

Antioxidant response of *Murraya koenigii* in moderating locomotive impairment in *Drosophila melanogaster* following Pharmaceutical wastes leachates

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Abstract - *Murraya koenigii* is thought to be an excellent source of antioxidant. The present study was intended to examine whether administration of aqueous extract of the Curry leaves (CuLE) (*Murraya koenigii* L.) do possess a protective effect against leachates induced toxicity in *Drosophila melanogaster*. Locomotor impairment is thought to be related to neurodegenerative disorder. Negative geotaxis assay has been extensively used to examine locomotor behavior. This behavioral assay is widely applicable for studying the role of genetic and environmental factors on fly behavior. The negative geotaxis assay has empirical support and presently attracts considerable attention. Flies exposed to multiple dose of leachate showed movement disorder. Curry leaves (CuLE) (*Murraya koenigii* L.) showed ameliorative effect against toxicity in *Drosophila* as seen in locomotor activity. The present study shows that Curry leaves (CuLE) exhibit varying degree of protection against leachates induced neurotoxicity.

Index terms- *Drosophila melanogaster*; Leachates; Behavior; Geotaxis; Climbing.

1. INTRODUCTION

Oxygen free radicals or activated oxygen has been implicated in diverse environmental stresses in animals and appears to be a common participant in most of the degenerative conditions in eukaryotic cells. Several studies carried out previously on different environmental toxicants i.e. metals, pesticides, solvents, industrial and municipal runoffs, in different model systems revealed increased oxidative stress caused by these xenobiotics [Dorts et al., 2009b, Singh et al., 2009, Bhargav et al., 2008, Siddique et al., 2008, Avci et al., 2005, Pandey et al., 2003, Livingstone et al., 2000]. From previous studies it was confirmed that industrial soil samples from few industrial sites in Rohtak contained heavy metal concentration (Pb, Zn, Cu, Ni, Cd) that exceed the calculated worldwide mean of unpolluted soil, indicating high level of pollution on the site of industries due to solid wastes dumped [Annu, 2016,2015,Urmilla, 2015].

Due to the presence of rich contextual literature, *Drosophila melanogaster* has been widely studied by many scientists to study neurodegenerative diseases such as Parkinson disease

to reveal the mechanism behind the disease [Feany and Bender 2000, Bonini and Fortini 2003, Lu and Vogel 2009]. Notwithstanding the conspicuous differences between *Drosophila* and humans, many genes and signaling pathways are conserved between them [Paricio, 2011]. Locomotive movement is related to nervous system [Sherrington, 1906]. Locomotion not only requires proper functioning body structures besides need neuronal mechanisms providing information from several sensory modalities. Neuronal action requires fine control for accurate motor output. Minor modification in this can be harmful for an animal mobility in organized manner. Due to the close relation between neuronal disorders and the musculoskeletal system, locomotive impairment and behavioral changes can be used diagnostically for genetic screening and characterization of disease models as well as drug screens. Additionally, a relationship between infection, immunity, and reproductive fitness in insects has been well established.

Several experimental and epidemiological evidences have revealed a strong correlation between dietary factors and prevention of many diseases. Phytochemicals have ability to relieve oxidative

stress [Park et al., 2012, Kim et al 2011, Melov 2002, Pandey 2009]. India has been proved to show diverse biological activities against neurodegeneration. Few of the recent studies have proved that the leaves of *M.koenigii* (L.) Spreng have memory and learning enhancing effects which in turn exhibited neuroprotection activities [Iyer, 2008]. The previous study also found that there was an increase in the acetylcholine (Ach) levels and decrease in the anticholinesterase (AchE) activity [Abdul, 2013, Bhandari, 2012]. Besides reducing brain cholinesterase activity, *M.koenigii* extracts significantly improved cognitive functions as evidenced by the significant increase in the memory scores of young and aged mice while the extracts reserved the amnesia induced by scopolamine (0.4 mg/kg i.p.) and diazepam (1 gm/kg i.p.). Since *M.koenigii* has a long history of being a neuroprotective plant, recent researchers have made attempts to exploit this medicinal plant to attenuate oxidative stress mediated neuronal dysfunctions.

