the extensive use of this plant as anticancer medicine, there is no scientific data available for phytochemical screening and genotoxicity of the extract of *C. roseus*. The present study aims to investigate the genotoxicity of this plant extract by *in vivo* protein profile analysis and acute toxicity test using *D. melanogaster* as a model organism.

Materials and Methods

Preparation of plant extracts and extraction yield calculation

Genotoxicity screening of *C. roseus* extracts in *D. melanogaster* was conducted during 2010 - 2011 at Calicut University Campus, Thenjippalam, Kerala, India. Healthy samples of *C. roseus* were collected from Calicut University campus and fresh plants were thoroughly washed and shade dried at room temperature for four days. The dried samples were subsequently transferred in hot air oven at temperature 60 °C, still constant weight. The bioactive compounds from the powdered plant materials were extracted using ethanol in Soxhlet apparatus.

Yield Calculation

Fresh Weight of the Plant	= 24.895gm
Weight of dry powder used for extraction	= 4.613gm
Weight of powder after Soxhlet extraction	= 3.682gm
Therefore actual weight loose:	= 0.931gm
Actual weight of ethanol extractable compound present in <i>Catharanthus roseus</i>	= 20.182%
1 kg bioactive compound /substance soluble in ethanol need	= 26.74 kg fresh plant biomass.

Spectrum analysis

Spectrum analysis of *C. roseus* extract was performed using UV/VIS spectrophotometer (Systronics double beam-2201) in the range 200-800nm. The presence of different compounds in the *C. roseus* plant extract was demonstrated in the spectrum, with wide range of compounds in different concentrations.

Experimental animal

The fresh culture of *D. melanogaster* was acquired from the infested fruits collected from the nearest fruit shop. They were reared continuously in the laboratory on the artificial diet at 25-34 °C temperature and 84-93% RH. Cultures were maintained in a beaker, the mouth of which was covered and secured with a piece of cloth and rubber band. Then the insect cultures were maintained for future use.

The culture media for *D. melanogaster* was prepared according to the formulation by Ashburner and Thomson (1978) [22]. The composition of standard culture media used in the present work is given below.

Wheat flour	:	75 Gms
Molasses	:	75 Gms
Baker's Yeast	:	15 Gms
Agar agar	:	15 Gms, for one litre

The above medium was boiled in a pressure cooker for thirty minutes and temperature of the media was brought to room and 2.5ml propionic acid was added as an additional mould inhibitor. The medium prepared was poured into sterile culture flasks and were capped with sterilized cotton plugs. Those culture flasks which were not used immediately were stored in a refrigerator at 4 °C. While preparing culture flask uniform surface area and depth were ensured in all trials.

Administration of *C. roseus* extract to *Drosophila melanogaster* larva

Healthy and uniform size *D. melanogaster* larvae (25 No. X 3sets) were collected and simultaneously exposed to three different concentrations of extract for studying the toxic and genotoxic effects of *C. roseus*. The battery trial experiments were conducted and three concentrations selected for the definite toxicity assay were 3, 1.5 and 0.1875 mg per ml ethanol extractable fraction from *C. roseus* dry powder and ethanol control and control. Three sets of experiment were run simultaneously and 1st and 2nd for demonstrating the genotoxic effects of *C. roseus* and 3rd set for stress protein induction experiments. During toxic exposure organism along with control groups were maintained 16/9 light and dark photoperiod till the completion of the work.

Protein profile analysis

Uniform size pupae were transferred in to eppendroff tube contains chilled homogenization tris buffer (p^H7.8) and homogenized samples were centrifuged at 10000 rpm at 4 °C for thirty minutes and subsequently equal volumes of samples were digested in loading buffer (2 ml glycerol, 2 ml 10% SDS, 0.2 mg bromophenol blue, 2.5 ml stacking gel 4X buffer, 0.5 ml 2-mercapto ethanol were added to water to a final volume of 10 ml and stored at room temperature) and digested samples were stored in freezer for SDS-PAGE analysis.

SDS-PAGE

The digested samples were subjected to 1D SDS-PAGE analysis using promega protocols USA [23]. Equal volume (50 ml) digested samples along with standards (Sigma USA) were loaded in each well. Electrophoresis was performed at constant current of 15 mA in stacking gel and 30 mA in separating gel.

Table 1: Standards used for SDS-PAGE

Sl. No.	Protein standards	Molecular weight
1	Myosin	212 kDa
2	α -2 Macroglobulin	170 kDa
3	β - Glycosidase	116 kDa
4	Transferrin	76 kDa
5	Glutamic dehydrogenase	53 kDa

After electrophoresis, gels were stained using Coomassie brilliant blue R 250 and subsequently destained till the complete removal of background stains. Destained gels were stored in 12% glycerol still documentation. Stained gels were documented using BIO-RAD molecular image Gel doc (GS 800), USA. Acquired gel image were subjected to further analysis such as molecular weight calculation comparing of tract with control profile were done using *quantity one* software.

