Comparative study of different nutraceuticals on chlorpyrifos triggered neurotoxicity in *Drosophila melanogaster*

Dissertation Report

Submitted in partial fulfilment for the award of degree of

MASTER OF PHILOSOPHY (ZOOLOGY)

Submitted by

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LOVELY PROFESSIONAL UNIVERSITY

SCHOOL OF BIOENGINEERING AND BIOSCIENCES
Department of Zoology

CERTIFICATE

This is to certify that ***Humera Hamid (11617269)*** doing Master of Philosophy project entitled **"Comparative study of different nutraceuticals on chlorpyrifos triggered neurotoxicity in *Drosophila melanogaster*"** under my guidance and supervision. To the best of my knowledge the present work is the result of her original investigation and study. No part of the thesis has ever been submitted for any other degree or diploma at any university. The report is fit for the submission and the partial fulfillment of the condition for the award of **MASTER OF PHILOSOPHY IN ZOOLOGY**.

Date: 27/4/2017

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DECLARATION

I hereby declare that the thesis entitled **"Comparative study of different nutraceuticals on chlorpyrifos triggered neurotoxicity in *Drosophila melanogaster*"** submitted for Master of Philosophy degree is entirely my original work and all ideas and references have been duly acknowledged. It does not contain any work for the award of any other degree or diploma at any university.

Date: 27/4/2017

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Humera Hamid (11617269)

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Chapter: 1

Terminology

ACR	Acrylamide
ATC	Acetylcholine
AchE	Acetylcholinesterase
BM	*Bacopa monnieri*
CNS	Central Nervous System
DTNB	Dithiobis (2-Nitrobenzoic acid)
DPPH	1,1,-Diphenyl-2-picryl-hydrazyl
EGCG	Epigallocatechin-3-gallate
GAE	Gallic acid equivalent
MAHs	Monocyclic aromatic hydrocarbons
O.D	Optical density
PI	Performance index
PQ	Paraquat
ROS	Reactive oxygen species
ROT	Rotenone
TPC & TFC	Total phenol & flavanoid content

Chapter: 2

Introduction

INTRODUCTION

Neurodegenerative diseases are a sub-group of human diseases with common characteristics. These diseases occur suddenly and without showing any symptoms during its development/ maturation in the central nervous system (Lu and Voge, 2009). The prominent character of these diseases is the loss of specific neuronal population in human and the prominent neurological disorders are Huntington's disease, Parkinson's disease, Alzheimer's disease, and multiple sclerosis. Alzheimer's disease as the most common disease of brain is the prominent cause of dementia. AD reveals as a gradual decrease of cognitive functions such as learning and memory has been proved clinically (De Strooper, 2003). Also by pathological means the disease is characterized by selective atrophy of frontal cerebral cortex and hippocampus. The triggering event in AD occurs by the accumulation of A β peptide that may underlie synaptic failure that results in the decline of cognitive function and neuronal dysfunction (Hardy and Selkoe, 2002).

The second most popular disorder of neurodegenerative disease is Parkinson's disease following Alzheimer's disease (Nass and Przedborski, 2008; Polymeropoulos *et al.,* 1996). Symptoms of Parkinson's disease by nature are motoric: bradykinesia, rigidity and flexed posture (Fahn, 2008; Jankovic, 2008) and most of the times patients also suffer from the symptoms that are non-motoric in nature including dementia, depression and disturbed sleep (Wilkinson and Lennox, 2005; Dickson, 2008). Selective loss of dopaminergic neurons of the *substantia nigra*, specifically in the *pars compacta* of the SN (Wilkinson and Lennox, 2005) that causes a reduction in the amount of dopamine available in that brain part (Wilkinson, 2005) results in neurodegeneration and lack of the significant treatment approaches that can arrest degeneration, among them most of prove fatal. These diseases are dominantly present in developed countries like America and Europe where effect was much more severe and the cause of these diseases are unknown or poorly understood. A number of previous studies had shown that environmental risk factors are directly or indirectly responsible for neurodegenration (Chaudhuri *et al.*, 2007). It is feasible/probable that the expression of genes in dopaminergic neurons, having relation with a familial Parkinsonism has roles in determining environmental toxicant susceptibility. Irregular release of dopamine, in particular to its release into the cytoplasm of dopaminergic neurons, results in oxidative stress that causes the instant death of neurons (Stokes *et al.,* 1999). Several treatments and techniques have been already evolved to cure this disease but transiently.

Human population has been suffered from several central nervous system (CNS) disorders which have built up the focus of applied scientific research on CNS and its pathology. Exclusively the area including CNS infections, psychiatric and neurological disorders, there is rising prevelance of neurodegenerative diseases (Shinomol *et al.*, 2011). The phytochemicals having significant beneficial medicinal merits are being used (Kumar, 2006) and information of phytochemicals developed from long-lasting knowledge of herbs originated from nature and traditional medicines has been endured for thousands of years in rising countries for instance India. So, with this better knowledge in current years it has improved interest in the use of herbal medicines in western countries (Sparreboom *et al.*, 2004). The Indian conventional system of medicines "Ayurveda" that is thousands of years old has illustrated numerous medicines and nutraceuticals, which are valuable for neurological diseases. These natural remedies include *Withania somnifera* (Ashwaganda), *Mucuna pruriens* (Zandopa), *polyphenols, Bacopa monnieri* (Brahmi), *Curcuma longa* (Curcumin). Among them we are going to evaluate efficacy of *Bacopa monnieri* as preventive/therapeutic agent for neurodegenerative diseases with other nutraceuticals like green tea and curcumin.

Brahmi or *Bacopa monnieri* is derived from word "Brahma", the "supreme creator" according to Hindu mythology of India. It has derived its name because of the characteristic found in it, as any product that boost the brain functioning and keeps it healthy. Brahmi largely referred by *B. monnieri* commonly known as water hyssop has been used as a traditional medicinal plant since years (Chopra *et al.*, 1956; Russo and Borrelli, 2005). *B. monnieri* displays invitro antioxidants and cell protection effects. In animals it inhibits acetylcholinestarase (AchE) inhibitory activity, activates choline acetyltransferase and increase cerebral blood. It interacts with the serotonergic and dopamine systems, but its main mechanism is concerned with promoting neuron communication. Various tests have been performed on neuroprotective effects of *B. monnieri* extract against various toxicants that includes aluminum, beta-amyloid, glutamate etc. Also *B. monnieri* extract restrain various components of the beta-amyloid induced oxidative cascade pathway that can lead to Alzheimer's pathology and diminish beta-amyloid levels in the brain of an Alzheimer's disease (AD) transgenic mouse model (PSAPP mice) (Dhanasekaran *et al.*, 2007). *B. monnieri* has also been used as memory enhancer, antianxiety, anti-epilepsy and against cognitive dysfunctions since primordial period (Ernst, 2006).

Natural substances that are present in extracts obtained from plants, fruits and vegetables such as olive oil, red wine and tea well-known as polyphenols (Butterfield *et al*., 2002; Higdon *et al.,* 2003). The primary role of polyphenols is to work as an antioxidant and provide cell resistance against oxidative stress through scavenging activity. Antioxidant properties of polyphenols play a therapeutic role in diseases of neurodegeneration like as Parkinson's disease (PD) and Alzheimer's disease (AD) (Gotz *et al.,* 1990; Halliwell, 1992). *C. longa* commonly known as curcumin derived from turmeric (*Haldi*) is a yellow curry powder, which has been used as food preservative and herbal medicine in India (Kelloff *et al.,* 2000). C. *longa* has also antioxidant and anti-inflammatory properties may protect against Alzheimer's disease (AD) pathology. The diverse neuroprotective activities of *C. longa* are still limited especially how *C. longa* influences neuronal proliferation.

Organophosphate compounds are a group of chemicals principally used as an insecticide and an industrial effluent. The primary role of toxic action of organophosphate compound is an inhibition of acetylcholinestarase (AchE) (Ecobichon, 1997; Lotti, 2001) which leads to intoxication of central and peripheral nervous system (Namba *et al*., 1971). An organophosphorus ester-induced chronic neurotoxicity (OPICN) syndrome has been suggested. The OPICN syndrome has been resulted from sub clinical doses of organophosphate's long term exposure (Canadas *et al*., 2005). Among organophosphate compounds chlorpyrifos is a broad spectrum pesticide that is widely used in agricultural purposes because of safe handling and easily available in markets. It is a neurotoxic compound causing inhibition of cholinesterase resulting in acute toxicity. Even though chlorpyrifos toxicity may affect means other than cholinesterase inhibition including mechanism for the inhibition of mitochondrial ATP production through uncoupling of oxidative phosphorylation that results in the ROS generation (Ishii *et al.,* 2004).

One well built approach for studying the mechanism of various diseases has been done by the use of animal models. Among invertebrate animals, *Drosophila melanogaster* has long been excellent model organism for biologists. A great advantage of *Drosophila melanogaster* is its rapid development under standard laboratory conditions (25^{O}C) (Bonini *et al*., 2003; Muqit *et al*., 2002; Driscoll *et al*., 2003). *Drosophila melanogaster* completes its life cycle in 10 days and can be cultured in laboratory by various rearing methods. *Drosophila melanogaster* has satisfied as a genetic model for a century. Because of its numerous advantages, it has colonized

research laboratories all over the world. It is reasonable concerning dietary and spatial requirements, simple observation and manipulation at various developmental stages, produces considerable number of off-springs. Thus, by these differential characteristics *Drosophila melanogaster* is considered as the model system of choice in order to study the biological phenomenon in diverse manner (Hugo and Peter).

Chapter: 3

Review Of Literature

REVIEW OF LITERATURE

Previously, several studies have been done on the neurodegenerative diseases that offer key features of each disease quality and to summarize nifty developments in their methodology and analytic outlook (Lu and Voge, 2009).