Negative geotaxis measure the ability of fly to climb vertically from the bottom of a container as a part of its inborn escape reaction. It was found to be sensitive to oxidative stress, stage of development and prior cold exposure [Hosamani, 2009, 2010, Llorens, 2007, Arking, 1990]. We hypothesize that the physiological changes that cause performance deficits in negative geotaxis assay are affected by stress, such that this assay can be used to detect decreases in health during stress. Accordingly, in the present study, we specifically addressed questions related to the possible neuropharmacological properties of CuLE against pharmaceutical wastes induced oxidative stress and neurotoxicity (impaired climbing behavior) employing wild strains of *Drosophila*.

2. MATERIAL AND METHODS

2.1 Rearing of flies

Wild-type *D. melanogaster* (Oregon K) flies and their larvae were reared at 21 ± 1 °C on standard *Drosophila* medium containing agar-agar, maize powder, sugar, yeast, Sodium benzoate and propionic acid. Additional yeast suspensions were provided for the healthy growth.

2.2 Industrial Soil and solid waste collection

Randomized sampling technique was used for collection of industrial soil and solid wastes for the assessment of toxicity [Houk, 1992]. Wastes from

pharmaceutical industries were collected from waste disposal site in the vicinity of Rohtak. Control soil samples were collected from the institute only. Five random samples were collected and mixed.

2.3 Leachate preparation from soil samples

For the preparation of 10% leachates leachates from soil and the industrial solid wastes at three different pH viz, 7.00 (in MilliQ water, neutral), 4.93 (5.7ml glacial acetic acid + 64.3ml IN NaOH + 930ml MilliQ water; low acidic) and 2.88 (5.7ml glacial acetic acid + 994.3ml MilliQ water; highly acidic) Toxicity Characteristics Leachate Procedure (TCLP) [Method-1310, USEPA, 1990] was used. The leachates (prepared at three different pHs) were referred to as N, M and H, respectively.

2.4 Preparation of extract of *Murraya koenigii*

Fresh leaves of *Murraya koenigii* were collected from different parts of Rohtak washed thoroughly and dried in hot air oven at 35° C and crushed to powder form. For the preparation of aqueous extract 10g was dissolved in 100 ml double distilled water and soaked overnight. Filtered and filtrate was rotary evaporated at 400°C for 30 minutes and dried to obtained greenish brown powder was stored at -20 ° until further use.

2.5 Experimental design

Newly emerged larvae (22 ± 2 hr) collected from synchronized egg collections were transferred to food vials containing different concentrations of the leachates prepared from pharmaceutical industrial waste and soil at different pHs and CuLE and allowed for flies to emerge. Virgin flies are collected and experiment was performed on 5 days old flies. Study is divided in three groups as mentioned below:

Group 1 Control (Standard diet)

Group 2 Leachates treated

Group 3 Leachates treated along with CuLE concentrations

The neurobehavioral assay performed was
1. Rapid Iterative Negative Geotaxis Assay

2.5.1 Negative Geotaxis assay

Negative geotaxis is a frequently used index of locomotor behavior in flies [Goddeeris, 2003, Morrow, 2004, Simon, 2006, Gargano et al., 2005].

Briefly, 50 treated and 50 control flies were transferred once a day in the early afternoon without anesthesia into 10 cm tall clean glass vials. Flies were allowed to acclimate to the environment, undisturbed, for 15-20 minutes. Flies were tapped down on the surface of the bench three times, ensuring that the tap is hard enough to knock down all the flies to the bottom of the vials. Climbing time was recorded by a stop watch as 10 sec, 30 sec, 60 sec post startle. All work was done on sex basis. Flies were then transferred without anesthesia back into their rearing vial.

