ASSESSMENT OF NEUROPROTECTIVE POTENTIAL OF QUERCETIN IN CADMIUM INDUCED NEUROTOXICITY- *IN SILICO*, *IN VITRO* AND *IN VIVO* APPROACHES

A Thesis Submitted to
Babu Banarasi Das University
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in

Pharmaceutical Sciences

by

Richa Gupta
M. Pharm (Pharmacology)

Co-Supervisor

Dr Manjul Pratap Singh
Senior Assistant Professor
School of Pharmacy
Babu Banarasi Das University
Lucknow – 226 028, India

Supervisor

Dr Vinay Kumar Khanna
Senior Principal Scientist
Developmental Toxicology Laboratory
CSIR – Indian Institute of Toxicology Research
Lucknow – 226 001, India

Babu Banarasi Das University
Lucknow – 226 028 (U. P.), India

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Sincerely dedicated
to
My loving Family

CONTENTS

CERTIFICATE

This is to certify that the thesis entitled **Assessment of Neuroprotective Potential of Quercetin in Cadmium Induced Neurotoxicity – *In silico, In vitro* and *In vivo* Approaches** submitted by Richa Gupta, for the award of degree **Doctor of Philosophy** by Babu Banarasi Das University, Lucknow is a record of authentic work carried out by her under our supervision. To the best of our knowledge, the matter embodied in this thesis is the original work of the candidate and has not been submitted elsewhere for the award of any other degree or diploma.

(Co Supervisor)

Dr Manjul Pratap Singh
Senior Assistant Professor
School of Pharmacy
Babu Banarsi Das University
Faizabad Road
Lucknow-226 208, India

(Supervisor)

Dr Vinay K. Khanna
Senior Principal Scientist
Developmental Toxicology Laboratory
Systems Toxicology & Health Risk Assessment Group
CSIR-Indian Institute of Toxicology Research
MG Marg, Lucknow – 226 001, India

Date:

DECLARATION

I, hereby, declare that the work presented in this thesis entitled **Assessment of Neuroprotective Potential of Quercetin in Cadmium Induced Neurotoxicity – *In silico, In vitro* and *In vivo* Approaches** in fulfillment of the requirements for the award of Degree of **Doctor of Philosophy** of Babu Banarasi Das University, Lucknow is an authentic record of my own research work carried out under the joint supervision of Dr Vinay K Khanna, Senior Principal Scientist, CSIR- Indian Institute of Toxicology Research, Lucknow and Dr Manjul Pratap Singh, Senior Assistant Professor, Babu Banarsi Das University, Lucknow.

I also declare that the work embodied in the present thesis is my original work and has not been submitted by me for any other Degree or Diploma of any university or institution.

Date: **Richa Gupta**

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Abbreviations

Abbreviations

Ach	:	Acetylcholine
AChE	:	Acetylcholinesterase
Ad libitum	:	(L) at one's pleasure
BSA	:	Bovine serum albumin
Bmax	:	Maximum number of binding sites
cAMP	:	Cyclic adenosine monophosphate
CAR	:	Conditioned Avoidance Response
ChAT	:	Choline acetyltransferase
CNS	:	Central nervous system
^{o}C	:	Degree centigrade
DA	:	Dopamine
DBH	:	Dopamine β-hydroxylase
DCFH-DA	:	2`,7`-Dichlorodihydrofluorescin Diacetate
DMSO	:	Dimethylsulphoxide
DOPAC	:	3,4-dihydroxyphenylacetic acid
EDTA	:	Ethylene diamine tetra acetic acid
EPN	:	Epinephrine
ETC	:	Electron transport chain
g	:	Gram
GABA	:	γ-aminobutyric acid
GSH	:	Reduced glutathione
GSM	:	Grip strength meter
H_2O_2	:	Hydrogen peroxide
HVA	:	Homovanillic acid
HRP	:	Horseradish peroxidase
i.p.	:	intra peritoneal
i.v	:	intra venous
Kd	:	Dissociation constant
kd	:	Kilodalton

kg	:	Kilogram
LD	:	Lethal dose
L-DOPA	:	L-3, 4-dihydroxyphenyl-alanine
M	:	Molar
mg	:	Milligram
ml	:	Milliliter
mm	:	Millimeter
mM	:	Millimolar
MMP	:	Mitochondrial membrane potential
mRNA	:	Messenger ribonucleic acid
N	:	Normal
NBT	:	Nitro blue tetrazolium
NADH	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
ng	:	Nanogram
nm	:	Nanometer
nM	:	Nanomolar
NO	:	Nitric oxide
NE	:	Norepinephrine
PBS	:	Phosphate buffered saline
PCA	:	Perchloric acid
PD	:	Postnatal day
pg	:	Pico gram
pmol	:	Pico mole
POPOP	:	1,4, bis 5-Phenyl oxazolyl benzene
PPO	:	2,5-Diphenyl oxazole
PUFA	:	Polyunsaturated fatty acid
QNB	:	Quinuclidinyl Benzilate
rpm	:	Revolutions per minute
ROS	:	Reactive oxygen species
RT-PCR	:	Reverse transcriptase-polymerase chain reaction

SDS	:	Sodium dodecyl sulphate
SEM	:	Standard error of mean
SLA	:	Spontaneous locomotor activity
TEMED	:	N,N,N tetra methylene diamine
TH	:	Tyrosine hydroxylase
V/v	:	Volume/volume
W/v	:	Wight/volume
μg	:	Microgram
μl	:	Microliter
μM	:	Micromolar
QNB	:	Quinuclidinyl benzilate
6-OHDA	:	6-Hydroxydopamine
^{3}H	:	Tritium
Cd	:	Cadmium
QUR	:	Quercetin

Preface

After the discovery of cadmium as an element in 1817, it attracted the attention for industrial use because of its non-corrosive nature. It has wide occurrence in the environment due to extensive anthropogenic uses and thus enhances the risk of human exposure in occupational settings. Besides, general population may also be exposed in non-occupational settings by consuming contaminated food and through air, soil and cigarette smoke. Although the first report of damaged lungs in cadmium exposed workers appeared in 1938, later health effects associated with *Itai-itai* disease aroused a great concern among the scientists. A number of epidemiological and experimental studies have been undertaken since then to understand the impact of cadmium on body organs and etiology associated in cadmium induced toxicity.

Although cadmium has been found to be carcinogenic and classified in the category of group IA chemicals according to IARC, disruption in the functioning of lungs, liver and kidney have also been reported extensively in cadmium exposed individuals. Due to long half life in the biological system (~ 18 - 20 years), it is cumulative in nature and

distributed in body organs. While peripheral neuropathy is widely recognized in cadmium exposed population, it has been found that cadmium easily crosses the blood brain barrier and affects the functioning of central nervous system. Risk of Alzheimer's disease has been associated with cadmium exposure as high cadmium levels in plasma, brain and liver have been detected in Alzheimer's patients. Acute exposure to cadmium has also been found to cause Parkinsonism. Increasing incidences of neurological and psychiatric disturbances associated with cognitive deficits on cadmium exposure in recent years is a cause of concern and reflect the vulnerability of brain while the exact mechanism is not known.

In view of increasing risk of cadmium induced neurotoxicity, a number of experimental studies have been carried out involving pharmacological agents and natural extracts to assess their protective potential. Quercetin, a polyphenolic flavonoid has wide occurrence in vegetables, fruits and many other dietary items. Although anti-carcinogenic, anti-inflammatory and vasodilating effects of quercetin are well documented, it has also been found to be effective in the management of nervous system disorders including Parkinson's and Huntington's disease. Further, protective effects of quercetin in chemical induced neurotoxicity are largely due to its antioxidant and free radical scavenging properties.

In view of this, the present study has been aimed to carry out *in vivo*, *in vitro* and *in silico* studies in an attempt to

i). unravel the molecular mechanisms associated with cadmium neurotoxicity focusing at the expression of the selected proteins involved in the process of synaptic transmission, oxidative stress and neuronal signaling and asses protective potential of quercetin, a flavonoid in rat brain

ii). screen the prophylactic, protective and therapeutic effect of quercetin in cadmium induced neurotoxicity in PC12 cells and

iii). elucidate the bimolecular targets involving docking studies

Experimental studies undertaken to decipher the molecular mechanisms associated with cadmium induced cognitive deficits exhibited decrease in the binding of cholinergic-muscarinic receptors and mRNA expression of cholinergic-receptor genes (M1, M2, M4) in frontal cortex and hippocampus on exposure of rats to cadmium (5.0 mg/kg body weight, p.o.) for 28 days as compared to controls. Cadmium exposure decreased mRNA and protein expression of ChAT and AChE and enhanced ROS generation associated with mitochondrial dysfunctions. Ultrastructural changes exhibiting mitochondrial damage both in frontal cortex and hippocampus assessed by transmission electron microscope and learning deficits monitored by Y-maze and passive avoidance response were also distinct in cadmium exposed rats. Enhanced apoptosis as evident by alterations in key proteins involved in pro and anti-apoptotic pathway and MAPkinase signaling was evident on cadmium exposure. Simultaneous treatment with quercetin (25 mg/kg body weight, p.o.) protects cadmium induced alterations in cholinergic-muscarinic receptors, mRNA expression of genes (M1, M2 and M4) and expression of ChAT and AChE. The protective effect on brain cholinergic targets was attributed to antioxidant potential of quercetin which reduced ROS generation and protected mitochondrial integrity by modulating proteins involved in apoptosis and MAPkinase signaling.

While investigating the molecular mechanisms associated with cadmium induced motor dysfunctions in an attempt to identify targets that govern dopaminergic signaling in brain involving *in vivo, in vitro* and *in silico* approaches, selective decrease in dopamine (DA)-D2 receptors was evident in corpus striatum of rats exposed to cadmium (5.0 mg/kg body weight, p.o.) for 28 days. There was no change in the expression of DA-D1 receptors in corpus striatum. Interestingly, cadmium induced decrease in DA-D2 receptors affected the post-synaptic PKA/DARPP32/PP1α and β-arrestin/Akt/Gsk-3β signaling concurrently in rat corpus striatum and PC12 cells. Pharmacological inhibition of PKA and Akt *in vitro* exhibit that both pathways are independently modulated by DA-D2 receptors and associated with deficits in motor activity. Ultrastructural changes in corpus striatum were distinct, exhibiting neuronal degeneration on cadmium exposure. Molecular docking studies *in silico* provided interesting evidence that decrease in DA-D2 receptors could be due to direct binding of cadmium at the competitive or non-competitive sites of dopamine

on DA-D2 receptors. Simultaneous exposure with quercetin (25 mg/kg body weight, p.o.) resulted to protect cadmium induced behavioral and neurochemical alterations. DFT studies suggest that quercetin has the tendency to form complex with cadmium and may be attributed to the metal chelating property of quercetin and thus reduced toxicity of cadmium. While investigating the prophylactic, protective and therapeutic effect of quercetin in cadmium induced neurotoxicity, studies were undertaken on differentiated PC12 cells *in vitro*. Protective and therapeutic effect of nano-quercetin was clearly evident. Interestingly, nano-quercetin was found to be more effective as compared to bulk quercetin in protecting cadmium induced dopaminergic alterations in PC12 cells. More interestingly, cadmium induced alterations in biogenic amines and increased expression of metallothionein (MT3) in brain regions were also found to be protected on simultaneous treatment with quercetin.