Withania somnifera (Ashwaganda) belonging to the Solanaceae family is having several multiple pharmacological properties and is well known for its disparate therapeutic uses in Ayurveda and Unani fields (Kulkarni and Dhir, 2008; Gokul *et al*., 2012). A recent design on *W. somnifera* showed its preventive chattels in *Drosophila melanogaster* that was unarmed to rotenone (ROT) and a suited modulation was found at variance with locomotor deficits, oxidative impairments and neurotoxicity (Manjunath and Muralidhara; 2015). In this context flies supplemented with *W. somnifera* showed protective phenotype in ROT urged on lethality and locomotory activity in adult males and furthermore showed attenuation in oxidative claim, mitochondrial dysfunction and neurotoxicity in *Drosophila*. This *W. somnifera* mediated release of flies was associated with decreased oxidative uphold, enhanced antioxidant defenses and weakened cholinergic function and elevated dopamine depletion (Coulom and Birman, 2004).

Tea consumption has bestowed with possible eventual neurological and pharmacological actions valuable to human wellness. Evidences prove that oxidative uphold resulting in reactive oxygen species generation plays a pertinent role in neurodegenerative diseases, supporting natural antioxidant polyphenols. The polyphenolic dietary supplements have enforcement on cognitive deficits in advance aged individuals. Green tea is made from the leaves of *Camellia sinesis* plant endemic in Asia. Studies revealed that green tea is biochemical compound containing rich amount of flavanoid also including catechins. One polyphenolic compound epigallocatechin-3-gallate (EGCG) that is found in most abundance in green tea has anticancer and antioxidant properities (Nanjo *et al*., 1996; Pannala *et al*., 1997). Also one study suggests that polyphenolic tea can be procured in the brain.

Previously, Ravikumar and Muralidhara (2009) described the neuroprotective efficacy of *Bacopa monnieri* (BM) at variance with rotenone urged on oxidative stress and neurotoxicity in *Drosophila melanogaster*. *Drosophila* male aged were treated with BM powder in the diet and exhibited reduced levels of endogenous oxidative markers *viz*., malondialdehyde,

hydroperoxide and protein carbonyl content. Despite of having this effect, it further propounds complete protection against rotenone (ROT) induced oxidative stress and substantially inhibits dopamine depletion in flies. Flies exposed to ROT along with *B. monnieri* shows decline prevalence of mortality and execute better in negative geotaxis assay.

Another study showed the Prophylactic treatment along with *Bacopa monnieri* leaf powder alleviates paraquat-induced oxidative disruption and lethality in *Drosophila melanogaster* (Ravikumar and Muralidhara, 2010). Environmental leak to oxidant inducing herbicide, paraquat (PQ) has been seen as an acute risk factor in the Parkinson's disease (PD). Adult male flies were exposed to PQ alone and showed 50% mortality in flies as PQ has been established to undertake intracellular reduction to a free radical form. Prophylaxis with *B. monnieri* extract shows better protection against PQ induced death rate. Moreover, oxidative impairment and mitochondrial dysfunction was observed among *Drosophila* exposed to PQ and after treatment with *B. monnieri* extract, it halts the oxidative uphold caused by induction of PQ and reinstate the activity of electron transport chain complex that clearly states its effect on mitochondria.

A report documented by Gupta *et al*., (2010) showed Chloropyrifos stimulated cell death and DNA damage in *Drosophila* through generation of reactive oxygen species. Choloropyrifos (CP) is an organophosphate pesticide used as agricultural purposes throughout the world for agricultural purposes. A study by Trasher *et al.*, (2002) showed that Choloropyrifos induces immunological disorders also it causes oxidative stress (Goel *et al*., 2005) and tissue damage. Reactive oxygen species (ROS) can cause chemical changes and modifications in DNA and nucleoproteins (Jaruga *et al.*, 1994) resulted DNA damage in the organism exposed to it.

Liu *et al.*, (2013) recently studied *C. longa* commonly known as curcumin or *Haldi* protects at variance with rotenone-induced neurotoxicity in the cell and *Drosophila* models of Parkinson's disease (PD). This neurodegenerative disease is induced due to the loss of dopaminergic nerve cells. In the study *C. longa* was used as a powerful antioxidant in rotenone based cell (mitochondrial complex I) and in *Drosophila* models in order to judge the protection against ROT induced neuronal toxicity. The study suggested that *C. longa* defenses against ROT induced toxicity *in vitro* (SHSY5Y cells) in humans as well as *in vivo* (in *Drosophila*) as it diminishes ROT induced cell death in SH-SY5Y cell (human neuroblastoma cells).

Report documented by Singh *et al.,* (2011) described that exposure to various monocyclic aromatic hydrocarbons (MAHs) like; benzene, toluene and xylene being used for various industrial purposes are directly or indirectly harmful to organisms. A *Drosophila melanogaster* model was used to evaluate the genotoxic and apoptotic activity of the above compounds and also simultaneously checked out the potential efficiency of two phytochemicals, named as curcumin and quercetin in attenuation to the toxicity induced by these chemicals. 1.0-100.0 mM of benzene, toluene or xylene exposure was given to third instar larvae of wild type *Drosophila melanogaster* (Oregon R^+) individually for time period of 12, 24 and 48 hrs for evaluation of their potential in apoptosis and genotoxicity. It was observed that organisms exposed to these chemicals showed raised apoptotic marker and genotoxicity upon concentration and time dependent manner. Also they observed the diminishing activity in cytochrome P450 activity, GST levels, and oxidative stress markers when flies were simultaneously exposed to chemicals along with quercetin and curcumin enrichment (Singh et al., 2011).

Chaudhuri *et al*., (2007) revealed correlation of environmental and genetic factors in Parkinson *Drosophila* model. Fatal loss of dopaminergic neurons is an indication of Parkinson's disease and it was found that environmental toxin, such as the herbicide paraquat, can show Parkinsonism in *Drosophila* that is related with loss of subsets of dopaminergic neurons. The study also showed that the male flies has exhibited increases incidence of Parkinson's disease and get earlier symptoms than females.

A recent study by Subramanian *et al*., (2014) on the role played by *B. monnieri* in regulating oxidative stress temporarily in clock mutant (cry^b) of *Drosophila melanogaster*. The flies were exposed to hydrogen peroxide (H_2O_2) or rotenone and the link between circadian clock and oxidative stress sensitivity was examined and also after exposure of flies to *B. monnieri* rhythmic reversibility was investigated. After treatment elimination of 24h rhythms in negative geotaxis, oxidative stress markers and antioxidants under oxidative stress were observed. Also, the reversibility of rhythms was noted in wild type flies treated with *B. monnieri* than cry^b flies.

A study done by Rauf *et al*., (2011) on Bacoside that contains B. monnieri extract reduces morphine as well as increased striatal dopamine and serotonin level. An extract of plant known as *n*-butanol (*n*Bt-ext BM) was found to contain Bacoside A (Bacoside A, Bacoside A3,

Bacoside II, Bacopasaponin C). Also, the extract of B. monnieri was used to study the effect on morphine induced hyperactivity as well as dopamine and serotonin levels as these play crucial role in opioid dependence and sensitivity. Before morphine treatment the mice were pretreated with saline or nBt-ext BM and after treatment locomotory activity was recorded. It was observed that *n*Bt-ext BM significantly lowered the locomotory activity in both of the treated groups. Further, it was also suggested that nBt-ext BM has an antidopaminergic/serotonergic effect.

Study focused on neuroprotective effects of phytochemicals against oxidative stress and neurotoxicity caused by paraquat as herbicide induced in *Drosophila melanogaster* was documented by Park *et al*., (2012). The study showed that paraquat exhibits LC_{50} at 24.7 mM to adult male flies of *Drosophila melanogaster* within a time period of 24h. After treatment with paraquat, exposure to dietary enrichment of quercetin, curcumin, *Sanguisorba officinalis* and *Zedoariae rhizoma* increased life span and enhanced motor activities of flies. Also, the dietary enrichment of phytochemical substances also decreases acetylcholinesterase activities that were previously enhanced by paraquat treatment.

A previous study on extracted leaves of *B. monnieri* on dietary enrichment in transgenic Parkinson's disease model of *Drosophila melanogaster* was reported by Siddique *et al*., (2014). The flies expressed normal human alpha synuclein (h-αS) in their neurons. Loss of dopaminergic neurons with time dependent manner and the accumulation of αS (Lewy bodies) have been documented in the flies with Parkinson's disease. Leaf extract was prepared and diets with final concentrations of 0.25, 0.50 and 0.1 µl/ml were prepared and flies were exposed for 21 days. Study was done on oxidative stress, climbing assay pattern and apoptosis in brain of flies. It was observed that flies delay in loss of activity pattern as well as climbing ability according to dose dependent and also that extract diminished the oxidative stress and apoptosis in comparison with untreated Parkinson's disease flies.

Prasad *et al.,* (2014) revealed the neuroprotective property of curcumin and geranoil in acrylamide (ACR) induced neurotoxicity in *Drosophila melanogaster*. The male adult flies were exposed for 7 days to ACR (5 mM) and also with or without curcumin and geranoil in the diet. It was observed that both of phytochemicals decrease the effect of ACR-induced mortality, recover the locomotory phenotype and reduce the increases levels of oxidative stress

markers in body/head regions. Also, the phytoconstituents decrease ACR –induced mitochondrial dysfunction and also rescued the dopamine levels in head/body regions.

Report documented by Sourajit *et al*., revealed the property of two different nutraceuticals curcumin and artemisinin that inhibits brain tumor, extends life span and rescues locomotory ability in *Drosophila melanogaster*. Brain tumor in *Drosophila melanogaster* is caused by deletion of tumor suppressor gene, *lethal(2)giant larvae* [*l(2)gl*] which causes brain tumor at larval stages. The efficiency of these phytochemicals was distinguished morphologically by measuring the sizes of brain of both treated and untreated larvae and as well as anatomically by observing at cellular level. It was found that both phytochemicals show antitumor effects individually and also in combination when exposed to larvae. Also, both of them showed prolonged life span and locomotory activity.

Silvia *et al.,* (2011) suggested a study about the wide spread role of green tea polyphenols in aging and against neurodegenerative diseases. Aging of brain and neurodegenerative diseases of the elderly persons are distinguished by oxidative stress and inflammation by irregular function of redox metals. Natural nutraceuticals including polyphenols play a pivotal role in diminishing the induced free radicals in the body. Epidemiological studies suggests that green tea containing the rich content of catechins and flavanoid helps in prevention of brain aging and reduce the occurrence of dementia, Alzheimer's and Parkinson's disease. These dietary enrichments are considered as cytoprotective agents that treat various brain diseases.