Results and Discussion

In the present study, we investigated the genotoxicity of the alcoholic extracts of *C. roseus*. Studying the effect of plant extract form is more appropriate since people have been found using only the crude extract for folk medicine. Working with plant extracts means working with complex mixtures of

biologically active compounds, some of the compounds in such a mixture can be genotoxic or antigenotoxic. So, screening of the genotoxic and antigenotoxic properties is important to predict the potential health hazards of using the plant for medicinal purposes. In order to fully understand the genotoxic potential of the alcoholic extracts of *C. roseus*, we performed *in vivo* protein profile analysis^[34]. The whole plant (*C. roseus*) ethanolic extraction of was performed using Soxhlet apparatus (incessant, 25cycles). The yield of Soxhlet extraction (25cycles) from dry *C. roseus* powder showed 20.182% weight loss in ethanol. The spectrum analysis results showed the presence of wide range of compounds with absorption at different wave length starting from 200 to 800nm (Fig.1). The yield (weight loss) and spectrum analysis results showed the presence of different pharmacologically active substance in the ethanol extract.

Toxicity trials with early phase of 3rd instar *D. melanogaster* larvae exhibit dose dependent response along with avoiding behavior. The battery toxicity bioassays were performed and the results were included in the (Table. 1). In present study early phase of 3rd instar *D. melanogaster* larvae were selected because this stage is remarkable with very limited movement and showed burrowing behavior. The very moderate feeding stage is ideal for toxicity bioassays.

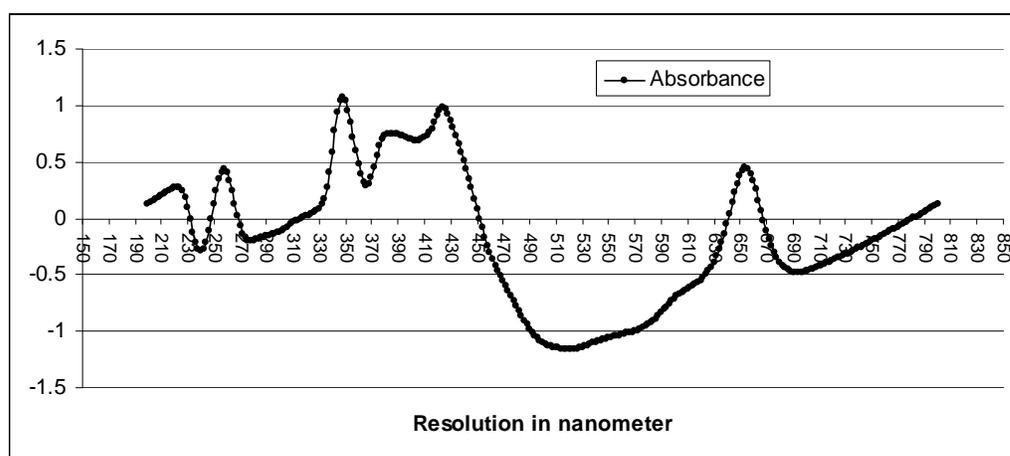


Fig 1: UV/VIS spectrophotometric analysis of plant extract of *C. roseus* showing different absorbance spectrum.

The doses selected for the present study were respectively 3, 1.5 and 0.1875mg/ml ethanol extractable bioactive compounds from *C. roseus*. Toxicant free control and ethanol control group was simultaneously maintained for deducting the normal environmental effects on *D. melanogaster* larval development. The doses selected for the toxicity bioassays and genotoxicity effects have a geometric relationship between doses, 1.5 is two times lesser than 3mg/ml and 0.1875 is eight times lesser than 1.5mg/ml and between 3 and 0.1875 mg/ml has sixteen times difference.

At the early stage (45 min) of toxic administration, the larvae were exhibit very unique behavioral progression in 0.1875 mg/ml group. The organisms tend to avoid the medium and creep on the wall of the container, this indicate the chemotactic response (Table. 1). In higher doses (3mg/ml) organisms fail to move from the point of release and after 24 hr only two pupae were reported and they failed fail to emerge in to adult. However in lower doses (0.1875mg/ml) all larvae migrate to the wall of the container and emerge successfully. Dose dependent pupae colour was recorded and included in the Table 2.

1-D SDS-PAGE protein analysis

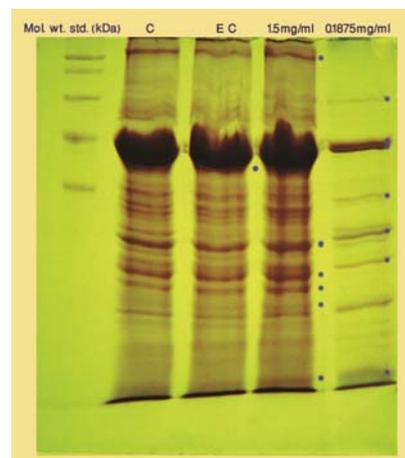


Fig 2: Electropherogram of protein profile in the pupae of *D. melanogaster* exposed to *C. roseus* extract

The electropherogram of one-dimensional protein profile of *D. melanogaster* pupae exposed *C. roseus* extract showed elicitation and disappearance of different stress related proteins and the results are demonstrated in Fig. 2 & 3. The protein profile between control group and ethanol control group were seemed almost similar protein expression. In treatments considerable changes were noticed in both the doses such 1.5 & 0.1875 mg/ml.