As neuroinflammation and autophagy have emerged as important mechanisms in chemical induced neurotoxicity and also in the progression of neurodegenerative diseases, effect of cadmium on the key targets involved in these processes and protective efficacy of quercetin were assessed. Increased expression of IBA1 and GFAP in hippocampus, frontal cortex and corpus striatum was evident on exposure of rats to cadmium exhibiting activation of glial cells. Further, activation of proinflammatory cytokines (TNFα, IL-1β and IL-6) associated with increased levels of NO and expression of both iNOS and nNOS was evident in all the three brain regions on cadmium exposure. Consequently, increase levels of NO and ROS may lead to the activation in the expression of MAPK and CamkIIα as observed on cadmium exposure may cause neuronal death. Cadmium exposure altered the expression of LC3II, p62 and Atg, marker proteins associated with the integrity of autophagy in rat hippocampus, frontal cortex and corpus striatum in *vivo* and also in SHYSY5Y cells *in vitro*. Using the specific pharmacological inhibitor (3MA) and activator (rapamycin) of autophagy, it was found that cadmium increased the autophagic flux and thus impaired the autophagic pathway that may lead to neurotoxicity. Interestingly, simultaneous exposure with quercetin was found to protect these changes.

The present study provides new information on understanding the cellular and molecular mechanisms associated with cadmium induced neurotoxicity. More interestingly, the potential of quercetin in protecting cadmium induced neurobehavioral alterations by modulating the signaling targets appear to be promising and provide a lead to further explore its role as a protective and therapeutic agent.

Chapter I

Introduction

Cadmium (Cd), one of the naturally occurring trace elements present in earth crust is distributed in the environment due to its extensive anthropogenic applications (Méndez-Armenta and Ríos, 2007; Wang and Du, 2013; Gomes de Moura and Ribeiro, 2017). Use of cadmium is common in smelting, mining and electroplating and cadmium salts are frequently used as color pigments in metals and plastics and in the manufacture of batteries and phosphate fertilizers (Järup et al., 1998; Järup and Åkesson, 2009; Satarug et al., 2011). Volcanic eruptions, burning of fossil fuel, incineration of municipal waste, recycling of cadmium plated scrap and electronic waste significantly contribute in the release of cadmium in the environment. Exposure to cadmium could occur through ambient air in urban areas close to industrial settings (Khade and Adholeya, 2009). High cadmium levels in drinking water have also been found in certain regions (Cai et al., 1995; Cerutti et al., 2003). As cadmium has high rate of transfer from soil to plants, vegetables, fruits and cereals have been found to be highly contaminated grown on cadmium contaminated soil. Interestingly, contaminated rice is one of the chief sources of

cadmium exposure in population at large in different regions across the world (dell'Omo et al., 1999). Besides, cigarette smoke is one of the potential sources of cadmium exposure (Wong and Klaassen, 1982; Satarug and Moore, 2004; Piadé et al., 2015). Human exposure to cadmium may therefore occur both in occupational and non-occupational settings and poses a serious risk to health and associated problems.

Ever since *Itai-itai* disease was associated with chronic cadmium poisoning in Japan in 1912 (Murata et al., 1969; Inaba et al., 2005), there has been a great impetus to assess the adverse health effects of cadmium. Cadmium is carcinogenic in nature and has been classified as class IA carcinogen according to IARC (Baan, 2010). Besides carcinogenic potential, it has been found to disrupt the functioning of lungs and liver in exposed individuals (Kolonel, 1976; Huff et al., 2007). Renal dysfunctions and decreased bone mineral density have been reported on exposure to cadmium in non-occupationally exposed individuals in a cross sectional study (Lemen et al., 1976). While monitoring the health outcomes, increased urinary levels of cadmium have been associated with hypertension, diabetes and diabetic nephropathy in different set of population (Bernard, 2008). The biological half life of cadmium in human body is around 18 – 20 years. Due to poor elimination, it is cumulative in nature and distributed in body organs (Méndez-Armenta and Ríos, 2007). A number of autopsy reports exhibit high accumulation of cadmium in body organs including brain (Chang et al., 2012; Hayashi et al., 2012). Cadmium easily crosses the blood brain barrier and thus brain is one of the soft target (Viaene et al., 1999). Toxic effects of cadmium are largely attributed due to its poor elimination from the body and thus high accumulation in tissues including brain (Shukla et al., 1996; Zalups and Ahmad, 2003; Gonçalves et al., 2010). Further, it has been found to affect the functioning of central and peripheral nervous systems adversely (Wong and Klaassen, 1982; Waalkes et al., 1992). Headache, vertigo and other neurological abnormalities including olfactory dysfunctions have been reported in cadmium exposed individuals (Marlowe et al., 1983). Cadmium exposure has been found to cause cognitive dysfunctions and affect the development of visual perception in children (Thatcher et al., 1982). Increasing incidences of neurological and psychiatric disturbances associated with cognitive deficits on cadmium exposure in recent years is a cause of concern and reflect

the vulnerability of brain. Although blood cadmium is considered to be an index to assess the extent of exposure, levels of cadmium have also been measured in urine, hair and other biological samples to monitor the neurological performance (Pihl and Parkes, 1977; Viaene et al., 2000). Epidemiological studies have found that exposure to cadmium may increase neuropsychological disturbances and memory deficits (Sanders et al., 2015). Children are more vulnerable to adverse health effects of cadmium since a negative correlation has been observed between maternal cadmium levels and performance IQ of children exposed during pregnancy. Risk of Alzheimer's disease has been associated with cadmium exposure as high cadmium levels in plasma, brain and liver were detected in Alzheimer's patients (Basun et al., 1990; Panayi et al., 2002). Involvement of cadmium in the formation of neurofibrillary tangles and amyloid beta peptides in experimental studies has further suggested its role in the pathogenesis of Alzheimer's disease. Numerous experimental studies have found that cadmium may cause brain cholinergic alterations and functional deficits (Carageorgiou et al., 2004). Motor dysfunctions have also been reported in humans on cadmium exposure (Okuda et al., 1997). Chronic exposure to cadmium has been found to cause peripheral neuropathy and affect psychomotor functions in occupational workers. In a cross sectional epidemiological study, cadmium levels in urine were inversely associated with visuomotor functions, peripheral neuropathy and disturbed equilibrium. Further, acute exposure to cadmium has been found to cause Parkinson's disease (Okuda et al., 1997). Interestingly, role of dopaminergic neurotransmission in the regulation of motor and reward system has been demonstrated (Beaulieu and Gainetdinov, 2011) and thus the possibility that cadmium may disrupt the functioning of brain dopaminergic system exists.

Cadmium may disrupt the metabolism of trace elements specially copper and zinc which play important role in the metabolism and kinetics (Antonio et al., 1999). It has been found that cadmium enhances the expression of metallothionein, a cysteine rich protein due to its high affinity in different organs including brain (Choudhuri et al., 1996; Hidalgo et al., 2001). A number of studies found that cadmium may decrease the calcium influx and inhibit the activity of Na^+, K^+-ATPase in the brain and thus affect the process of neurotransmission and energy metabolism respectively (Abdalla et al., 2014).

Alteration in synaptic transmission due to changes in the levels of brain biogenic amines and their metabolites on exposure to cadmium have been reported in experimental studies (Hastings et al., 1978; Lafuente et al., 2001). Interestingly, these changes were associated with neurobehavioral abnormalities (Andersson et al., 1997; Lafuente et al., 2001; Minami et al., 2001; Lafuente et al., 2003; Ashok et al., 2014). It has been found cadmium easily targets mitochondria in brain and other tissues and affects its functional integrity (Beal, 1996; Wang et al., 2004; Chang et al., 2013). Increased oxidative stress associated with enhanced apoptosis are potential mechanisms, largely accepted in cadmium induced neurotoxicity (Shaikh et al., 1999; Nemmiche et al., 2007; Liu et al., 2009). Further, a line of evidences suggest inflammation as a crucial mechanism in neurodegenerative diseases including Parkinson's and Alzheimer's disease and in chemical induced neurotoxicity (Liu et al., 2003; Griffin, 2006; Rogers et al., 2007; Hirsch and Hunot, 2009; Amor et al., 2010; Chen et al., 2016) (Mosley et al., 2006). Activation of microglia enhances production of inflammatory cytokines which in turn disrupt the signaling cascade and affect the neuronal integrity (Liu and Hong, 2003; Frank-Cannon et al., 2009; Lehnardt, 2010). Although cadmium has been found to affect the glial architecture in brain (Streit et al., 2004; Vargas et al., 2005; Qin et al., 2007; Tilleux and Hermans, 2007), the mechanism and targets associated with cadmium mediated neuroinflammation are not known. Besides inflammation, alteration in basal autophagy may also contribute in the pathogenesis of neurodegenerative diseases (Zhou et al., 2011b; Hensley and Harris-White, 2015). As autophagy and apoptosis work in a coordinated manner to regulate the cell survival and death (Maiuri et al., 2007; Eisenberg-Lerner et al., 2009; Kang et al., 2011; Zhou et al., 2011a; Gordy and He, 2012), any toxic insult or stress lead to the activation and accumulation of damaged proteins inside the cell and contribute towards the autophagy mediated cell death.

In view of increasing risk of cadmium induced neurotoxicity, a number of experimental studies have been carried out involving pharmacological and natural extracts to assess their protective potential. Flavonoids present in plants as glycosides have attracted much attention in recent years due to their high bioactivity, largely associated with their anti-oxidant potential (Unsal et al., 2013). Among flavonoids, quercetin, a class of flavonol is

widely present in vegetables, fruits, tea and many other foods (Formica and Regelson, 1995; Murota and Terao, 2003). The content of quercetin however varies in different dietary products (Wach et al., 2007). High levels of quercetin are also present in medicinal plants - *Ginkgo biloba* and *Hypericum perforatum* (Sultana and Anwar, 2008). High antioxidant capacity of quercetin has made it popular over other established antioxidants - vitamin C, vitamin E and β-carotene. Potential of quercetin to chelate transition metals has been found to protect iron induced Fenton's reaction (Cheng and Breen, 2000; Leopoldini et al., 2006). Although anti-carcinogenic, anti-inflammatory and vasodilating effects of quercetin are well documented, it has been found effective in the management of nervous system disorders (Chopra et al. 2000; Pereira et al. 1996; (Ho et al., 2013). Protective role of quercetin in cognitive deficits has been demonstrated in several experimental studies (Kumar et al. 2008; Liu et al. 2006; Singh et al. 2003; Yao et al. 2010). Interestingly, dopaminergic dysfunctions in animal models of Parkinson's disease and Huntington's disease have been found to be protected by quercetin (Sandhir and Mehrotra, 2013; Chakraborty et al., 2014). Quercetin has also been found effective to protect chemical induced neurotoxicity (Sharma et al., 2013; Chander et al., 2014). Further, promising effects of quercetin to modulate physiological functions in preclinical studies have strengthened its use in clinical situations in the management and treatment of many diseases (Linde et al., 1996; Okamoto, 2005).