Report documented by Anjum *et al*., (2010) studied the toxicological estimation of chlorpyrifos and Neem extract (Biosal B) in 3rd instars larvae of *Drosophila melanogaster*. The larvae were exposed to both the compounds and it was found that they were melanized and failed to pupate. LC_{50} was possessed to be higher in Neem extract in comparison with chlorpyrifos. Exposure of *Drosophila melanogaster* with theses toxic compounds demonstrate that chlorpyrifos is very powerful pesticide than Neem extract and also these compounds inhibit the cholinesterase in treated flies as compared to control.

A recent study by Lee *et al*., (2016) on repeated exposure to chlorpyrifos toxicity transforms hippocampal expression of neuropeptide and neurotrophins. To examine the chlorpyrifos neurotoxicity, adult male long Evan (IE) rats were given enrichment of chlorpyrifos at 3 or 10/mg/kg/d for 21 days. After enrichment, they measured mRNA and non-

coding RNA expression by RNA-seq, microarray analysis in the CA1 region of hippocampus. Also, immunohistochemistry was used to find out the change induced by chlorpyrifos in protein expression. A sign of cholinergic toxicity was not found in any doses of chlorpyrifos provided to rats, however following 21 days of exposure there was a decline in cholinesterase activity to 58% or 13% control in rat hippocampus groups administered by 3 or 10 mg/kg/d.

Study of Mandel *et al.*, (2005) suggests that reactive oxygen species and inflammation resulting from oxidative cascades plays an important role in age related cognitive dysfunction and loss of neurons in neurodegenerative diseases. Tea flavanoid containing catechins have been revealed to have radical scavenging, anti-inflammatory and iron chelating properities to protect death of neurons in animal models. In addition to the antioxidant activity of catechins they have a pivotal role in signal modulation pathways and mitochondrial function.

A report documented by Prasad *et al.*, (2014) investigated the potency of geranoil to reduce acrylamide (ACR)-induced mitochondrial dysfunction, neurotoxicity and oxidative stress in rat model in comparison with that of curcumin. Growing rats were administered with ACR for 4 weeks and they show typical symptoms of neuropathy, after ACR administration same rats were provided with oral supplements of phytochemicals. The result showed that both the phytochemicals mitigate ACR-induced oxidative stress by decreasing levels of malondialdehyde reactive oxygen species and resume the decreased glutathione levels in brain.

Chapter: 4

Rationale and Scope of study

RATIONALE AND SCOPE OF STUDY

Organophosphate compounds nowadays used widely in industries, agriculture and household products that cause neurotoxicity by inhibiting acetylcholinesterase which is responsible for the termination of neurotransmitter acetylcholine. The termination that leads to various neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and many more related to neurotoxicity. Due to side effect nature, modern based psychoactive drugs have met with restrained success. The demand for herbal substances based on therapeutic information in recent times has been increased at an alarming rate in both developed and under developed countries that focuses multiple pathways to improve mental capabilities without causing multi-factorial disease. The rationale of this study was to evaluate the comparison of different nutraceuticals *viz., B. monnieri*, green tea and curcumin on neurotoxicity triggered by the powerful insecticide chlorpyrifos in a model organism *D. melanogaster*. The aim of this study was to focus on the most effective nutraceuticals among *B. monnieri*, green tea and curcumin. As from centuries curcumin is being consumed in a daily routine as a rich spice and these days green tea has also become a routine drink for the people who got to know its antioxidant properities. Also, *B. monnieri* which is known to play the neuroprotective role can also be consumed daily as of it is available at Ayurvedic stores and is cost effective. Scope of this study was to enlighten the use of these nutraceuticals for their advantages to may human related neurodegenerative diseases which needs further investigation and can be explored by promoting their properities so that they can also be used against genotoxicity and further more.

Chapter: 5

Objectives of Study

OBJECTIVES OF STUDY

- To examine and compare anti-oxidant capacity, total phenol and total flavanoid content of nutraceuticals (*viz.*, *Bacopa monnieri,* green tea and curcumin.
- To analyze the comparative effect/s of *Bacopa monnieri*, green tea and curcumin at organismal level (negative geotaxis/climbing assay) in chlorpyrifos treated *Drosophila melanogaster.*
- To study the comparative effects of *Bacopa monnieri*, green tea and curcumin at cellular level (measurement of acetylcholinestarase (AchE) in chemical induced organisms.

Chapter: 6
Material and Methods

MATERIAL AND METHODS

Fly stock: *Drosophila melanogaster* (Oregon R+).

Drosophila culture media:

Maize, sulfur free sugar, Agar-agar (Cat#033004, CDH), Yeast Granules (baker's yeast), [all procured from local marker, Jalandhar, Punjab].

Chemicals: Methyl-p-Hydroxybenzoate (Cat# MCR-4809, MOLYCHEM), Propionic acid (Cat#3463, LOBA chemie).

Nutraceuticals: ***B. monnieri***, Green Tea, *C. longa* [Curcumin (Cat#020031, CDH)].

Total phenolic and flavanoid content: Sodium carbonate (Cat#431, TITAN BIOTECH LTD), Folin-ciocalteu's reagent (Cat#835020, CDH), Gallic acid (Cat#0391000500 LOBA chemie), Aluminum chloride (Cat#0089600500, LOBA chemie), Quercetin (Cat#8902729586051, HIMEDIA), Methanol (Cat#0019505000, LOBA chemie), Potassium acetate (Cat#0532200500, LOBA chemie).

DPPH scavenging test: Methanol, DPPH (Cat#0000226382 HIMEDIA), L- Ascorbic acid (MOLYCHEM).

Negative geotaxis assay: Chlorpyrifos (ALDRIN TC), Dimethyl sulfoxide (Cat#0012300500, LOBA chemie), Diethyl ether (Cat#0010300500, LOBA chemie).

Acetylcholine assay: NaH_2PO_4 (Cat#0585800500, LOBA chemie), Na_2HPO_4 (Cat# Art-5971, LOBA chemie), DTNB [Dithiobis 2-Nitrobenzoic Acid (Cat#000213528, HIMEDIA), Acetylcholine (Cat#005500010, LOBA chemie).

EQUIPMENTS REQUIRED:

Digital balance (Ohaus), Visible Spectrophotometer (Systronic), Vortex, Heating mantle (REMI), Centrifuge (REMI), B.O.D incubator (REMI), pH meter.

DROSOPHILA CULTURE AND REARING:

Drosophila (Oregon R^+) fly stock was bought from D.A.V College Chandigarh Punjab and the culture media was prepared according to Singh *et al*., 2009, 2010, 2011 and composition of media is as under:

1 unit food (360 ml):

Maize powder	17	gm
Water	360	ml
Sulphur free sugar	15	gm
Agar Agar	1.5	gm
Yeast	6	gm
Methyl paraben salt	1.5	gm (mixed with 3-4 ml 90% ethanol)
Propionic acid	1	ml

PROCEDURE:

Take 200ml of tap water in 1 litre beaker and boil it, after boiling add to it 1.5 gm of Agar media and leave it for 5 minutes. Now add maize and sugar already soaked in 100 ml of water and stir it continuously and boil it for 10-15 minutes. After thicknesses add yeast previously soaked in 60 ml of water and stir it, cook for 15 minutes. Add 2-3 ml of methyl paraben in boiling food. Stop flame and add 1 ml of Propionic acid, mix it well and pour in conical flasks/vials. Wipe out all the moisture from the vials/conical flasks before transferring flies.

SAMPLE PREPARATION:

***Bacopa monnieri* (Brahmi):**

Two types of *Bacopa monnieri* (Brahmi) were used for this study that includes Kashmiri Brahmi and other one was Commercial Brahmi (Purchased from Patanjili store, Jalandhar). Kashmiri Brahmi was purchased from Agro Food Processing Emporium Peerbagh, Srinagar, J&K and was identified by plant taxonomist Dr. Arbeen Ahmad Bhat, Assistant Professor, Lovely Professional University, Phagwara Punjab.

One gm of each *B. monnieri* (commercial, Kashmiri) was added in 10 mL of distilled water individually and was soaked for 24 hrs. Next, day other 1 gm of each *B. monnieri* (commercial,

Kashmiri) was added in 10 ml of distilled water and was individually boiled for 10-15 minutes. Both the boiled as well as soaked *B. monnieri* were centrifuged at 3000 rpm for 10 minutes and after that supernatant was poured in another centrifuge tube for evaluation (Hosamani and Muralidhara, 2009).

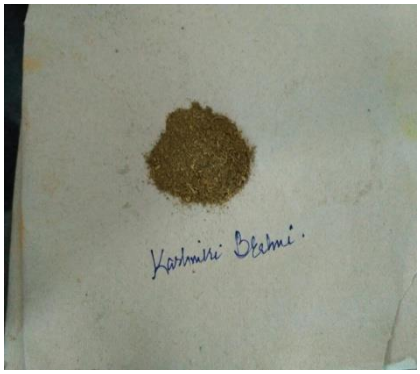


Fig.; 1(a) KASHMIRI *B. monnieri*

Fig., 1(b) COMMERCIAL *B. monnieri*

<u>Green tea</u>:

Green tea from Bud White Company was purchased through online. 2% green tea was prepared by boiling 0.2 gm of green tea in 10 ml of distilled water for 5 minutes at 95^0C using thermometer for accurate temperature. After 5 minutes it was left to cool for 2-3 minutes at room temperature and the extract was filtered through Whatman's filter paper and the filtrate was used for further evaluation (Unachukwu *et al.*, 2010)

Fig., (2) GREEN TEA

***C. longa* (Curcumin):**

Curcumin was available in the labarotory of Lovely Professional University Phagwara, Punjab in the department of Bioengineering and Biosciences.

Final concentration of 0.1mM solution was prepared by dissolving 36.8 mg of curcumin in ml of dimethyl sulfoxide (DMSO) as of it is insoluble in water and the solution was used for evaluation.