2.6 Statistical analysis

All data are analyzed by two way ANOVA followed by post hoc test for multiple comparison $p < 0.05$ was considered statistically significant. Prism computer program (GraphPad version 8.0) was used for statistical analysis.

Table 1: a) Represent % of males flies climbed

Male groups	% climb in 10 sec	% climb in 30 sec	% climb in 60 sec	Male groups	% climb in 10 sec	% climb in 30 sec	% climb in 60 sec
Control	82	14	4	PM 1%	62	26	12
PN 0.5%	80	18	2	PM 2%	58	32	10
PN 1%	72	24	4	PH 0.5%	54	36	10
PN 2%	74	22	4	PH 1%	38	50	12
PM 0.5%	78	18	4	PH 2%	26	42	32

Table 1: b) Represent % of female flies climbed

Female Groups	% climb in 10 sec	% climb in 30 sec	% climb in 60 sec	Female groups	% climb in 10 sec	% climb in 30 sec	% climb in 60 sec
Control	78	16	6	PM 1%	66	24	10
PN 0.5%	76	20	4	PM 2%	54	28	18
PN 1%	70	22	8	PH 0.5%	56	32	12
PN 2%	70	24	6	PH 1%	32	54	14
PM 0.5%	74	22	4	PH 2%	24	48	28

Pharmaceutical waste, N: prepared in neutral solvent (~pH 7.00) PN; M: prepared in mildly acidic solvent (~pH 4.93) PM ; H: prepared in highly acidic solvent (~pH 2.88) PH

Figure 1 revealed the response of males/females towards leachates exposure it shows declines in climbing performance with increase in concentration and decrease in pH. At low concentration it shows statistically nonsignificant effect $p > 0.05$ on climbing

3. Results

3.1 Pharmaceuticals waste toxicants declines negative geotaxis

Climbing is a strong and reproducible behavior. Leachates treated flies resulted in severe locomotor impairment as apparent from the negative geotaxis assay. Maximum flies remain at the bottom of the glass vial. Values are expressed as Means \pm SEM. Data is analyzed by two way ANOVA (statistically significant $p < 0.05$) followed by post hoc test. Table 1 depicts the percentage (%) of flies climbed in particular time. Even a small dose, lead to 34% reduction in climbing capacity and higher doses reduces it by 68% in males and among females it decrease from 28% to 69% in acidic pH. At different concentration and at different time interval flies shows significant effect on % climbing.

activity. Much effect has been seen on performance of females as compared to males. The decline in climbing exhibits a dose-dependent response, with highest concentration exposure worsening the climbing deficits.