Despite considerable investigations on cadmium neurotoxicity and moreover enhanced risk to develop Alzheimer's and Parkinson's disease, the exact mechanism by which it causes brain cholinergic and dopaminergic alterations and affect associated functions is not well characterized. Also, the exact mechanism and targets associated with the protective potential of quercetin in modulating cadmium induced neurotoxicity are not understood. The present study has there been aimed to carry out *in vivo*, *in vitro* and *in silico* studies in an attempt to

i. unravel the molecular mechanisms associated with cadmium neurotoxicity focusing at the expression of the selected proteins involved in the process of synaptic transmission, oxidative stress and neuronal signaling and asses protective potential of quercetin, a flavonoid in rat brain

ii. screen the prophylactic, protective and therapeutic effect of quercetin in cadmium induced neurotoxicity in PC12 cells

iii. elucidate the bimolecular targets involving docking studies.

Chapter II

Review of Literature

2.1 Cadmium

Cadmium, one of the naturally occurring transition heavy metal is present in the earth's crust. Pure cadmium is soft, malleable, ductile, bluish-white divalent metal found in natural sources with the ores of lead, zinc and copper. As per an estimate, about 0.1 part per million of cadmium is found as an impurity in Zn or lead deposits and therefore cadmium is produced as a byproduct during zinc and lead smelting (Järup and Åkesson, 2009). In periodic table, cadmium belongs to the 12^{th} group and exhibits similar properties like zinc and mercury. Cadmium is found in +2 oxidative state and has eight isotopes in which two are radioactive. Cadmium is insoluble in water however, its salt like cadmium chloride and cadmium sulfate are soluble in water. In surface and groundwater, cadmium may exist as hydrated ion or as ionic complexes with other inorganic or organic substances. The physicochemical properties of cadmium are briefly described in Table – 2.1

Table 2.1. Physiochemical Properties of Cadmium

Colour	**:**	Silver white metal
Atomic No	**:**	48
Atomic Weight	**:**	$112.4gmor^{-1}$
Density	**:**	$8.7gm^3$ at 20^oC
Melting point	**:**	321^oC
Boiling Point	**:**	767^oC
Wander wall Radius	**:**	0.154nm
Electronic Shell	**:**	$(kr)1d^105S^2$
Ionic Radius	**:**	0.097nm
Energy of First ionization	**:**	$866kJmol^1$
Standard potential	**:**	0.402V

(Farron et al., 2012)

2.1.1 Cadmium – Applications, Contamination in the Environment and Exposure Sources

Cadmium has wide occurrence in the environment due to its anthropogenic uses (Fassett, 1975). Use of cadmium is common in nickel–cadmium (Ni-Cd) batteries and as colour pigment in metals and plastics. It is also used in smelting and refining of metal ores (Méndez-Armenta and Ríos, 2007). As cadmium is resistant to corrosion, it is preferred over other metals in electroplating. With such increasing application of cadmium, US geological survey has shown increment in worldwide cadmium production till 2020 (Figure – 2.1). It is also used in surface mount devices as chip resistors, infrared detectors and semiconductor. Besides, volcanic eruptions, burning of fossil fuel, incineration of

municipal waste, recycling of cadmium-plated scrap and electronic waste significantly contribute in the release of cadmium in the environment.

High levels of cadmium as a contaminant in water and soil has been reported in various parts of the globe (Fassett, 1975). Exposure to cadmium could also occur via air and house dust in cadmium contaminated areas (Nordberg, 2004; Hogervorst et al., 2007). The picture that cadmium is accumulated in top agricultural soil has been associated with risk assessment related to cadmium exposure by several European and Asia pacific countries. In a cross sectional study in rural areas of China to assess dietary exposure to cadmium, (Zhu et al., 2016) found higher concentration of cadmium in rice and vegetables (65.31% and 23.75%) as compared to standard concentrations of cadmium in China. Further high levels of cadmium were found in soil sample in Cracow (Poland) (Aleksander-Kwaterczak and Rajca, 2015). In another study, higher levels of cadmium were found in fruits and vegetables in Guangzhou, China (Li et al., 2006). Further, Padungtod and co-workers in an interesting review reported a clear picture of cadmium contamination in food, water and soil and associated with the risk of exposure in population residing near mining area in Thailand (Chantana Padungtod). High cadmium levels in soil samples have been linked with high contamination of cadmium in rice (80 % higher) as compared to standard one in a case study. Levels of cadmium ranged quite high from 0.4 to 70.5 ppm in soil samples collected near zinc smelting units (Staessen et al., 1994). Interestingly, vegetables grown in the area was found to be highly contaminated with cadmium (upto 11.8 ppm). (Svoboda et al., 2000) also found high levels of cadmium in mushrooms in forested area near a copper smelter. Interestingly, high levels of cadmium in mushroom were associated with high cadmium levels in soil (0.25 to 10 ppm). Likewise many more study carried out worldwide in European countries, Thailand, Japan and China showing high level of cadmium in soil, water and food (Cai et al., 1995; Chatham-Stephens et al., 2013; Wang and Björn, 2014).

Higher levels of cadmium in the soil collected from different places near sewage disposal areas was reported in a study from Coimbatore, Tamilnadu. The levels (3 mg/kg)

detected in the soil were 3 – 4 times higher than the standard levels (Radha et al., 2014). High contamination of cadmium in ground water in Assam has been reported (Borah et al., 2009). In a recent study, Akhand and co-workers found high cadmium contamination in Kolkata city (near 20 km stretch on the Bidyadhari River) (Akhand et al., 2016). (Dahiya et al., 2005) found high levels of cadmium in chocolates and candies available in local markets of suburban areas of Mumbai.

Due to the hyperaccumulating characteristics of *Nicotiana tabacum*, cadmium is a major component of tobacco leaves. Cadmium content in tobacco leaves normally ranges between 1 and 2 μg/g dry weight resulting in 0.5–1 μg Cd/cigarette. Interestingly, high cadmium concentrations in tobacco leaves is independent of the soil-cadmium content. Cigarette smoke has been identified as one of the potent source of cadmium exposure (Satarug and Moore, 2004). Furthermore, cadmium oxide generated during smoking either is deposited in lung tissues or absorbed into the systemic blood circulation of smokers (Ellis et al., 1979). It results in 4 - 5 times higher cadmium levels in blood and 2 - 3 times greater amount of cadmium in their kidneys than non-smokers (Sharma et al., 1983; Piasek et al., 2001).

Due to the highly soluble nature of cadmium compounds as compared to other metals, they are readily taken up by plants resulting in storage in crops (Satarug and Moore, 2004). Rice, vegetables and cereals are the main source of dietary exposure to cadmium in humans. Although crustaceans and molluscs have been found to accumulate large amount of cadmium from contaminated aquatic environments, levels of cadmium are less in meat products and fish (Bolam et al., 2016; Galimberti et al., 2016). Thus, human exposure to cadmium could occur both in occupational and non-occupational settings. However, the general population is exposed to cadmium largely through contaminated food.

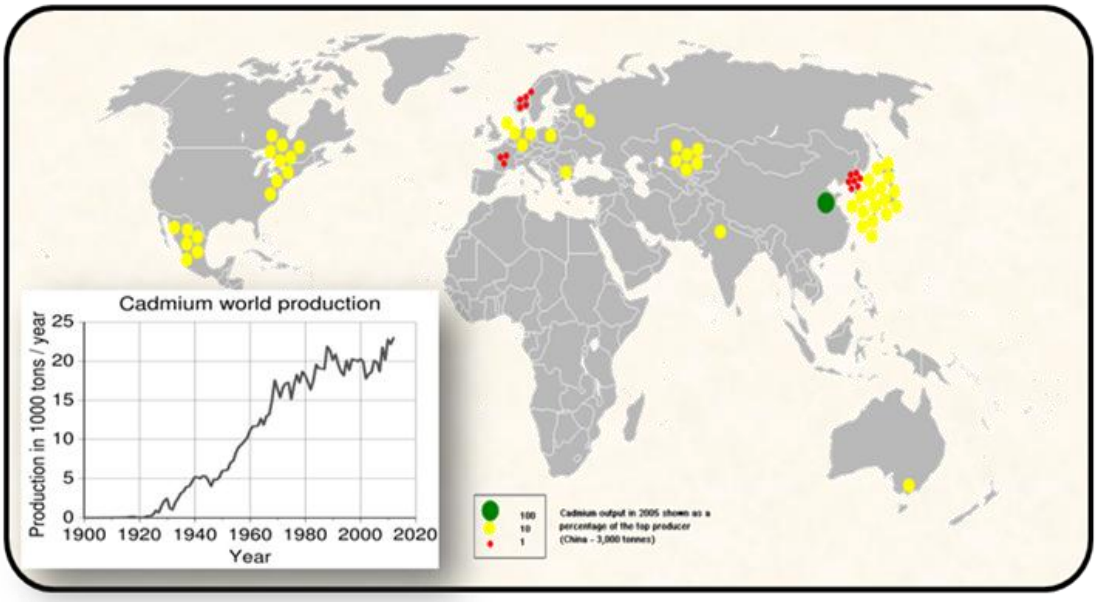


Figure – 2.1. Epidemiological Burden of Cadmium & World Over Production (UUSG mineral Book, 2015)

2.1.2 Cadmium - Exposure Guidelines

With the abundance of cadmium in the environment, there is a lot of concern to protect the population from its adverse health effects. Exposure standards have been set by health agencies to protect the general population from excess cadmium exposure through various sources. The permissible / safety limits of cadmium in drinking water and food have been summarized in Table – 2.2.