Fig., (3) CURCUMIN

TOTAL PHENOLIC AND FLAVANOID ASSAY

Determination of total Phenolic content: (Baba and Malik, 2015)

The total polyphenol content (TPC) was determined by using Gallic acid as standard for 6 extracts simultaneously. *B. monnieri* (Kashmiri/Commercial), green tea, curcumin (0.05 ml) from each extract was diluted in 2.95 ml of distilled water to make the volume of 3 ml in each separate tube and the each extract was taken in triplicates. Now 0.2 N Folin-ciocalteu's reagent was prepared and from this reagent 5 ml concentration was added in each test tube and was incubated for 5 minutes at room temperature. Following 5 minutes 2.0 ml 7.5% of sodium carbonate was added in each tube. The absorbance was measured at 650 nm following 30 minutes of incubating at room temperature. The standard curve was set up by 20-100 µg/ml solution of Gallic corrosive in methanol. The aggregate phenolic content was measured in reference to Gallic acid standard curve.

DPPH Radical Scavenging Assay: (Zhu *et. al.*, 2006)

The characterization of DPPH as a free radical molecule is based on the diminution of stable DPPH after accepting hydrogen from a cancer preventing compound. Radical scavenging activity of extracts [*(B. monnieri* (Kashmir/Patanjili), green tea and curcumin] against stable DPPH was determined spectrophotometrically. A 0.1 Mm solution of DPPH

solution was prepared by dissolving 3.9 mg of DPPH in 100 ml of methanol. DPPH was diluted in 50% methanol to obtain the optical density (OD) between 0.8–1.0. Different concentrations of extracts [(*B. monnieri* (Kashmir/commercial), green tea and curcumin] were added to every 2 mL of DPPH solution (Mensor *et al.* 2001). The solution was incubated for 30 minutes at room temperature and discoloration of the purple to yellow colour of DPPH solution was measured at 520 nm using a visible spectrophotometer. 2ml of DPPH solution was taken as a control and methanol was taken as blank for this assay. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following relationship

$$\% \text{ scavenging activity} = \frac{A_{520}(\text{control}) - A_{520}(\text{sample})}{A_{520}(\text{control})} \times 100$$

TREATMENT PROTOCOL

Treatment groups:

Freshly enclosed/emerged flies were used for experiment.

Group 1: control/untreated.

Group 2: Chlorpyrifos treated.

Group 3: Chlorpyrifos treated+*Bacopa monnieri* (Brahmi)

Group 4: Chlorpyrifos treated+green tea.

Group 5: Chlorpyrifos treated+curcumin.

Chlorpyrifos Treatment:

Stock concentration of 2000 ppm was calculated from 20% EC chlorpyrifos. 10 µl of chlorpyrifos from stock concentration was mixed with 990 µl of Dimethyl sulfoxide (DMSO) and after that the final concentration was taken as 1 ppm. 1 ppm was prepared by taking 25 µl from stock solution and was mixed with 50 ml food (Gupta *et al*., 2010).

Chlorpyrifos + *Bacopa monnieri* Treatment:

Chlorpyrifos exposed flies were provided with several concentrations (1, 0.1 and 0.01%) of *B. monnieri.* Both Kashmiri and commercial *Bacopa monnieri* were used and were screened in order to govern whether the treatment has any effect on the survival of experimental flies. Although, for the better study only one final concentration of Commercial *Bacopa monnieri* viz., 0.01% (50 µl) per 50 ml of medium was chosen as optimum concentration (Hosamani and Muralidhara, 2009).

Chlorpyrifos + Green tea Treatment:

25µl of chlorpyrifos and 0.01% (50 µl) final concentration of green tea were added to 50 mL of food and after that the flies were exposed to it.

Chlorpyrifos + Curcumin Treatment:

0.1 mM of stock concentration was prepared and the final concentration was taken as 0.1%. 25µl of chlorpyrifos and 0.1% (50 µl) of curcumin was added to 50 ml food and flies were exposed to it (Singh et al., 2011).

NEGATIVE GEOTAXIS ASSAY

Climbing assay was performed according to the procedure of Sharma et al., (2012). Twenty treated adult flies per treatment taken in triplicates were transferred in a vertical plastic cylinder (16 cm length × 2 cm diameter). Flies that crossed the 10 cm line within 20 s from the time they were tapped from bottom of the cylinder were counted. The climbing counts denote the mean percentage of flies that crossed the 10 cm line out of the total number of flies per experiment. The scores represent the mean of number of flies above 10 cm (n_{top}) and below 10 cm (n_{bot}), expressed as the percentage of total number of flies (n_{tot}). Performance index (PI) was also calculated for each experiment as;

$PI = ½[(n_{tot} + n_{top} - n_{bot})/n_{tot}]$.

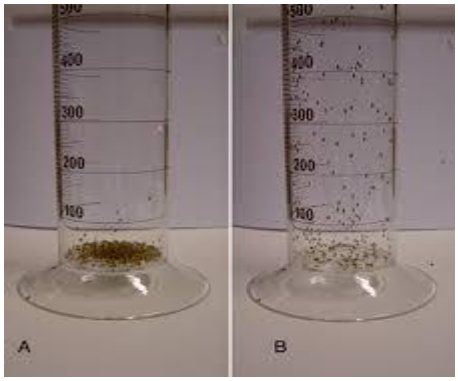


Fig., 4: **MEASURING CYLINDER**

BIOCHEMICAL ASSAY

ACETYLCHOLINESTARASE ACTIVITY:

Preparation of Chemicals:

1) 0.1M phosphate buffer:

Solution A: 1.41 gm of sodium dihydrogen phosphate (NaH_2PO_4) was dissolved in 100 ml of distilled water.

Solution B: 1.56 gm of disodium phosphate (Na_2HPO_4) was dissolved in 100 ml of distilled water.

77.4 ml of solution A was added to 22.6 ml of solution B to get the desired pH 7.0 or 8.0.

2) DTNB reagent: 0.1mM of DTNB [Dithiobis (2- Nitrobenzoic acid)] reagent was chosen for this study. It was prepared by dissolving 39.6 mg of DTNB with 15 mg of sodium bicarbonate ($NaHCO_3$) in 10 ml of 0.1M phosphate buffer (pH 7.0).

3) Acetylthiocholine (ATC):

0.1mM of acetylcholine was prepared by dissolving 21.67 mg of acetylthiocholine was dissolved in 1 ml of distilled water. Acetylcholinesterase (AchE) is the primary cholinesterase in the body. It is an enzyme that catalyzes the breakdown of acetylcholine and of some other choline esters that function as neurotransmitters. This enzyme present predominantly at neuromuscular junctions and in chemical synapses of the cholinergic type, where its activity serves to terminate synaptic transmission. Acetylcholinesterase (AchE) catalyzes the hydrolysis of neurotransmitter acetylcholine into choline and acetic acid and AchE activity was measured by following detailed protocol of (Srikumar, *et al*., 2004). Estimation of AchE activity also called as Ellman's method named after George Ellman who introduced this method in 1961. Esterase activity is measured by acetylcholine (ATC) which is used as an artificial substrate. The breakdown of ATC by AchE releases thiocholine and is allowed to react with –SH reagent (DTNB) 5, 5´-Dithiobis- (2- Nitrobenzoic acid, a yellow colour anion with an absorption at 412 nm). Extinction coefficient of thio-nitro benzoic acid is 1.36 × 104/molar/centimeter and its concentration is then detected by UV spectrophotometer which is used to determine AchE activity. Thirty adult flies were used per treatment in triplicates for this experiment. After 6 hrs

of exposure to the treatment food the treated flies transferred into empty vials (14 cm length × 2 cm diameter) were placed in a freezer for 10 minutes. After that the flies were weighed (average 95 mg per 30 flies) and 10% homogenate was made by crushing the flies in 950 µl of 0.1M phosphate buffer. The homogenate mixture was then centrifuged at 10000 rpm for 10 minutes at 4^0c. 100 µl of supernatant was then added to cuvette containing 650 µl of 0.1M phosphate buffer (pH, 7 or 8) and 25 µl of DTNB. The contents of cuvette were mixed thoroughly and absorbance was measured at 412 nm. 5 µl of substrate i.e., acetylcholine was added and change in absorbance was recorded for a period of 3 minutes at an interval of 1 minute.

STATISTICAL ANALYSIS

The data in this study was stated as mean ± S.D for triplicate samples. The statistical comparison was performed by independent/unpaired t-test using online GraphPad software (Prism 7). If the *p*-values are 0.05 or less, the results will be considered statistically significant.

Chapter: 7

RESULT & DISCUSSION

TOTAL PHENOLIC CONTENT:

The total phenolic content (TPC) of [(*B. monnieri* (2 Kashmir/ 2 commercial)], green tea and C.longa (curcumin)] was determined by Folin-ciocalteu's assay by using Gallic acid as standard phenolic compound. A standard curve of Gallic acid in range of (20-100 µg/ml) with a coefficient of determination (R^2) value was equal to 0.963 (fig. 5).