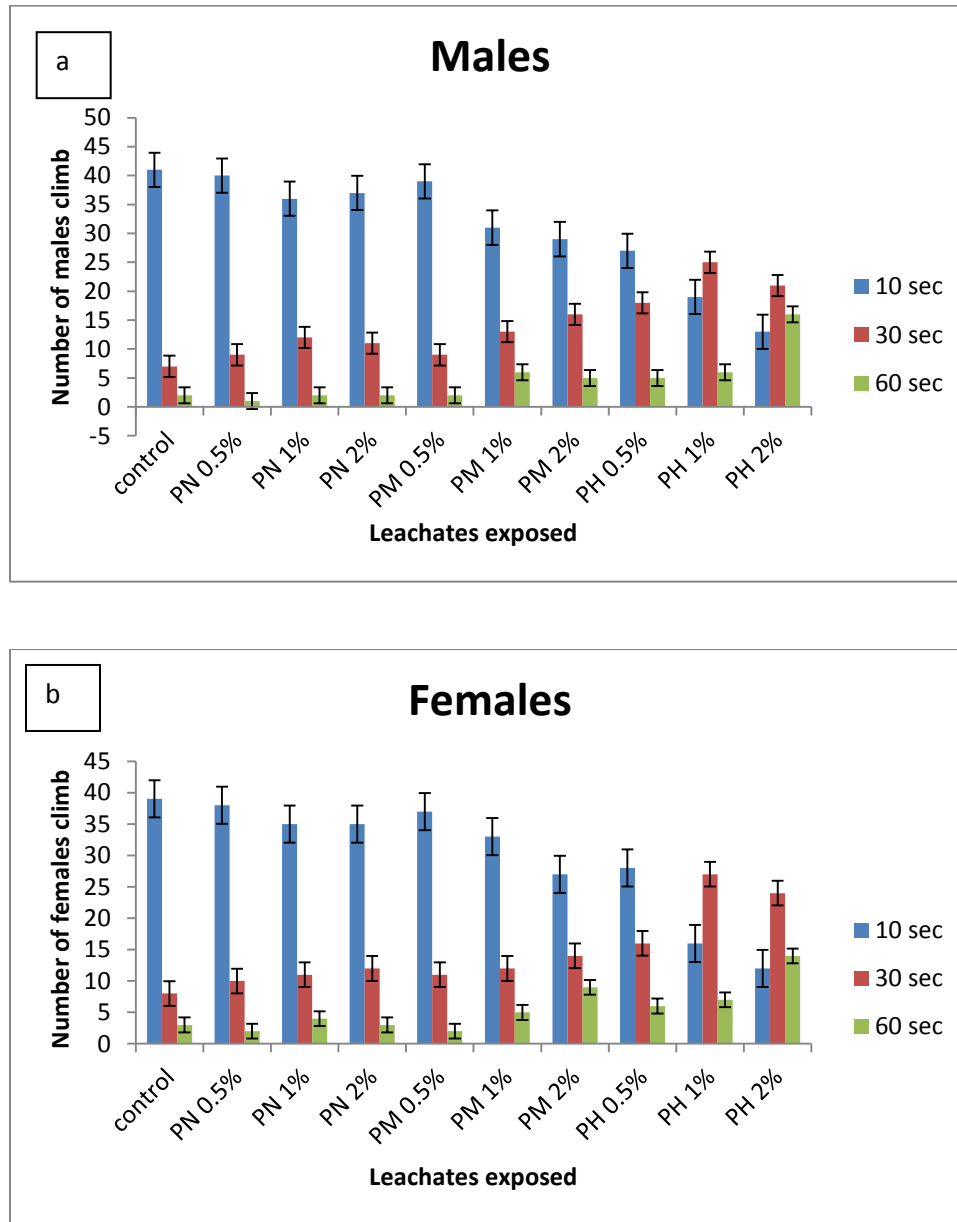


Figure 1(a,b) Number of leachates exposed males/female flies climbed. Values are expressed as Mean \pm SE in each group.

3.2 CuLE treatment improves negative geotaxis

Supplementation of antioxidant was able to relieve the locomotor impairment caused by leachates exposure. Higher concentration of CuLE in male flies at 30 sec shows non-significant effect $P > 0.5$. F value

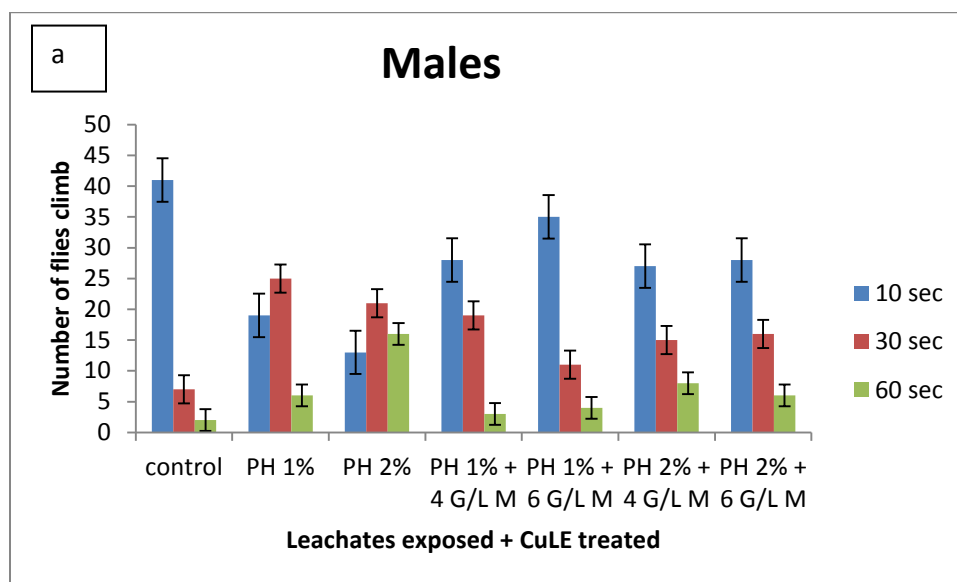
was found to be $F(4, 8) = 482.1$. Maximum effect was observed in acidic leachates with higher concentrations. Table 2 represents % of flies treated with CuLE climbed in particular time. Comparison of the data by leachates treated versus CuLE showed overall significant differences. Higher concentration