Table -2.2. Cadmium - Exposure Guidelines

Agencies	Year	Permissible limits
World Health Organization (WHO)/FAO	1988	400-500µg/week
	1993	7 µg-kg $^{-1}$ b.w./week
- Drinking water	2010	5.8 μg·kg−1 b.w./week
- Food	2004	3 μg/L
	2004	10-40 µg

Occupational Safety and Health Administration	2006	TWA (PEL): 5 µg/m3 (fumes)
-In Air (at workplace)	2006	2.5 µg/m3
National Institute of Occupational Safety and Health (NIOSH)	2006	9 mg/m^3
US Environmental Protection Agency	1999	1 x 10^{-3} mg/kg/day
- Food	1999	0.005 mg/L.
- Water		
Consumer Product Safety Commission	2010	0.1 μg·kg−1 b.w./day
Australian water quality guideline (in fresh water)	1992	Australian water quality guideline,
Ministry of Public Health of China	2012	0.20 mg/kg (in food)
Codex	2008	0.40 mg/g (in rice)

(Wang et al., 2013)

2.1.3 Cadmium - Health Consequences

Ever since the endemic *Itai-itai* disease (intense pain), clinically characterized by renal dysfunction and osteoporosis and osteomalacia was associated with chronic cadmium poisoning in Japan (Inaba et al., 2005; Nair et al., 2013), there has been a significant interest to understand the health effects of cadmium. High occurrence of cadmium in the environment has further enhanced the risk of several diseases. Cadmium has been classified as a Group IA carcinogen by the International Agency for Research on Cancer (IARC) and ranks at 7^{th} position in the priority list of hazardous substances according to Agency for Toxic Substances and Disease (ASTDR 2015). Occupational exposure to cadmium has been found to cause lung cancer. Further, cancers of prostate, kidney, liver, urinary bladder, pancreas, testis, stomach and hematopoietic system have also been reported (Kolonel, 1976; Nawrot et al., 2006). Chronic exposure to cadmium at low levels may lead to end-stage renal failure, liver failure and other complications like cardiovascular disorders and metabolic disorders (Schwartz et al., 2003; Järup and Åkesson, 2009; Messner and Bernhard, 2010). Fracture of bones is quite common in

individuals on chronic exposure to cadmium. Immunosuppresion, hypertension, diabetes, myocardial infarction and ocular disorders have been frequently reported on cadmium exposure in population studies (Satarug et al., 2010). Use of cadmium in different applications and its effect on different body organs has been shown in Figure – 2.2. Besides, brain has been found to be a vulnerable target of cadmium (Méndez-Armenta and Ríos, 2007). Effect of cadmium on neurobehavioral functions in exposed population has been summarized further.

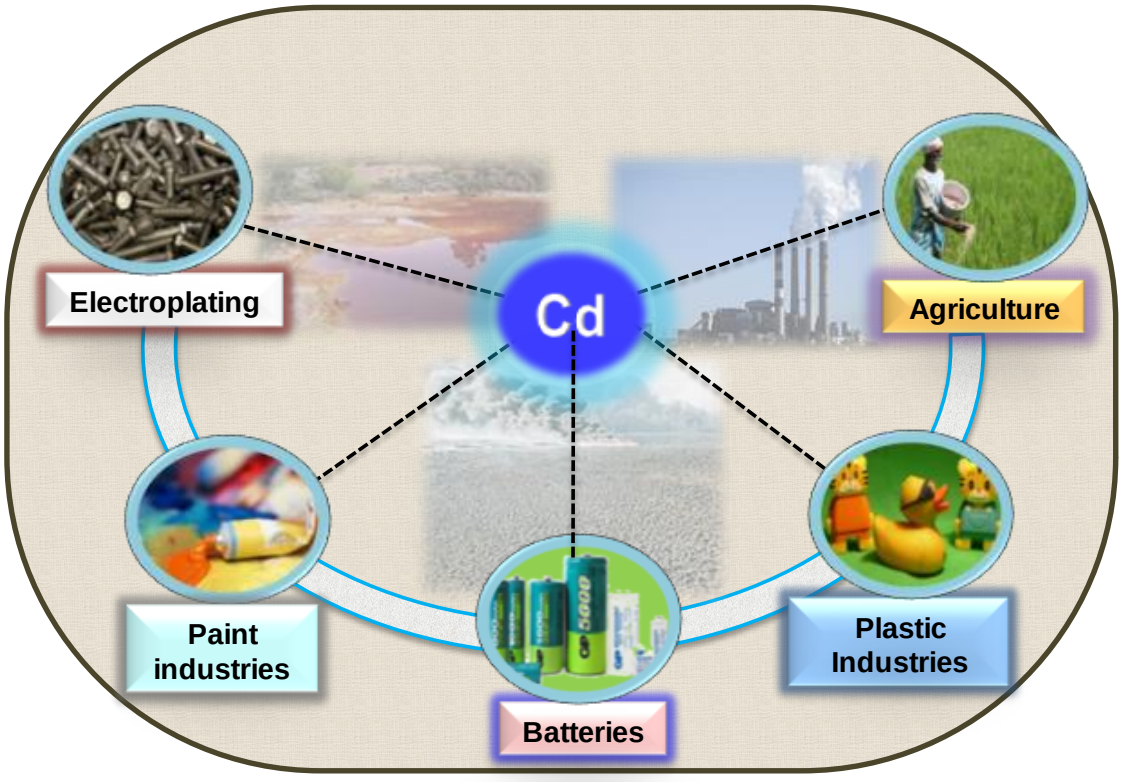


Figure – 2.2. Cadmium in the Environment- Natural & Anthropogenic Sources

2.1.4 Cadmium - Epidemiological and Clinical Evidences Associated with Neurobehavioral Toxicity

Acute cadmium exposure has been associated with symptoms of Parkinsonism (Okuda et al., 1997). High levels of cadmium in Alzheimer's brain (Panayi et al., 2002) aroused a

great concern to assess the impact of cadmium on neurobehavioral abnormalities. In a study on occupational workers exposed to cadmium, peripheral neuropathy and dysfunctions of the autonomic nervous system with impaired visuomotor functions has been reported (Viaene et al., 2000). Headache, vertigo, olfactory dysfunctions, impaired vasomotor functions, peripheral neuropathy, decreased equilibrium, decreased ability to concentrate, and learning disabilities have been reported (Shukla et al., 1996). Interestingly, these changes were found to be dose dependent (Viaene et al., 2000). Children have been found to be more vulnerable to deleterious effects of cadmium as compared to adults. High levels of blood cadmium were found to be correlated positively with incidences of hyperactive and impulsive behavior in a study on young males and females (Jabłońska et al., 2012). Exposure to cadmium has been found to affect the functions of the nervous system. Prenatal exposure to cadmium resulted to causes neurodevelopmental disorders in infants in a recent study (Wang et al., 2016). A number of studies have been carried out to assess effect of cadmium exposure at different stage of life and identify the window that is more vulnerable. In a study to assess the IQ and cognitive parameters, emotional problems in children (7 – 8 years) on prenatal exposure to cadmium were found to be high (Sioen et al., 2013). Higher cadmium exposure in cord blood (> 0.6 µg/L) was associated with a 4-point Full-Scale IQ deficit at preschool age after adjustment for cord blood Pb levels in a Chinese birth cohort study (Tian et al., 2009). Although placenta limits the transfer of cadmium from mother to fetus after the first trimester, high cord blood cadmium in infants was suggested due to high maternal exposure. Dysfunctions in attention, learning and memory have been associated with elevated level of cadmium in blood or urine by a number of studies. Ciesielski in 2012 showed the correlation of high urinary Cd level with learning disability. The epidemiological study carried in battery workers showed the impairment in oxidative stress that is associated with ALS due to reduced brain antioxidant system especially SOD activity (Bar-Sela S et al., 2002).

2.1.5 Cadmium - Experimental Studies Associated with Neurobehavioral Toxicity

In view of increasing risk of cadmium, induced neurobehavioral alterations, experimental studies involving *in vitro* and *in vivo* models have been carried out extensively to identify molecular targets associated. Further effect of cadmium has also been assessed in developing rat brain. Number of studies carried out showed that the developmental neurotoxicity in rats offspring exposed to prenatally to cadmium (Tian et al., 2009). In another study, prenatal exposure of cadmium evoked the amphetamine induced dopamine release in corpus striatum (Nowak et al., 2006). (Antonio et al., 1999) reported that exposure to cadmium exposure during developmental period resulted to affect the process of synaptic neurotransmission in rats. Interestingly, changes in serotonin and dopamine levels were associated with alteration in spontaneous motor activity ((Antonio et al., 1999). Another study carried out by (Notarachille et al., 2014) showed the effect of cadmium on beta amyloid. An emerging study carried out in 2005, where cadmium exposure in mice during cerebral oligemia showed a significant affect in memory processes in Y-maze and in passive avoidance test. (Łukawski et al., 2005). Study carried out by Del pino et al., 2015 suggests the death of cholinergic neurons in basal forebrain area following cadmium exposure. Further, cadmium exposure is also known to accumulate amyloidal beta formation (Li et al., 2012). Further, long-term cadmium exposure in rats showed the significant behavioral and brain enzymatic changes. Cadmium exposure showed the memory impairment and anxiety like behavior in rats, and also causes the disruption in AChE and Na+,K+-ATPase activities (Gonçalves et al., 2010). Studies carried out by Rai et al., 2013 linked the cadmium exposure with myelin deformation and optic nerve dysfunctions. In another study, short term or acute exposure of cadmium dose dependently elicit the neurobehavioral and neurochemical alteration in rats (Haider et al., 2015). Besides rodent models, fresh water crab also showed the oxidative stress and cell death following cadmium exposure (Wang et al., 2011). Further, study carried out by (Jin et al., 2011) showed the increase in the oxidative stress as well as immunotoxicity in brain following acute exposure of cadmium in zebrafish.