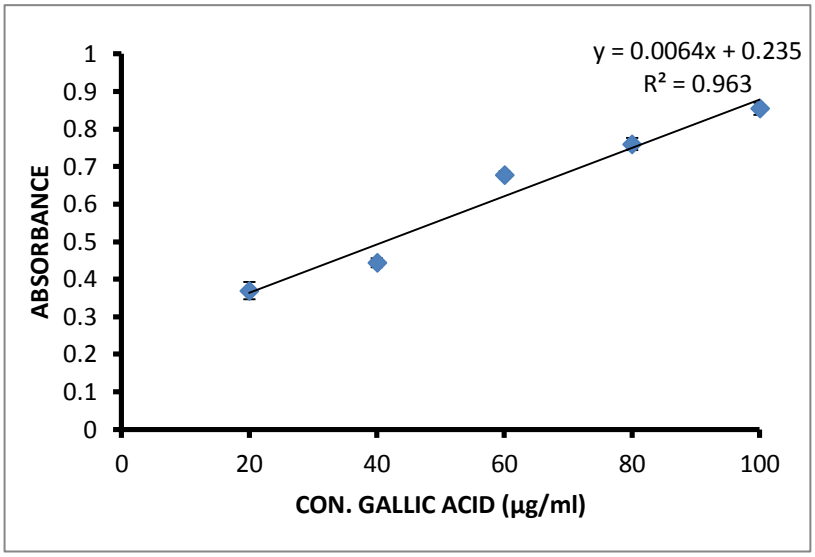


Fig., 5: Standard calibration curve of total phenolic content

Table 1: Total phenolic content (mean ± sd,n =3)

Sample	**Sample (µl)**	**total phenolic content mg/GAE/g**
Soaked Kashmiri *B. monnieri*	SKB (50 µl)	3.16 ± 1.858
Boiled Kashmiri *B. monnieri*	BKB (50 µl)	4.220 ± 2.682
Soaked commercial *B. monnieri*	SCB (50 µl)	1.3867 ± 0.252
Boiled Commercial *B. monnieri*	BCB (50 µl)	4.3707 ± 0.455
Green Tea	Gr. tea (50 µl)	99.667 ± 3.547
curcumin	(1 µl)	2.834± 0.069

The total phenolic content (TPC) was judged in two types of *B. monnieri* samples (Kashmir region and another commercial one), green tea and *C.longa* (curcumin) and shown in table 1. *B. monnieri* (Kashmiri and commercial), green tea and curcumin elicited 3.16±1.858, 4.220±2.682, 1.3867±0.252, 4.3707 ± 0.455, 99.667±3.547, 2.834±0.069 mg/GAE/g respectively (shown in table 1). In the present study Kashmiri (soaked and boiled) and commercial (soaked and boiled) *B.monnieri* in aqueous extract showed the total phenolic content as of 3.16±1.858, 4.220±2.682, 1.3867±0.252, 4.3707 ± 0.455 mg /GAE /g. In this context previous report showed that quantity of the total phenolic content in methanolic extract of *B. monnieri* possesses 24.75mg/GAE/g (Jain *et al*., 2017). Another study has showed that the phenolic content of *B. monnieri* in ethanolic and aqueous extract as of 3.18 and 3.71 mg/GAE/g (Mukherjee *et al*., 2011) Despite of having different extracts there is no or less variation in comparison to the previous study.

Polyphenols play a pivotal role in scavenging free radicals and are consumed in our daily routine life and the phenolic content of green tea aqueous extract in this study is 99.67±3.547 mg/GAE/g. From previous study it has been investigated that the phenolic content in methanolic extract of green tea possesses 24.3 mg/g (Taheri *et al*., 2011). Another study reported the phenolic content of green tea using Gallic acid as positive control as 7.72 mg/GAE/g dry tea. Variation in results might be due to having different solvents for tea extraction.

Spices and herbs are good source of phenolic compounds like carotenoids, flavanoids that are rich in antioxidants. In the present study the phenolic content of curcumin is 2.834±0.069. From the previous study it was found that the phenolic content in curcumin was 0.6789 mg/GAE/g [(67.89 mg/GAE/100g extract)] (Maizura *et al*., 2011). Another study used the two solvents at their different concentrations i.e ethanol (60% and 80% respectively), and methanol (60% and 80% respectively). The phenolic content of curcumin was 6.787 mg/GAE/g, 7.457 mg/GAE/g and 0.538 mg/GAE/g, 6.824 mg/GAE/g. The higher phenolic content was found in ethanol (80%) (Tanzeela *et al*., 2015)

On comparing our nutraceuticals on the basis of total phenolic content green tea has the highest phenolic content and soaked commercial *B. monnieri* the least.

TOTAL FLAVANOID CONTENT:

Flavanoid are the group of polyphenols that reduce the effect of free radicals that cause chronic stress related to human and animal diseases. A standard curve of Quercetin was in range of (2-10 µg/ml). The total flavanoid content was determined by Quercetin equivalent/g of extract as standard curve with (R^2) equal to 0.974 (fig. 6).

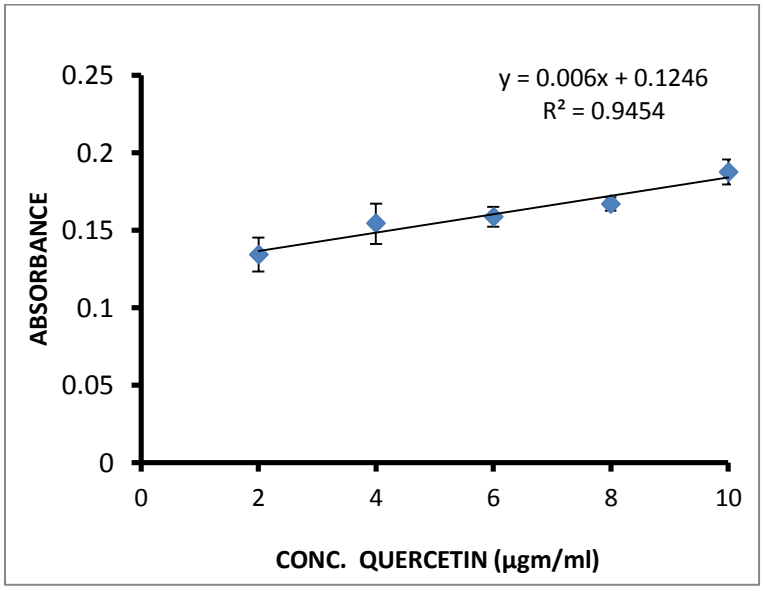


Fig., 6: Standard calibration curve of total flavanoid content

Table 2: Total flavanoid content (mean ± sd,n =3)

Sample	**Sample (µl)**	**total flavanoid content mg/QE/g**
Soaked Kashmiri *B. monnieri*	SKB (50 µl)	2.066 ± 0.553
Boiled Kashmiri *B. monnieri*	BKB (50 µl)	3.6561 ± 0.635
Soaked Commercial *B. monnieri*	SCB (50 µl)	1.4 ± 1.1112
Boiled Commercial *B. monnieri*	BCB (50 µl)	4.1 ± 0.0724
Green Tea	Gr. tea (50 µl)	7.633 ± 2.532
Curcumin	(1 µl)	1.166 ± 0.069

The total flavanoid content (TFC) was judged in two types of *B. monnieri* samples (Kashmir region and another commercial one), green tea and curcumin and shown in table 2. *B. monnieri* (Kashmiri and commercial), green tea and curcumin elicited 2.066±0.553, 3.6561±0.603, 1.4±1.1112, 4.1±0.0724, 7.633±2.532, 1.166±0.069. In the present study Kashmiri (soaked and boiled) and commercial (soaked and boiled) *B.monnieri* in aqueous extract showed the total flavanoid content as of 2.066±0.553, 3.656±0.603, 1.4±1.1112, 4.1 ± 0.0724 mg/QE/g. In this context previous study suggested that the flavanoid content of *B. monnieri* in terms of using Gallic acid as a standard at 10 µg was calculated as 6.3 GAE and at 1000 µg it increased to 29.66 GAE (Jain *et al*., 2017). Another report was investigated and it was found that the flavanoid content in methanolic extract of *B. monnieri* was 24.36 mg/QE/g (Alam *et al.*, 2012).

Flavanoids are secondary metabolites that are necessary for growth and development. The flavanoid content of green tea in this study was 7.633±2.532. However from the previous studies it was found that the phenolic content of green tea in aqueous extract was 23.17 mg/QE/g (Bansode, 2015).

The flavanoid content of curcumin in this study was found as 1.166±0.069 and data from the previous studies did not provide any information for its flavanoid content.

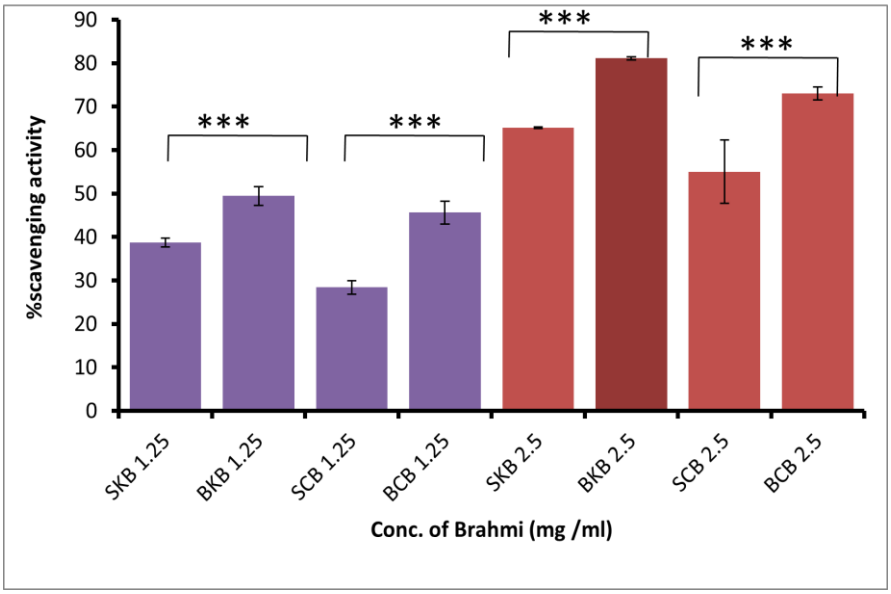
On comparing our nutraceuticals on the basis of total flavanoid content green tea has the highest phenolic content and curcumin the least.

***1, 1– Diphenyl-2-picryl-hydrazyl* (DPPH) Radical Scavenging Assay:**

DPPH is an oxidizing radical that can be reduced by the antioxidants. Nutraceuticals which includes [*B. monnieri* (2 Kashmir/ 2 Commercial), green tea and curcumin] are rich source of antioxidants. Ascorbic acid was used as standard at concentration of 5-10 µg/ml

A) *B. monnieri* (Brahmi):

The percentage scavenging effect of *B. monnieri* on DPPH free radical was enhanced with the increase in concentration of each extract from 1.25–2.5 mg/ml that is clearly shown in figure 7(a) and Table (3). The percentage inhibition was varying from 28.39% - 81.13% of *B. monnieri* (Brahmi) extract.