Table 2 a.b) Represent % of male/Female leachates exposed flies climbed;

Male Groups	% climb in 10 sec	% climb in 30 sec	% climb in 60 sec
PH 1% + 4 G/L CuLE	56	38	6
PH 1% + 6 G/L CuLE	70	22	8
PH 2% + 4 G/L CuLE	54	30	16
PH 2% + 6 G/L CuLE	56	32	12

Female groups	% climb in 10 sec	% climb in 30 sec	% climb in 60 sec
PH 1% + 4 G/L CuLE	56	34	10
PH 1% + 6 G/L CuLE	68	26	6
PH 2% + 4 G/L CuLE	54	38	8
PH 2% + 6 G/L CuLE	64	24	12

Pharmaceutical waste, N: prepared in neutral solvent (~pH 7.00) PN; M: prepared in mildly acidic solvent (~pH 4.93) PM ; H: prepared in highly acidic solvent (~pH 2.88) PH ; CuLE: *Murraya koeingii*,



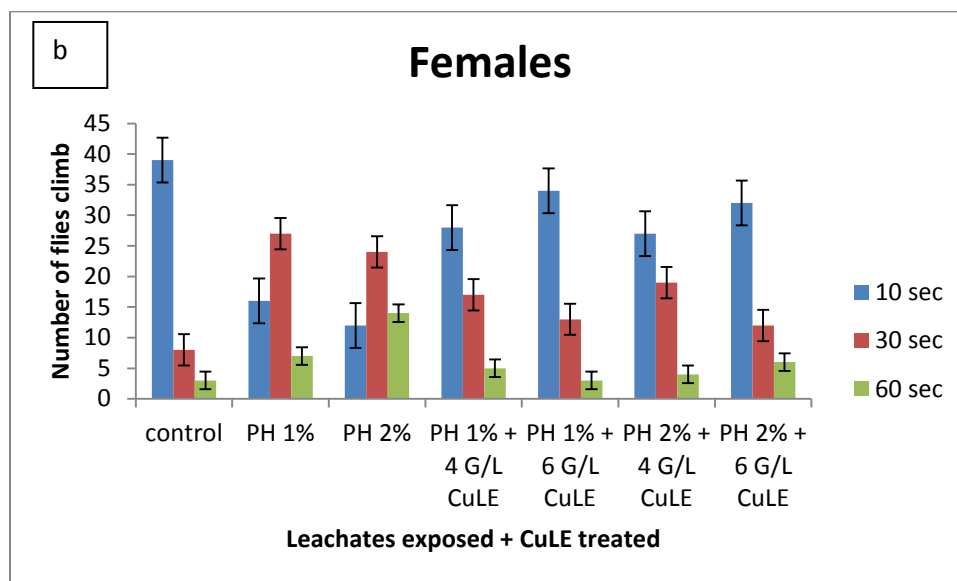


Figure 2(a,b) Number of leachates exposed along with CuLE treated female flies climbed; Values are expressed as Mean \pm SE in each group.

of CuLE ameliorates leachates effects and proves to reduce oxidative stress induced neural dysfunction (Figure 3, 4).