Brain of chicken is also found to be affected following cadmium exposure (Zhang R et al., 2016). Various *in vitro* studies carried out by using various neuronal cell line and primary rat neuronal culture showed that apoptotic mediated cell death by various

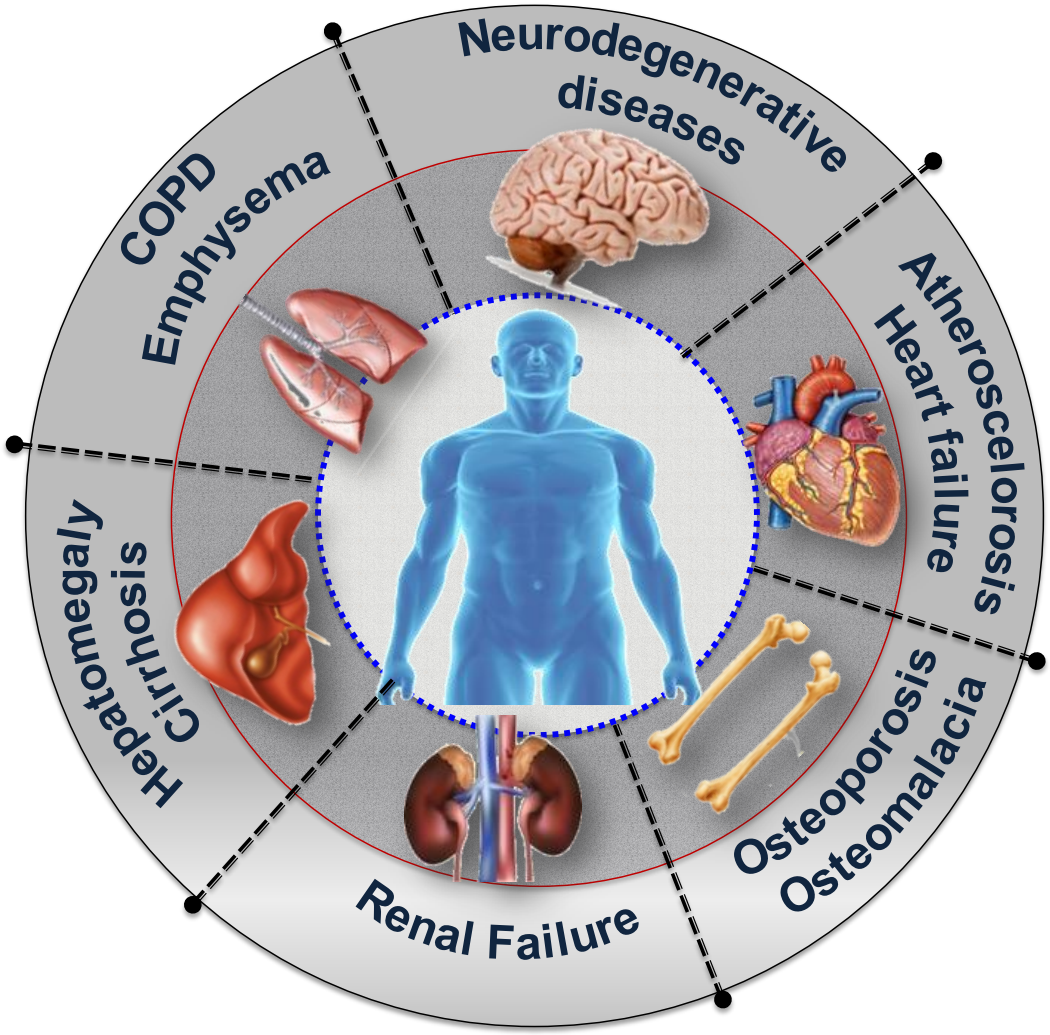


Figure – 2.3. Health Effects of Cadmium

signaling pathway like via targeting the calcium, ERK, c-Jun signaling, GADD32 (Kim and Sharma, 2004; Chen et al., 2011; Tvermoes et al., 2011; Kim et al., 2013b; Yuan et al., 2013).

2.1.6 Cadmium Neurotoxicity – Cellular and Molecular Mechanisms

A number of studies have been carried out by number of investigators where they showed the disturbances in the cellular antioxidant system associated increase in reactive oxygen and nitrogen species, changes in the metabolism of biogenic amines, neurotransmitter amino acids.

2.1.6.1 Oxidative Stress and Subsequent ROS Generation

Although various studies carried out epidemiologically and experimentally suggests the neurotoxic effect of cadmium and its severity in the brain. But the mechanism by which cadmium exert the neurotoxicity is not studied well. Although the generation of free radical species and imbalance in antioxidant system have been suggested the potential mechanism of cadmium induced neurotoxicity by various researchers (Lopez et al., 2006; Nemmiche et al., 2007). Recent study carried out by Agnihotri et al., 2015 that brain is most susceptible to cadmium induced oxidative stress. The oxidative impairment in terms is characterized by increased ROS and RNS production; increase the levels of LPO and subsequent reduction in total thiols, GSH and other antioxidant enzyme such as SOD, CAT, GST, GR, GPx etc. Cadmium is known to play with antioxidant system, known to alter the level of SOD, GSH and catalase and also shown the potent interaction body defense system. A number of studies carried out *in vivo* and *in vitro* suggests that cadmium primarily affects the antioxidant systems that decrease the activity of SOD and GSH increases LPO in liver, kidney, brain and other body organs (Shukla et al., 1996; Méndez-Armenta et al., 2003; El-Demerdash et al., 2004). So, cadmium after reaching in the human body involved in producing the redox like reaction and generates the free radicals in the brain. Experimental studies carried out suggests that oxidative stress as a potential mechanism in developing brain exposed to cadmium as increase in ROS generation and increase levels of LPO was observed following cadmium exposure (Carageorgiou and Katramadou, 2012). Further, adult rats exposed to cadmium also showed the increase level of LPO and decrease levels of GSH in corpus striatum and cerebral cortex (Pal et al., 1993). As a mechanism, cadmium primarily interacts with the

cellular GSH, having the strong affinity to bind with the thiols groups. Swiss albino mice that received 4 mg CdCl2/kg body weight orally for three days also found to enhance the lipid peroxidation and protein carbonylation in brain while taurine prevents such changes (Sinha et al., 2008). Study carried out shows that cadmium has strong binding capacity with cellular GSH (Cuypers et al., 2010). In an another study, cadmium exposure to albino rats for 30 days significantly increases the ROS generation and thus affecting the other signaling cascade like calcium signaling etc (Kumar et al., 1996). In vitro studies carried out using various neuronal cell lines also potentially support that cadmium has primarily affects the oxidative stress, which in turn diminishes the activity of antioxidatant enzymes. Cd-induced oxidative stress may lead to activation of death mechanism and proinflammatory cytokines especially COX 2 that are associated with the neuronal death while these changes was protected with NAC and celecoxib in a mouse neuroblastoma cell line HT4 (Figueiredo-Pereira et al., 2002)

2.1.6.2 Interaction of Cadmium with Metallothionein

Cadmium is known to have strong affinity with metallothionein, a low molecular weight protein isolated first from the cortex of horse kidney (Margoshes and Vallee, 1957). Metallothionein contains the strong metal binding property and the high cysteine content with two binding chain, known as α &β chains. Putative role of metallothionein in cadmium metabolism in brain and other body organs specially liver and kidney has been demonstrated. Out of different isoforms of metallothionein, an isoform MTIII is highly expressed in the brain. It is known to serve as growth inhibitory factor in brain and plays major role in the development, organization and programmed cell death of brain cells (Thirumoorthy et al., 2011). Potential role of metallothionein in removing the trafficking of cadmium in renal and hepatic systems has been suggested (Sabolić et al., 2010). Involvement of metallothionein in neurodegenerative diseases has been reported. Expression of metallothionein has been found to be down regulated in Alzheimer's disease (Uchida et al., 1991). Neurons in choroid plexus also express MT proteins. (Nishimura et al., 1992) found linkage of cadmium exposed younger rats (1–3) with

increased immunostaining of metallothionein proteins in ependymal cells and choroids plexus epithelium. Based on this, role of MTs in cadmium toxicity was suggested. In another study, increase in the levels of lipid peroxidation in brain on cadmium exposure was found reduced by dexamethasone and associated with increase in mitochondrial content (Méndez-Armenta et al., 2003). Choudhuri et al. (1995) found variable and increased expression of metallothionein in brain of mice at developmental stage. Interestingly, the expression was found to vary with cadmium accumulation (Choudhuri et al., 1996). Further, under the condition of active gliosis increased expression of MT was observed in various brain regions (Giralt et al., 2001). *In vitro* studies on primary astrocytes exhibited positive role of cadmium to enhance expression of metallothionein (Rising et al., 1995).As MT III in brain has been suggested to have free radical scavenging activity, effective role in aging brain and neurodegenerative diseases like Alzheimer's disease has been postulated (West et al., 2008; Howells et al., 2010; Santos et al., 2012)

2.1.6.3 Cadmium and Mitochondria

The mechanism of cadmium induced primarily explained and supported by the oxidative stress and metallothionein proteins, however number of studies carried out recently strongly revealed the potential role of mitochondria in cadmium mediated neurotoxicity and causing the neurodegenerative diseases.

Mitochondria is a major source of bioenergetics. There are convincing reports exhibiting that disruption in the mitochondrial matrix may generate reactive oxygen species by activating the intrinsic mitochondrial apoptotic pathway leading to cell death. A close link of mitochondrial dysfunctions in the etiology of neurodegenerative disorders like Alzheimer's disease and Parkinson's disease has been demonstrated extensively (Baloyannis, 2013). Alterations in the functional integrity of mitochondria have also been