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Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 7(a): % DPPH radical scavenging activity of Brahmi (Kashmiri/commercial)

In this study the IC_{50} value for boiled Kashmiri *B. monnieri* (BKB) and soaked Kashmiri *B. monnieri* (SKB) is 1.265 mg/ml and 1.6814 mg/ml. The IC_{50} value for boiled commercial *B. monnieri* (BCB) and soaked commercial *B. monnieri* (SCB) is 1.395 mg/g and 2.193 mg/g. Previous studies showed that the IC_{50} value for Brahmi was 0.45 mg/ml and 0.764 mg/ml using ascorbic acid as standard (Alam *et al*., 2012 and Mukherjee *et al*., 2011). From the above studies it was found that the less the IC_{50} value more will be the scavenging activity. Here the variation in the present study is due to sample extraction in different solvents and the fresh leaves were used for their study.

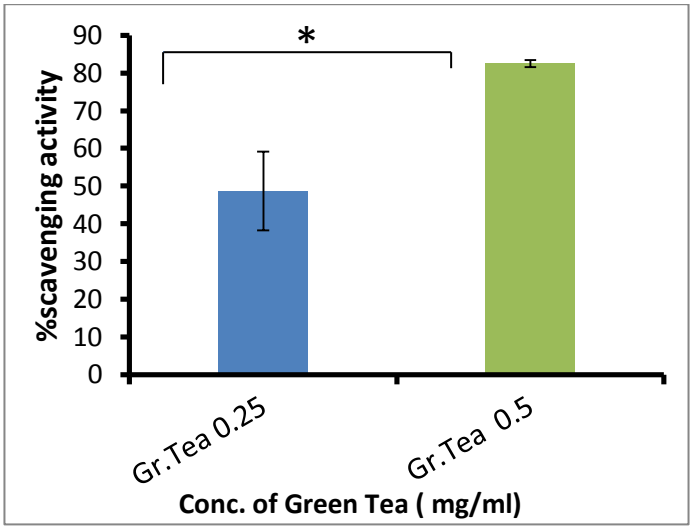
Table 3(a): DPPH radical scavenging activity of *B. monnieri* (Kashmiri/commercial) (mean±sd, n=3)

Brahmi (µl)	**Con.mg/ml**	**%Scav. activity**	**IC 50**
Soaked Kashmiri *B.monnieri* 25	1.25	38.696 ± 0.9844	1.6814
Soaked Kashmiri *B. monnieri* 50	2.5	65.123 ± 0.1768	
Boiled Kashmiri *B. monnieri* 25	1.25	49.421 ± 1.562	1.265
Boiled Kashmiri *B. monnieri* 50	2.5	81.1343 ± 7.287	
Soaked Commer *B. monnieri* 25	1.25	28.395 ± 2.199	2.193
Soaked Commer *B. monnieri* 50	2.5	55.015 ± 0.347	
Boiled Commer. *B. monnieri* 25	1.25	45.640 ± 2.624	1.395
Boiled Commer. *B. monnieri* 50	2 .5	73.032 ±1.504	

On comparing the Kashmiri and commercial *B. monnieri* the highest scavenging activity was found in boiled Kashmiri *B. monnieri* (81.13±7.28) with an IC_{50} value of 1.265.

B) Green Tea:

The percentage scavenging effect of green tea on DPPH radical was raised with increase in the concentration of each extract from 0.25–0.5 mg/ml that is clearly seen in the figure 7(b). The percentage inhibition was varying from 48.66 % - 82.47 % of green tea extract.



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 7(b): % DPPH radical scavenging activity of green tea.

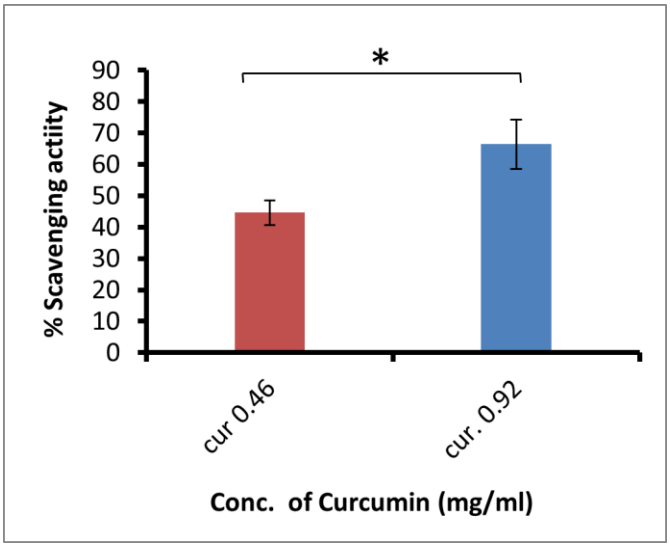
The present study showed the % scavenging activity of green tea varies from 48.66 % - 82.47% at concentration of 0.25 – 0.5 mg/ml and the IC_{50} value for green tea is 0.2569 mg/ml. From the previous reports it was found that green tea has the % inhibition activity of 80.3 % in methanolic extract (Taheri *et al.*, 2012). Another study reported that the IC_{50} value for green tea on using Gallic acid and Ascorbic acid as a positive control was calculated as 0.02326 mg/ml. One more study was investigated and it was found that the green tea has the IC_{50} value as 0.0195 mg/ml (Manian *et al.*, 2008).

Table 3(b): DPPH radical scavenging activity of green tea (mean±sd, n=3)

Conc.mg/ml	% Scav. Activity	IC50
Gr.Tea 0.25	48.66 ± 10.416	0.2569
Gr.Tea 0.5	82.47 ± 0.973	

C) Curcumin:

The percentage scavenging effect of curcumin was increased on DPPH radical as the concentration of each extract was raised from 0.46–0.92 mg/ml shown in fig. 7(c). The percentage inhibition was varying from 44.63% - 66.46 % of curcumin extract.



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 7(c): % DPPH radical scavenging activity of curcumin

The present study suggested that the % scavenging activity of curcumin varies from 44.63±3.903% - 66.46±7.88% at a concentration of 0.46 – 0.52 mg/ml and the IC_{50} value of curcumin 0.545.Studies revealed that the curcumin has % scavenging activity of each sample against DPPH ranging from 55.60 to 71.64% at a concentration 0.001- 0.005 mg/ml. The IC_{50} value was found to be 0.00108 mg/ml (Borra *et al.*, 2013). One more study of (Maizura *et al.*, 2011) revealed that the curcumin has the % scavenging activity of 64.6 ± 2.4%

Table 3(c): % DPPH radical scavenging activity of curcumin (mean±sd, n=3)

Conc. mg/ml	% Scav. Activity	IC 50
0.46	44.63±3.903	0.545
0.92	66.46±7.883	

On comparing the above results for Brahmi, green tea and curcumin for DPPH we found that

The green tea has the highest scavenging activity with an IC_{50} value of 0.2569 and boiled Kashmiri Brahmi has the least with IC_{50} value of 1.265

Climbing/Negative geotaxis assay:

Negative geotaxis assay was performed according to the treatment protocol per triplicates for 6hrs, 24hrs and 48hrs. During this time interval locomotory activity was determined among the untreated control flies and treated flies [chlorpyrifos (chlor.), chlor+Brahmi, chlor.+Gr.Tea, chlor.+ Curcumin] shown in table [4 (a, b and c)]

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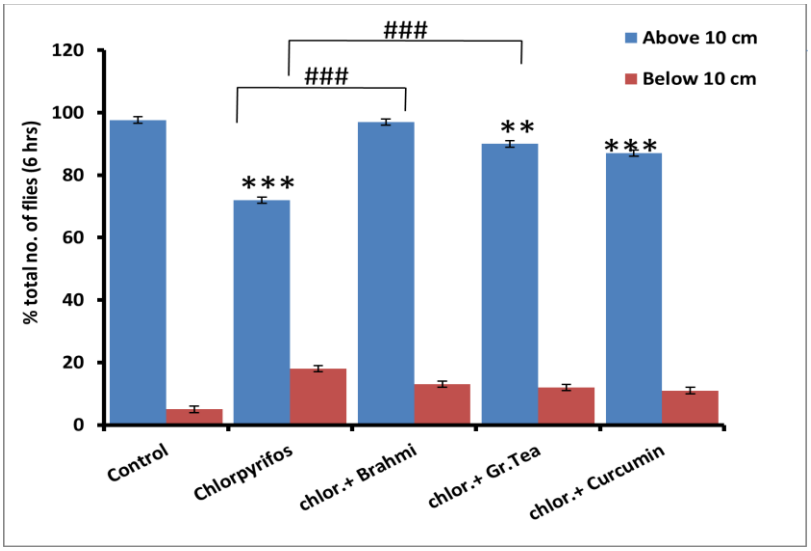
After 6 hrs:



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 8(a):% no. of flies above 10 cm (n^{top}) and below 10 cm (n^{bot}) after exposure of 6 hrs.