4. DISCUSSION

Alternative medicine in management of different diseases is gaining in importance and emerging as an extensive field of research for the drug development industry. Different dietary factors and nutritional components are emerging in future therapeutics either as magical healers or as protective shields in ensuing fatal diseased conditions.

The present study aims to establish that aqueous leaf extract of curry plant (a popular South-Asian spice herb) has the potential to protect against leachates induced neurological disorder. The decline in locomotion exhibits a dose-dependent response, with highest concentration exposure worsening the locomotion. Highest concentration of CuLE shows much effect on reversing the effect of pharmaceutical waste leachates. Highest concentration of CuLE proves to be more beneficial and highly acidic pH 2.88 shows maximum reduction in locomotory activity in *Drosophila*. As the pH falls it results in solubility of many metal ions that are insoluble in neutral pH.

The content of total anti-oxidant activity of *M. koenigii* leaves was found highest (2691 μ mol of Ascorbic acid/ gm) amongst all green leafy vegetables [The Wealth of India]. Summarizing the

earlier conducted large body of studies, it is now apparent that exposure of environmental chemicals to organisms may lead to exposure-effect responses like decline in the population [Houlahan et al., 2000], deficiency in development, instabilities in behavior [del Carmen Alvarez and Fuiman, 2005], organ malformations [Klumpp et al., 2002], various ailments in the exposed organisms [Zelikoff et al., 2002] and DNA damage [Siddique, 2005, 2008]. Studies on industrial soil samples from Rohtak proved to contain heavy metals like Pb, Zn, Cu, Ni, Cd [Annu, 2016,2015, Urmilla, 2015]. Experiments conducted on animals indicated that pb is both genotoxic [Shaik et al., 2006] and carcinogenic [Fowler et al., 1994], Ni [Haugen et al., 1994] and Cd [Elinder and Jarup, 1996] are carcinogenic, Cu generated free radicals when present in free form and produces ROS that causes damage to biomolecules like DNA, protein, and lipid [Galaris and Evangelou, 2002].

Mutation in few genes can reduce severe motor dysfunction in flies in negative geotaxis assay. In those cases, flies ability to climb the walls in

response to gravity upon agitation decreases. Negative geotaxis is robust behavioral assay to measure changes in locomotor or learning and memory capacities arising from genetic or environmental manipulation.

The use of *Drosophila* and the negative geotaxis assay provides an inexpensive and reliable method to identify genes involved in locomotive defects and subsequently screen candidate drugs for phenotype rescue. *Drosophila* has a central nervous system containing orders of magnitude fewer neuronal and glial cells than in vertebrate central nervous systems, yet they share the same types of neurotransmitter systems such as GABA, glutamate, dopamine, serotonin, and acetylcholine, and they are able to perform complex behavior, including sexual displays, social behavior, and learning [Botella, 2009].

We finally hypothesize that the changes in physiological parameters that causes performance deficits in negative geotaxis assay are somehow affected by leachates induction, such that this assay can be used during toxicity to detect decreases in different health consequences. Secondly we observed that CuLE prove to be a positive regulator to enhance climbing activity in leachates fed flies.

5. CONCLUSION

Negative geotaxis analysis are fast and modest method of evaluating behavioral shortfalls and quantitatively point out locomotor, learning and memory capabilities in *Drosophila*. This assay can be beneficial in exhibiting human neurodegenerative conditions in *Drosophila*. The current study on *M.koenigii* (L.) Spreng leaves against acute toxicity of pharmaceutical wastes aimed to bring a glance of light on different doses, dose frequencies, duration of treatments, results, and methods used by previous researchers for different activities of carbazole alkaloids proved to be beneficial. This study might prove *M.koenigii* leaf powder has neuropharmacological properties against pharmaceutical wastes induced oxidative stress and neurotoxicity.

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