reported

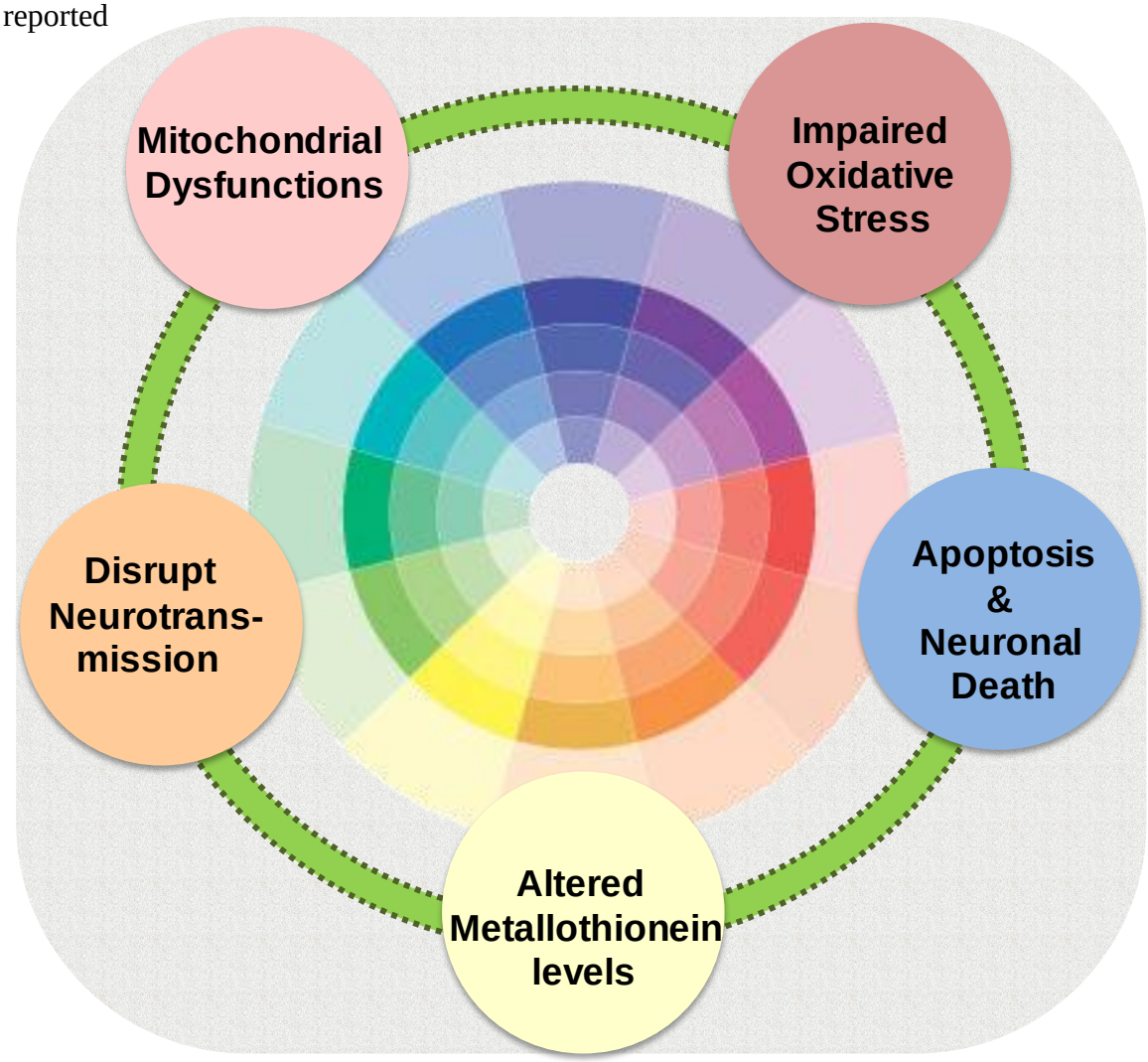


Figure 2.4 Possible Mechanisms of Cadmium Neurotoxicity

extensively on exposure to neurotoxicants (Chang et al., 2013). Cadmium has been found to affect the activity of mitochondrial complexes which are important for cell respiration (Belyaeva et al., 2012). Interestingly, impairment in the activity of mitochondrial complexes was found to enhance ROS generation and apoptosis due to activation of caspase cascade associated with release of cytochrome c. (Lopez et al.

2006) *In vitro* studies carried out using rat cortical neuron culture showed the imbalance in mitochondrial matrix with lead to generation of ROS and LPO in these cells (Lopez et al., 2006). Cadmium induced Apoptosis cell death in primary Rat Cerebral Cortical Neurons culture is also mediates through mitochondrial mediated calcium signaling. In a study carried out by Miccade et al., 1993 showed an important aspect of cadmium induced mitochondrial dysfunctions. They identified the site of inhibition of the mitochondrial transport chain following exposure to cadmium. Studies carried out by Yuan et al., 2015 showed the role of mitogen-activated protein kinase in cadmium mediated toxicity via targeting to the mitochondrial apoptotic pathway, where they uses the primary rat cerebral cortical neurons at dose of 10µM for 24 hr. They found a significant increase in the (Bcl-2/Bax) ratio and increased the percentage of apoptotic cells, release of cytochrome c, cleavages of caspase-3 and PARP, and nuclear translocation of apoptosis-inducing factor. The effect of cadmium on the mitochondrial electron transport chain and related complexes was also assessed using pc12 cell line as a model, where altered respiratory function, and mitochondrial membrane potential was found on dose and time dependent manner following cadmium exposure(Belyaeva et al., 2012). Chang et al., 2013 found the cadmium mediated apoptosis in pancreatic beta cells through mitochondrial mediated pathway. At a dose of 5 and 10µM of cadmium a significant decrease in the cell viability, and increased annexin V-Cy3 binding, increased intracellular reactive oxygen species generation and malondialdehyde production and induced mitochondrial dysfunction (the loss of mitochondrial membrane potential and the increase of cytosolic Cyto C, the decreased Bcl-2 expression, increased p53 expression, poly (ADP-ribose) polymerase (PARP) cleavage, and caspase cascades was found. A study carried out by Li et al., 2003 suggest a potential mechanism of cadmium mediated apoptosis through directly opening of MPT by cadmium. Study carried out by (Dorta et al., 2003) in Brazil proposed sequence of events for cadmium-induced mitochondrial impairment. They isolated the mitochondria form rat liver and showed that cadmium exposure even at dose of 5 µM for 0–25 min impairs the mitochondria. A sequential process proposed starting from the interaction of thiols in the mitochondrial membrane,

which stimulates the cation's uptake followed by opening of mitochondrial permeability transition that causes the alteration in mitochondrial ATP levels.

2.1.6.4 Cadmium and Cellular Death - Mechanisms

Reports carried out previously strongly suggest that cadmium mediated cell death but the signaling cascade involved in these processes is not fully understood. But the major reports involved showed the involvement of apoptotic cell death in cadmium mediated cell death. Apoptotic that is defined as the systematic programmed cell death and is generally govern by two pathways either by activating the death pathway or by activating the intrinsic mitochondrial pathway. Cadmium induced apoptotic in mediating the neuronal death is given by various reports (Wätjen and Beyersmann, 2004; Jung et al., 2008). The studies carried out using rat model suggest that involvement of p38 MAPK in cadmium mediated apoptotic cell death in brain endothelial cells. Further, in an another study besides the apoptotic mediated cell death, cadmium also induces the neuronal cell death by GADD32 leading to activation of ROS (Kim et al., 2013b). Cadmium via depletion of gluthaionone causes the astrocytic cell death (Im et al., 2006).

Various in vitro studies carried out using neuronal cell lines and primary culture given a glimpse of cadmium mediated apoptotic cell death by involving the ASK1, MKK4, JNK, c-Jun, and caspase-3 (Kim et al., 2005). Besides the studies, the exact mechanism and pathway involved in cadmium mediated apoptotic neuronal cell death is poorly understand. More concaving studies needed to be done to assess the signaling molecules involved cadmium mediated neuronal death. However, recently some studies carried out in 2016 showed the MAPkinase involvement in cadmium mediated apoptotic cell death via activating mitochondrial pathway is also studies (Yuan et al., 2015).

2.1.7 Role of Neuroprotectant in Cadmium Induced Neurotoxicity

With the wide occurrence of cadmium in the environment and with the extensive uses of cadmium in industries, agriculture and daily life, human are highly exposed to cadmium

and consequences are quite serious. With such a great concern and associated health effects, there is lots of concern whether cadmium neurotoxicity could be protected.

Talking about cadmium neurotoxicity, which also imposes the serious neurological and psychological disorders, especially neurodegenerative disorders like Alzheimer's and Parkinson's. Various case study reports and clinical reports suggests that the role of cadmium in development of such diseases. With this serious concern, it becomes quite necessary to manage the levels of cadmium, and reducing the neurotoxicity of cadmium by using various natural and pharmacological agents.

2.1.7.1 Herbal and Natural Agents Used

A number of experimental studies carried out showed the involvement of various natural and herbal agents in mitigating the cadmium induced neurotoxicity. These studies primarily involved the flavonoids like hesperidin, rutin, Resveratrol and quercetin. Further, other natural agents like epigallocatechin, grape seed, proanthrocyaninin and curcumin has been used in combating cadmium induced neurotoxicity (Karaca and Eraslan, 2013; Gong et al., 2015; Winiarska-Mieczan, 2015).

There is abundant evidence, widely been accepted the potential role of natural agents in showing the protection against the neurodegenerative diseases like PD, AD, and ALS (Youdim et al., 2002; Mandel et al., 2006; Apetz et al., 2014; Choi and Choi, 2015) and are believed to be effective in blocking the neuronal injury and death. The mechanism by which they showed the protecting effect is either by building or restoring the antioxidant balance in body or by modulating the various cell signaling cascades like such as the PI3 kinase (PI3 K)/Akt and mitogen-activated protein kinase (MAP kinase)signaling pathways. However, in case of heavy metal toxicity, they bind the metal with its coordinate, as they are having the metal chelating and free radical scavenging properties. (Daniel et al., 2004). Number of experimental studies carried out showed the potential role of natural agents against cadmium induced toxicity. In a recent study, carried out by Liu et al., 2015 suggests the role of resvetrol in preventing the cadmium induced neuronal cell death by modulating the ERK and JNK1/2 pathways. Further, epigallocatechin- 3

gallate, which is abundant in green tea, is also found to be effective in cadmium induced neuronal death by preventing the mitochondrial lipid peroxidation. ((Abib et al., 2011). Also, *in vivo* and *in vitro* studies carried out suggest the role of various natural agents in cadmium induced improvement in cholinergic functions as well as in learning and memory. It has been shown that the mechanism by which L-cysteine protects the is by modulating the total antioxidant status, acetylcholinesterase (Carageorgiou et al., 2004). *Dendropanax morbifera Léveille* also mitigates cadmium induced increase in oxidative stress in rat hippocampus (Kim et al., 2014). protocatechuic acid is also known to modulate the Na+/K+-ATPase, cholinergic and antioxidant enzymes and prevents the cadmium induced neurotoxicity (Adefegha et al., 2016). One of the important carried out recently shown the attenuation of cadmium-induced neurotoxicity through the inhibition of oxidative damage and tau hyperphosphorylation by L-cysteine. They showed that treatment with L-cysteine reduces the cadmium level in mice brain.

2.1.7.2 Pharmacological Agents

Besides the natural agents, studies on no of pharmacological agents has also been done by many investigators in protecting the cadmium induced neurotoxicity and neuronal death. Study carried out by Li M et al., 2016 showed the role of melatonin in antagonizing the cadmium induced neurotoxicity by activating the transcription factor EB-dependent autophagy-lysosome machinery in neuronal cell lines(Li et al., 2016). Further, they carried out the study another study in 2016 where they shown the prevention of cadmium induced neurotoxicity by antagonizing the abnormal mitochondrial dynamics by blocking calcium-dependent translocation of Drp1 to the mitochondria. Celastrol, a pentacyclic triterpene, has wide implications in antioxidant, anti-apoptotic, anti-inflammatory, anti-carcinogenic and anti-obesity properties, prevents cadmium-induced neuronal cell death by blocking mTOR pathway and by modulating the AMP-activated protein kinase (Zhang et al., 2017). Studies has also shown that ceftriaxone plus sulbactam with VRP1034 also shows the protective role in cadmium

induced neurotoxicity (Dwivedi et al., 2012). Use of napridine has also done in mitigating the cadmium induced neurotoxicity (Bağirici et al., 2001).

2.1.7.3 Flavonoids as Promising Agents in Cadmium Induced Neurotoxicity: Role of Quercetin

Recently, potential of flavonoid to modulate neuronal function and to prevent brain from toxic insults and to flight against age-related neurodegeneration has been widely studied. The flavonoid, a class of polyphenols, has been accepted to have potent free radical scavenging and antioxidant as well as metal chelating properties (Vauzour et al., 2008). Flavonoids, which have wide occurrence in the nature and have the major constituents of fruit, vegetables and beverages (Nijveldt et al., 2001; Dajas et al., 2013). Flavonoids are known to reflect the neuroprotective potential by modulating the two integrity either they act by interacting with various signaling protein leading to the inhibition of necrotic or apoptotic mediated cell death. Secondly, they act to promote the neurogenesis and cell survival pathways. Further, flavonoid has also been potentially identified as potent therapeutic agents against various neurodegenerative diseases like Alzheimer's and Parkinson's (Zhu et al., 2007). Flavonoid may also exert beneficial effects on learning and memory and prevent cognitive losses in aged and young ones. They are also known to inhibit the inflammatory targets. No of epidemiological and experimental studies carried out suggests the role of flavonoid in cadmium induced toxicity (Morales et al., 2006a; Morales et al., 2006b; Renugadevi and Prabu, 2010).