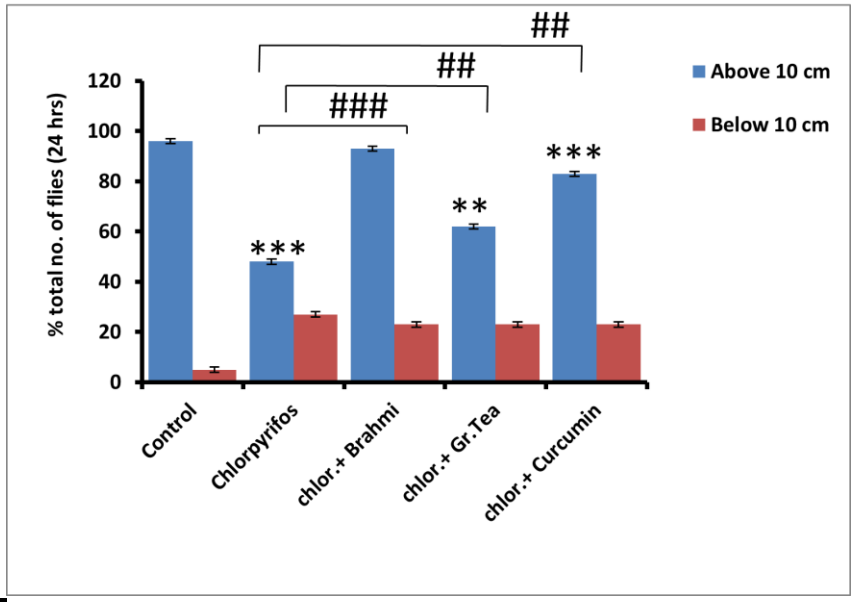
Data observed in a negative geotaxis among chlorpyrifos revealed time dependent locomotor deficits. After exposure of flies for 6 hrs, 97% of control untreated flies were

able to cross 10 cm within 20 seconds, while as in chlorpyrifos treatment, flies show significant decrease in climbing ability and also significant increase in mortality. Flies co-treated with *B. monnieri*, green tea and curcumin improved the performance of flies. With *B. monnieri* and green tea treatment more number of flies show negative geotaxis (97% and 90%) and the significant effect was also found in curcumin (86%) indicating clearly their neuroprotective effect

Table 4(a): % no. of flies above 10 cm (n^{top}) and below 10 cm (n^{bot}) after exposure of 6 hrs of treatment. (mean±sd, n=3)

	% Total no. of flies (6 hrs)				
	Control	**Chlorpyrifos**	**Chlor.+Brahmi**	**Chlor.+Gr.Tea**	**Chlor.+ Curcumin**
Above 10 cm	97±2.309	71±2.88	97±1.154	90±7.637	86±5.77
Below 10 cm	4± 0.028	18±2.88	13±1.66	12±2.88	11±2.88

After 24 hrs:



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

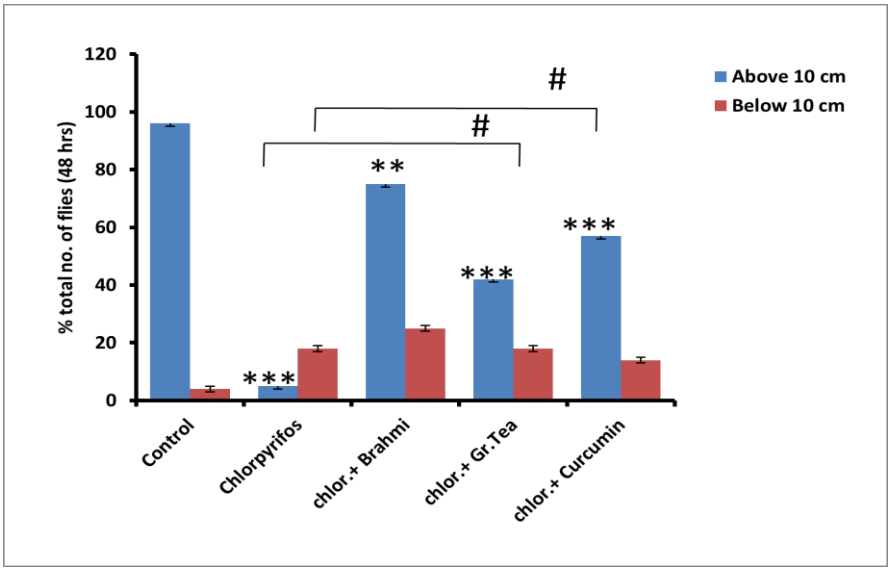
Fig., 8(b): % no. of flies above 10 cm (n^{top}) and below 10 cm (n^{bot}) after exposure of 24 hrs of treatment.

The flies exposed for 24 hrs revealed that among untreated control, 95% of flies were able to cross 10 cm within 20 seconds, while as in chlorpyrifos treatment flies show decreased tendency in climbing ability and also increased the mortality rate among flies. Flies co-treated with *B. monnieri*, green tea and curcumin improved the performance of flies .With *B. monnieri* treatment 93% flies were able to cross 10 cm within a specific time period. The green tea treated flies show synergetic effect as there was higher mortality rate as compared to *B. monnieri* and curcumin after 24 hrs while as the locomotor deficit was improved as more number of flies show negative geotaxis (62%). Flies treated with curcumin show better locomotor ability as compared to green tea (82%)

Table 4(b): % no. of flies above 10 cm (n^{top}) and below 10 cm (n^{bot}) after exposure of 24 hrs of treatment (mean±sd, n=3).

	% Total no. of flies (24 hrs)				
	Control	**Chlorpyrifos**	**Chlor.+Brahmi**	**Chlor+Gr.Tea**	**Chlor.+ Curcumin**
Above 10 cm	95±0	48.33±2.88	93±5.77	62±2.88	83±2.88
Below 10 cm	5± 0	26.6±2.88	23±2.88	23±1.154	23±1.52

After 48 hrs



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 8(c): % no. of flies above 10 cm (n^{top}) and below 10 cm (n^{bot}) after exposure of 48 hrs of treatment.

After exposure of flies for 48 hrs it was observed that among the untreated control, 95% of flies were able to cross 10 cm within 20 seconds, while as in chlorpyrifos treatment large number of flies showed capability to stay at bottom of measuring cylinder. Only 5% were able to cross the 10 cm within 20 seconds and also there was higher mortality rate among flies. Flies co-treated with *B. monnieri*, green tea and curcumin improved the performance of flies .With *B. monnieri* treatment 75% flies were able to cross 10 cm within a specific time period. The green tea treated flies show significant synergistic effect as there was higher mortality rate as compared to *B. monnieri* and curcumin after 48 hrs while as the locomotor deficit was improved as fewer number of flies show negative geotaxis (42%). Flies treated with curcumin show better locomotor ability as compared to green tea and lesser mortality (57%).

Table 4(c): % no. of flies above 10 cm (n^{top}) and below 10 cm (n^{bot}) after exposure of 48 hrs of treatment (mean±sd, n=3).

	% Total no. of flies (48 hrs)				
	Control	**Chlorpyrifos**	**Chlor.+Brahmi**	**Chlor.+Gr.Tea**	**Chlor.+ Curcumin**
Above 10 cm	95±2.30	5±0.577	75±5	42±7.63	57±5.77
Below 10 cm	4±1.733	18±2.88	25±5	18±5.77	14±1.732

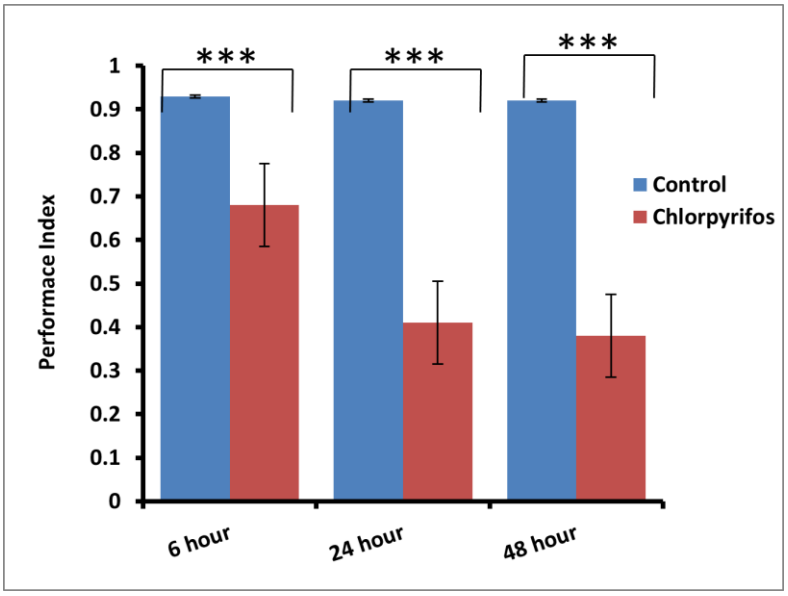
In the present study the negative geotaxis assay revealed the time dependent exposure of flies to various treatment groups for 6, 24 and 48 hrs respectively. After comparing the locomotory ability and lesser mortality of various treated flies for specific time duration, it was found that the *B. monnieri* showed better negative geotaxic behaviour clearly suggests its neuroprotective role and lower mortality rate among flies treated with *B. monnieri* clearly showed the presence of bioactive compounds in it. It was also revealed that green tea also increases the locomotor ability among chlorpyrifos induced flies but there was a significant synergetic effect which causes the higher mortality among flies as compared to *B. monnieri* and curcumin. Previous study suggested that the rotenone induced 48% of mortality among flies and caused locomotor deficits as 95% of flies were not able to reach the top of cylinder for 1 minute. With *B. monnieri* treatment more number of flies (45-65%) showed better negative geotaxic behaviour (Hosamini and Muralidhara, 2009). Another study documented that the flies exposed to paraquat increased locomotor deficits, while as untreated flies were able to reach 7 cm height of test tube. In contrast flies pre fed with curcumin before treatment of paraquat showed higher locomotor activity (Park *et a*l., 2012). There is no study on the evidence of negative geotaxis in green tea that might be because of its synergistic effect.

PERFORMANCE INDEX

The Performance Index for each experiment was calculated by;

$PI = ½[(n_{tot} + n_{top} - n_{bot})/n_{tot}]$

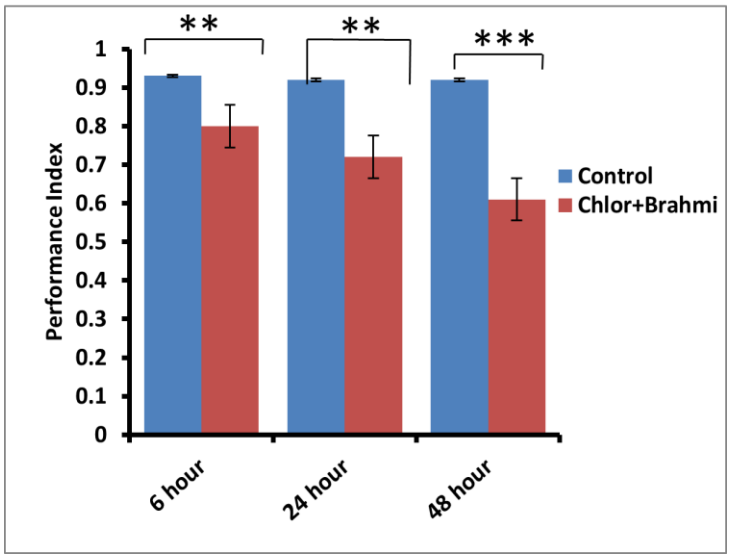
Chlorpyrifos treated:



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 9(a): Performance Index of flies treated with chlorpyrifos.