In lower doses (0.1875 mg/ml) five new proteins with apparent molecular weights 109, 45, 36, 28 and 11kDa were expressed. Disappearance many proteins especially higher

molecular group and lower molecular group were recorded in lower dose and the results are presented in Fig. 3 & 4. The incidence of higher molecular weight protein 109 kDa expressions recorded in 1.5mg/ml group and become concentrated in 0.1875mg/ml group. The intensity many proteins increased in higher dose as result of quick stress response and this may related with retention time in intoxicated media. In higher dose of *C. roseus* the larvae fail to move as part of avoiding behavioral progression. However in lower dose animals can sense the presence of toxic compounds and exhibit moderate movement on early phase.

Table 2: The behavioral and toxic response of *Drosophila melanogaster* larvae exposed to different doses of *C. roseus* extract

Treatments	No. of Larvae introduced	After 45 min	After 12 hrs		After 24 hrs			After 48 hrs		After 72 hrs
		Position	No of pupation	Position	No of pupation	Colour	Position	No of pupation	Position	No of adult flies
Control	25	Sub- surface	5	In bed	7	Light brown	6 in bed and 1 on the edge	10	9 in bed, 1 on edge	25
Ethanol control	25	Sub- surface	7	6 in bed and 1 on wall	13	Brown	all in the bed	25	6 on bed, 4 on edge	25
3mg/ml	25	Sub- surface	0	In bed	2	Dark brown marks at centre	In bed	2	In bed	Fail to emerge
1.5mg/ml	25	Sub- surface	15	2-face and edge	23	Dark brown	7in bed and 16 on walls	25	4 on bed remaining on the wall	25
0.1875mg/ml	25	Creeping in the wall	13	8 in wall and 5 in surface	19	Dark brown	All on or near wall	25	All on wall	25

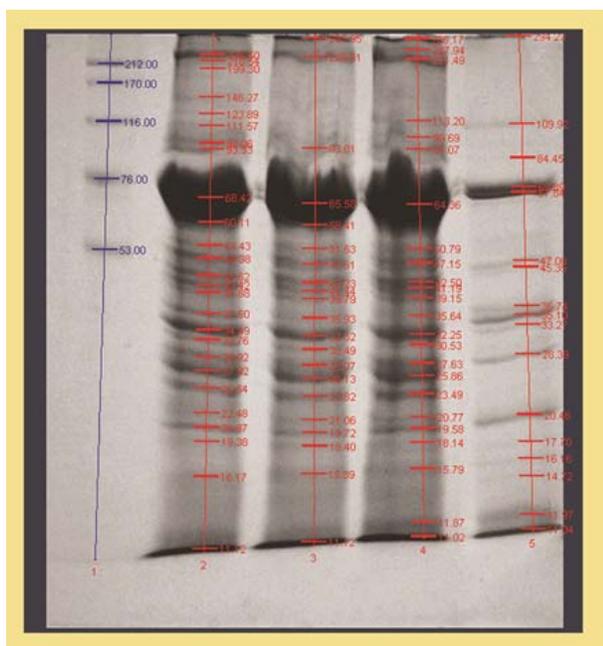


Fig 3: Electropherogram of *D. melanogaster* pupae protein profile showing the molecular weight in KDa, quantity one data.

The present protein profile analysis exhibits a dose dependent genotoxic effects with stress protein expression. During intoxication animals exhibits various types of behavioral progression and chemo tactic response. The study results demonstrated that the protein profile analysis of *D. melanogaster* is an ideal tool for evaluating genotoxicity assessment of several medicinal plant compounds of therapeutic use. Administration of vincristine for the treatment of cancer lacks the usual myelotoxic effects exhibited by other anti- cancer drugs, but it is limited by its

neurotoxicity [24, 25]. In higher doses such as 3 and 1.5 mg/ml larvae fail to move. Similar effects were already being reported by authors worked on vocal fold paralysis caused by the vinca alkaloid [26, 27]. The cellular response or heat shock response is involved in protecting organisms from damage due to exposure to wide variety of stressors, including elevated temperatures, UV light, heavy metals and xenobiotics. The stress proteins entail the rapid synthesis of a suite of proteins, referred as stress proteins, which has very specific function in cells. The present study showed appearance of different stress proteins during the toxic exposure and which protect and reduce the genotoxic effects. Hence the current study indicates higher doses of ethno pharmaceuticals cause very strong impact on organism and is similar to that of any xenobiotics.

Conclusion

The present study clearly showed that alcoholic extracts of *C. roseus* have genotoxic activity. Hence, further studies should be conducted to identify the active compounds responsible for causing genotoxicity to ensure the safe use of using *C. roseus* for medicinal purposes. The present finding may help to predict the genotoxicity assessment and potential health risk of using *C. roseus* as an anticancer and ant diabetic drug.

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