2.2 Quercetin

Quercetin, a major class of flavonoid, belongs to a group of polyphenols. Because of its interesting chemical and biological properties, quercetin has found to be effective against many disease like cardiovascular, anti arithmetic, anti-inflammatory, anti carcinogenic and neuroprotective activities (Coskun et al., 2005; Murakami et al., 2008; Zahedi et al.,

2013). Several line of in vitro and in vivo studies suggests the potent role of quercetin in neurodegenerative disease especially Alzheimer's and Parkinson's (Zbarsky et al., 2005; Ansari et al., 2009). Other than that, the protective role of quercetin in traumatic brain injury, ischemic preconditioning and hypoxic models has also been established (Su et al., 2003; Yang et al., 2014). Several in vitro and in vivo studies have shown that antioxidant molecules that scavenge free radicals may mitigate the increase in markers for oxidative stress, and attenuate cellular damage, thus restoring normal cellular homeostasis and physiological function. The quercetin in terms has wide potential to against various neurological and neurodegenerative diseases.

2.2.1 Sources of Quercetin

Since quercetin belongs to the flavonoid group, they are widely distributed in variety of fruits and vegetables like apples, berries, citrus and spinach and other leafy vegetables. Quercetin is the most abundant in all flavonoid group, which is widely distributed in various vegetables, fruits and beverages (Formica and Regelson, 1995; Okamoto, 2005) mainly as glycosides. Quercetin is the major bioflavonoid in the human diet. Onion, kale, broccolis, lettuces, chilli peppers and tomatoes are the prime vegetables imparting as quercetin sources. Among the fruits, while varieties of fruits like apples, along with the strawberries, grapes, apricots are the richest sources of quercetin. Some beverages like tea, coffee and red wines are also contributed as quercetin supplement in nature. (Day and Williamson, 2001; Olthof et al., 2003). Highest concentration of quercetin was reported in red wines and in tea infusions (Hertog et al., 1993). Besides, medicinal plants like *G. biloba, B. monniera* and *C.pluricaulisare* also known to provides as quercetin source (Bhandari et al., 2007).

The distribution of quercetin may vary according to environmental factors, geographical distribution and others. Hertog et al 1993 performed a study where they calculated flavonol intakes in seven different countries and found that different geographical distribution of quercetin in varieties of food like wine was found as the major source of

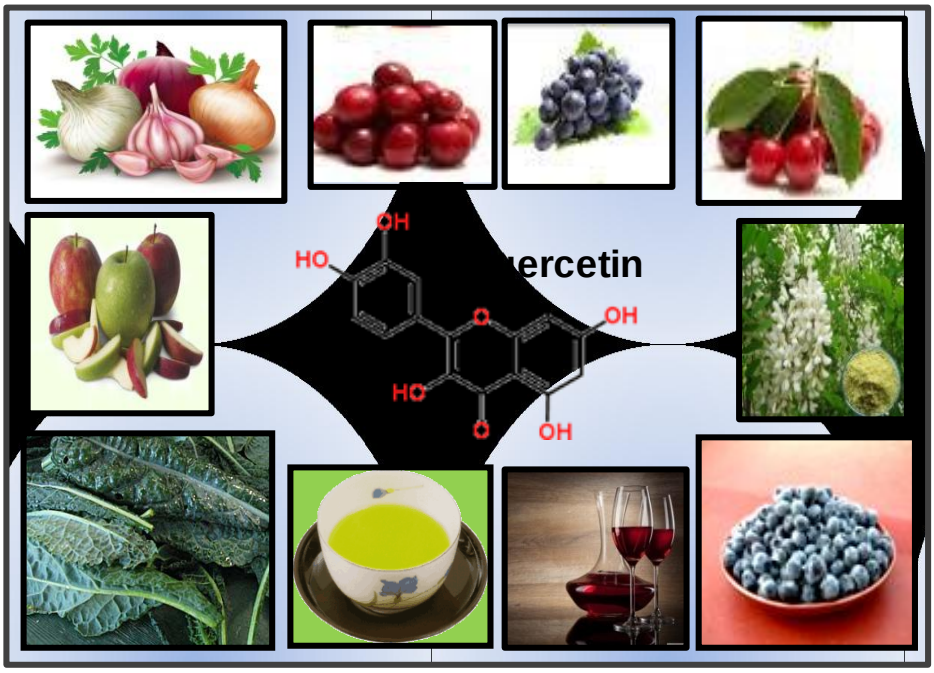


Figure – 2.4. Distribution of Quercetin

Quercetin in Italy, while onion and apples contributed most in the United States, Finland, Greece, and former Yugoslavia. Other than that, tea was the predominant source of quercetin in the Netherlands and Japan (Hertog et al., 1995). Further, similar kind of study was carried out by (Häkkinen et al., 1999) where they estimated the various source of quercetin in various fruits and vegetables in different regions. The estimated safe intake of quercetin in United State was to 22 mg/d, of which 73% to 76% was quercetin (values are for women and men)(Sampson et al., 2002).

2.2.2 Biosynthesis &Chemistry

Quercetin is the major flavonoid present in our diet. It has already been established that fruits and vegetables has been used in treatment of various neurodegenerative diseases. Flavonoids are produces in the plant by shikimic acid pathway. Flavonoids are consist of a three-ring structure having A, B and C rings in its structure and are further classified in to various subclasses (depending on the degree of oxidation in ring C). Quercetin belongs

to flavonols class known as 3,3',4',5,7 -pentahydroxyflavanone. It is having the OH group attached at positions 3, 5, 7, 3', and 4'. It also having the singlet oxygen in conjugation with double bond, gives it structural uniqueness and that is why having the superiority with the other members of flavonoid. Quercetin is a aglycone glycosides. It is of brilliant yellow color and having the solubility in alcohol and lipids, partially soluble in hot water and entirely insoluble in cold water. Addition of sugar molecule at position 3' in ring C makes it water soluble.

2.2.3 Bioavailability & Metabolism of Quercetin

Quercetin is generally present in the fruits and vegetables in form of glycosides. So, upon oral intake of quercetin, the glycoside quercetin are rapidly hydrolyzed by the enzyme of β-glycosidase in small intestine producing the algycone form of quercetin, which are known to be readily absorbed from intestine. Metabolism of quercetin generally takes place via first pass metabolism. Quercetin generally undergo methylation by catechol-O-methyltransferase (COMT), sulfation by sulfotransferases (ST) and glucuronidation by UDP-glucuronyltransferases (UGT)(Williamson et al., 2000)). Upon passing through the first pass metaboilism, where it undergoes O-methylation, glucuronidation, and/or sulfation to form its conjugates quercetin-3-glucuronide, quercetin-30-sulfate and iso-rhamnetin-3-glucuronide. (Hollman and Katan, 1997; Murota and Terao, 2003).

Studies have shown that bioavailability of aglycone quercetin is poor. An epidemiological study carried out suggests that aglycone quercetin in more bioavailable as to glycone quercetin. The studies shows that bioavailability of quercetin was found to be affected by various factors. Erlund I, Kosonen, performed an study in cross over settings in healthy volunteers with low doses of quercetin aglycone and quercetin- 3-rutinoside and found the interesting outcome that bioavailability of both forms of quercetin was some but the inter individual variation was more in case quercetin- 3-rutinoside, in woman's quercetin

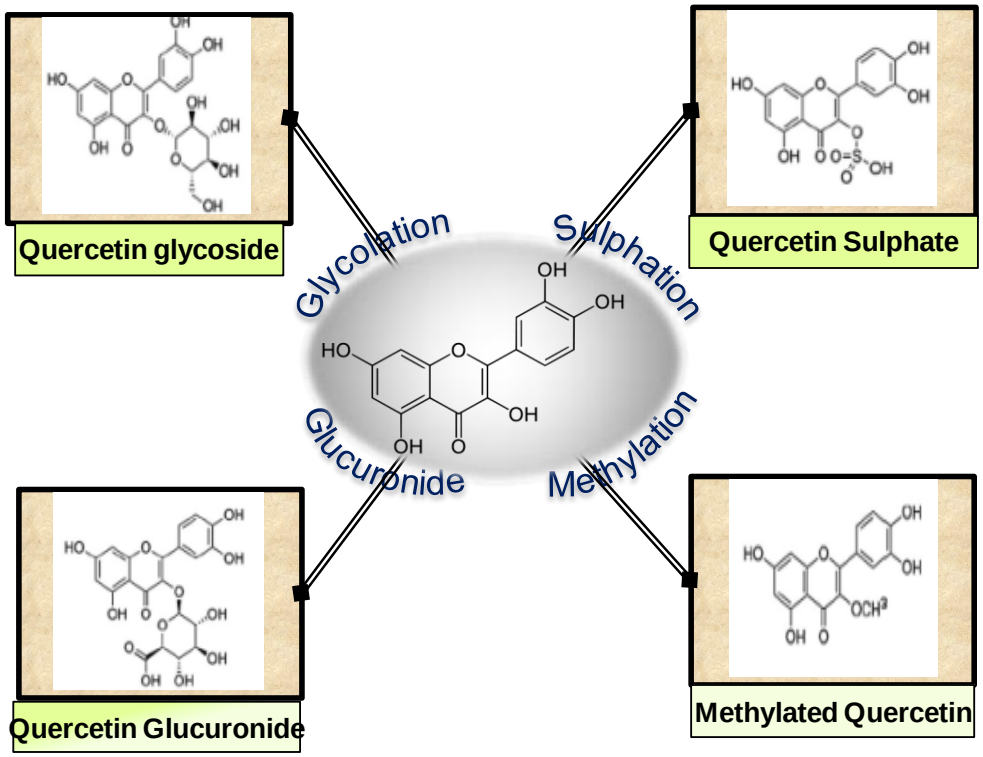


Figure – 2.5. Metabolism of Quercetin

- 3-rutinoside was more bioavailable (Erlund et al., 2000), the studies carried out by Walle et al., 2001 suggests of aglycone form of quercetin is more bioavailable as to glycone(Walle et al., 2001).