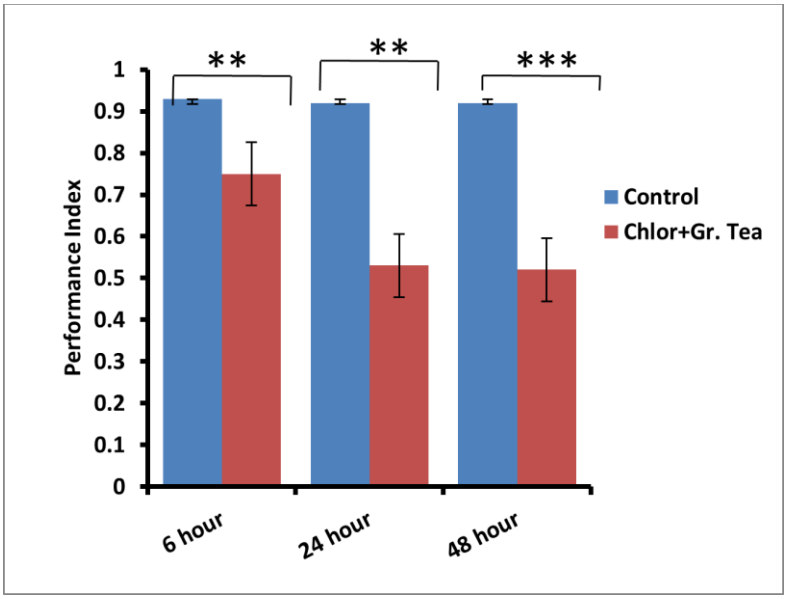
Chlorpyrifos+ Brahmi treated:



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 9(b): Performance Index of flies treated with chlorpyrifos+Brahmi.

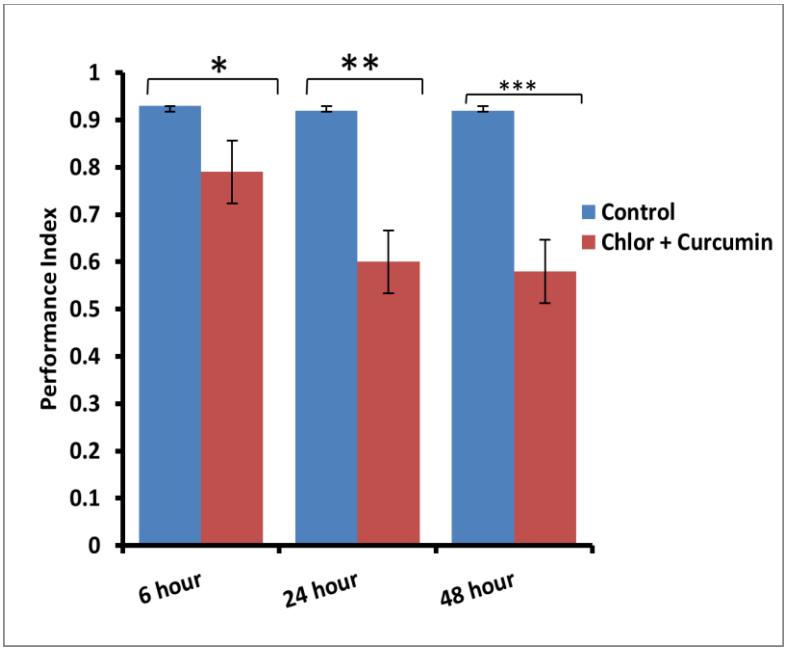
Chlorpyrifos+green tea:



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 9(c): Performance Index of flies treated with chlorpyrifos+green tea.

Chlorpyrifos+curcumin:



Each value was stated as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 9(d): Performance Index of flies treated with chlorpyrifos+curcumin.

In the present study the performance index was calculated in each experiment for 6, 24 and 48 hrs as shown in figures 9(a, b, c and d) and table (5). After comparing the control and treated flies the performance index was found higher in *B. monnieri* treated flies as compared to green tea and curcumin exposed flies.

Table 5: Performance Index of each experiment for negative geotaxis assay (mean±sd, n=3).

Performance Index			
	6 hrs	**24 hrs**	**48 hrs**
Control	0.92±0.02	0.92±0.025	0.92±0.02
Chlorpyrifos	0.675±0.025	0.41±0.184	0.38±0.160
Chlor.+Brahmi	0.825±0.025	0.72±0.057	0.61±0.052
Chlor.+Gr. Tea	0.746±0.141	0.53±0.028	0.52±0.025
Chlor.+ Curcumin	0.79±0.123	0.6±0.04	0.58±0.057

Activities of acetylcholinesterase (AchE):

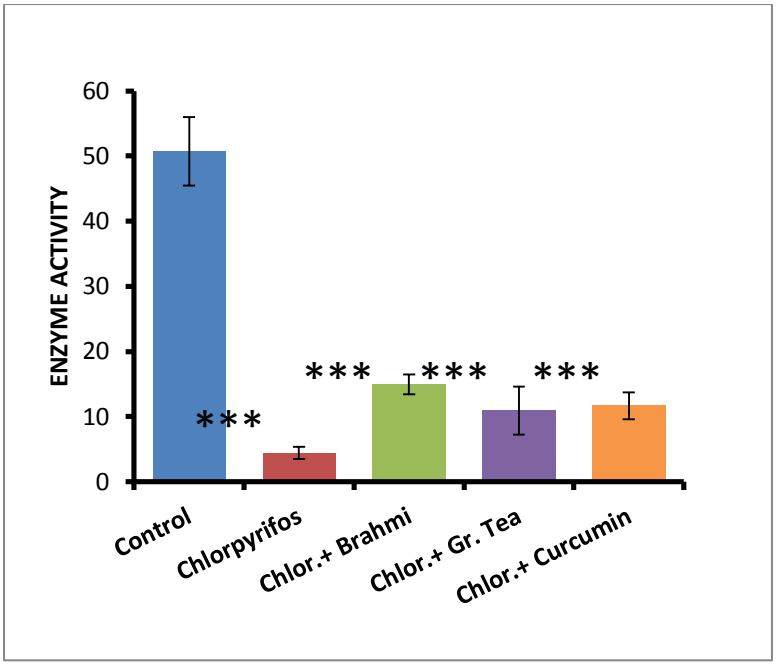
It was determined as described earlier (Srikumar, *et al*., 2004). To the reaction mixture containing 650µl of 0.1M phosphate buffer and 25µl of 5,5-dithiobis 2-nitrobenzoic acid (DTNB), 100µl of homogenate sample and 5 µl of acetylcholine was added and change in absorbance was monitored at 412 nm for 3 minutes at an interval of 1 min. Enzyme Activity was calculated by the following formula;

$R = 5.74 \times 10^{-4} \times A/CO.$

Where, R = Rate in moles of substrate hydrolyzed/minute/gm/tissue

A = Change in absorbance/minute.

CO = Original concentration of tissue (mg/ml).



Each value was expressed as mean±sd (n=3,) *#p≤0.01, **##p≤0.001, ***###p≤0.0001 indicate extremely statistically significant difference at (p≤0.05).

Fig., 10: Acetylcholinesterase activity in the fly brain of control and treated groups

Tab., 6: Acetylcholinesterase activity in the fly brain of control and treated groups (mean±sd,n=3)

	Enzyme Activity (6 hrs) (moles/min/g)
Control	50.73×10^{-4} M ± 5.23
Chlorpyrifos	4.38×10^{-4} M ± 0.93
Chlor.±*B.monnieri*	14.92×10^{-4} M ± 1.51
Chlor.± Gr.tea	10.88×10^{-4} M ± 2.065
Chlor.±Curcumin	11.67×10^{-4} M ± 3.69

In the present study it was found that in chlorpyrifos treated flies the AchE activity was dramatically reduced (4.38×10^{-4} M ± 0.93) in comparison to the control flies (50.73×10^{-4} M ± 5.23). The flies treated with Chlor.± *B. monnieri* (14.92×10^{-4} M ± 1.51), Chlor.± Gr.tea (10.88×10^{-4} M ± 2.065) and Chlor.±Curcumin (11.67×10^{-4} M ± 3.69) showed the AchE activity reduction as compared to control but elevation in comparison with the chlorpyrifos. On comparing the three nutraceuticals it was revealed that there was not so much variation among the nutraceuticals in inhibiting the chlorpyrifos effect. Despite, it was found that the *B.*

monnieri elevates most of the AchE activity by inhibiting the chlorpyrifos that has dramatically reduced the AchE activity in flies.

Previous studies showed that the activity level of AchE enzyme in rotenone-exposed flies treated with B. monnieri was marginally increased (Hosamini and Muralidhara, 2009).

Another study showed that the flies treated with dietary supplement curcumin prior to paraquat treatment reduced AchE activity (Park *et al.*, 2012).

Chapter: 8

Conclusion and future scope

CONCLUSION AND FUTURE SCOPE

Phytoconstituents having antioxidant properities are widely used for protective and therapeutic treatment to various neurodegenerative disease based symptoms. Our present study has aimed to describe the organismal and biochemical treatment in a *Drosophila* model system exposed to chlorpyrifos. We demonstrate here the corroboration on neuroprotective effect of three different nutraceuticals viz., *B. monnieri*, green tea and curcumin against the chlorpyrifos induced toxicity that caused the locomotor deficits and reduce the acetylcholinesterase activity in the flies. We presented here the antioxidant activity levels of three different nutraceuticals that prevents free radicals and suppress oxidative stress. Treatment with tested phytoconstituents to the flies exposed to chlorpyrifos elevates survival rate, impairs locomotory behaviour and also increases the enzyme activity in the ganglia of flies indicating that these substances possess neuroprotective role. Further this study can be explored by evaluating the cause of synergistic effect of green tea that causes the significant mortality. Also, the jumping assay and impulsive effect on different concentrations can be added to the organismal study. Besides acetylcholinesterase activity, butrylcholinesterase activity, depletion of R.O.S levels and modulating the effects of these nutraceuticals on mitochondrial function can be evaluated in future.

Chapter: 9

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