2.2.4 Pharmacological Properties - Quercetin

2.2.4.1 Anti inflammatory Properties

Quercetin has been shown to exert the potent anti inflammatory properties in various studies. Lipopolysaccharide-induced nitric oxide and tumor necrosis factor-α production has been found to be inhibited by the quercetin in murine macrophages (Ghosh, 1999). Several very recent randomized crossover clinical trials been demonstrating that quercetin supplementation quercetin may contribute to the cardioprotective effects of tea possibly by improving endothelial function and reducing inflammation (Dower et al., 2015). Further, quercetin also inhibits the in vitro production of enzymes usually induced

by inflammation (Kim et al., 1998; Lee et al., 2010) but the mechanisms behind the anti-inflammatory properties of quercetin are not clearly understood but may be exerts these affects by modulating the various signaling cascade.

2.2.4.2 Cardio protective properties

Quercetin has been found to show the cardioprotective properties. Flavonoids have been found to possess the wide spectrum of biological activities, which may have positive cardiovascular diseases. Quercetin protects against coronary heart disease by reducing the mortality caused by LDL, further it has having the potent activity to inhibit the platelet aggregation (Chopra et al., 2000) (Edwards et al., 2007) Epidemiological studies carried out showed an improvement in the coronary heart disease (CHD) on the consumption of red grape polyphenol extract rich in quercetin (Lekakis et al., 2005). Clinical trials carried out related to quercetin showed that quercetin at a dose of 150mg/kg reduces the systolic blood pressure and LDL levels (Egert et al., 2009).

2.2.4.3 Anticancer Properties

Various studies carried out recently showed the antiproliferative effects on various types of cancer. Mechanism by which quercetin exhibits such changes are mainly by regulating the cellular growth and by arresting the cells during various cell cycle phases apart from the antipoliferative properties. Studies carried out exhibit the potential of quercetin in leukemia cell lines L1210, breast cancer cells (Kim et al., 2013a), ovarian cancer cells (Scambia et al., 1990), and gastric cancer cells (Yoshida et al., 1990).

2.2.4.4 Quercetin – Role in Neurological Diseases

The effect of Quercetin was also studied in neurological disorders such as Alzheimer's disease and depression in humans and in preclinical studies.

2.2.4.4.1 Anti - Ischemic Properties

Quercetin has been found to be effective in several ischemic models (Su et al., 2003). The several processes like increase reactive oxygen species, altered cellular signaling processes, exitotoxicity are the contributing factor in the ischemic cell death processes. A number of studies carried out suggest the potential role of quercetin. Dong et al., 2014 showed the protective role of quercetin by modulating the oxidative stress in rat model of subarachnoidal hemorrhage, where the quercetin at a dose of 50mg/kg significantly reduces the levels of MDA, activity of caspases and enhanced the activity of SOD and GSH. In another study, the protective role of quercetin has been observed in traumatic brain injury via targeting to mitochondrial biogenesis. Quercetin at dose of 50mg/kg significantly attenuated TBI-induced neurological impairment and improved cognitive functions. Besides, quercetin in TBI reduced the expression of LC3, caspase-3 and Bax levels and thus imparting the potential role in TBI.

2.2.4.4.2 Alzheimer's Disease

models. Alzheimer's pathology is characterized by extracellular deposition of beta amyloid-Aβ and intracellular accumulation of tau proteins. The possible mechanism behind may be associated with the increase in oxidative stress and triggered inflammatory response. The quercetin being the potent free radical scavenger and having the strong antioxidant properties has been found to be a promising agent in Alzheimer's. Experimental studies carried out showed the Neuroprotective Effects against Aβ Aggregates in Hippocampal Neurons (Godoy et al., 2016). Neuroprotective effect of quercetin at a dose of 25 mg/kg 3 months decreases extracellular β-amyloidosis, tauopathy, astrogliosis and microgliosis in the hippocampus and the amygdala in transgenic AD model mice (Sabogal-Guáqueta et al., 2015). Further, one of the metabolite of quercetin that is quercetin-3-O-glucuronide significantly reduced β-amyloid (Aβ) in primary neuron cultures isolated from Tg2576 AD mouse model. The treatment of quercetin also significantly improved synaptic transmission and long-term potentiating in hippocampus (Ho et al., 2013). The quercetin having the low bioavailability has limits its clinical applications. Although quercetin has already been reported in Alzheimer's

disease, however nano formulations of quercetin improves the oral absorption and bioavailability of quercetin showed the higher improved the cognition and memory impairments and also reduces the GFAP activity in hippocampus (Puerta et al., 2017) Interestingly, PLGA nanoformulation of quercetin potential use in Alzheimer's disease models.

2.2.4.4.3 Parkinson's Disease

Parkinson's disease has known as the second most common neurodegenerative disease over worldwide with etiology of alpha synuclein protein aggregates. The mechanism implicated in the pathogenesis of PD includes abnormal protein aggregation, oxidative stress, mitochondrial dysfunction and inflammation. However, the exact mechanism related to is not assessed. Evidence from various epidemiological and animal studies exhibit that natural flavonoid may reduce the risk to the Parkinson's disease burden. Implications of quercetin in Parkinson diseases have been established well (Haleagrahara et al., 2011). Recently studies carried out by (El-Horany et al., 2016)showed the protective role of quercetin in rotenone model of Parkinson's disease by modulating the autophagy. Further ameliorative effect of quercetin at dose and time dependent manner in 6-hydroxydopamine (6-OHDA)-induced rat model of Parkinson's disease has also been shown (Mu et al., 2016). Further, the enhanced dopamine levels in striatum and increased mitochondrial activity following the combined exposure of fish oil and quercetin has been observed in chronic rotenone rat model (Joseph, 2015; Mu et al., 2016). Quercetin has been found to protect cell death in rotenone model of Parkinson's disease by regulating the mitochondrial activity (Karuppagounder et al., 2013). Besides, several studies carried out recently focused on the protective potential of quercetin in Parkinson disease model.

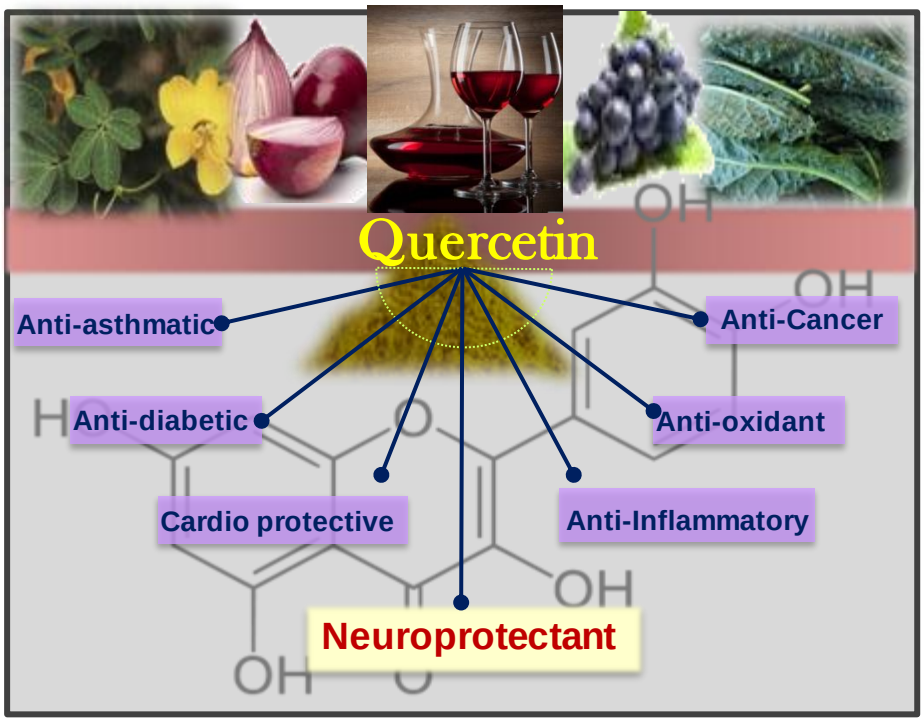


Figure – 2.6. Pharmacological Properties of Quercetin

2.2.4.5 Chemical Induced Neurotoxicity

Increased concern over worldwide to the impact of toxic agents on human health and the quality of life, brain has been shown to be particularly vulnerable to chemical insult . The clinical manifestation of such chemicals is quite extensive that may be associated with the increased risk to neurobehavioral toxicities. In consideration the fact, flavonoid has gained the large attention to combat the chemical induced neurotoxicity. Many of the investigators have established protective effects of quercetin in against chemical induced neurotoxicity. Quercetin at dose of 50 and 100 mg/kg consecutively for 5 days reduced the behavioral alteration in lead exposed rats.(Chander et al., 2014). The combine exposure to quercetin and chromium improved the learning and memory in F1 generation mice by decreasing the massive oxidative stress (Halder et al., 2016). In another study, quercetin reduces the oxidative stress and alters the mitochondrial activity in aluminum-exposed rat for 12 weeks. The decrease in the ROS levels and mitochondrial DNA

oxidation was observed in different brain regions following quercetin exposure (Sharma et al., 2013). Further, protective effects of quercetin (QE) against acryl amide induced neurotoxicity has also been established (Uthra et al., 2017). However, despite the considerable investigation on protective potential of quercetin, the exact molecular target has not been identified to its clinical implications in various neurological disease treatments. Further, more precise studies are to be needed to identify the mechanism and target that can be suitable to develop quercetin as a therapeutic entity in various neurodegenerative diseases and moreover besides the animal studies, clinical studies are to be needed.

Chapter III

Materials & Methods

3.1 Chemicals

Cadmium, quercetin, atropine sulphate, haloperidol, Triton-X-100, DCFH-DA, JC1 dye, MDC (monodansylcadervarine), DMSO, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and cresyl violet were purchased from Sigma-Aldrich, USA. The primary monoclonal antibodies - anti-rabbit choline acetyltransferase (ChAT), anti-rabbit tyrosine hydroxylase (TH), anti-rabbit Bcl2, anti-rabbit Bax, anti-rabbit Caspase-3, anti-rabbit Dopamine transporter (DAT), anti-rabbit vesicular monoamine transporter (VMAT-2), anti-rabbit protein kinase A (PKA), anti-rabbit DARPP32, anti-rabbit PP1α and anti-rabbit β- actin were purchased from Cell Signaling Technology, USA. Polyclonal antibodies – anti-rabbit dopamine D1 receptor and anti-rabbit dopamine D2 receptor were procured from Abcam, U.K while anti-rabbit PKCβ1 was purchased from Cell Signaling Technology, USA. Secondary antibodies – (goat anti-rabbit IgG- HRP, goat anti-mouse IgG-HRP) were also procured from Cell Signaling Technology, USA. Radioligands - ^{3}H-Quinuclidinyl benzilate (specific activity - 42 Ci/mmol) and ^{3}H-Spiperone (specific activity - 18.5 Ci/mmol) were purchased from PerkinElmer, USA. Rapamycin (Tocris Biosciences), 3-methyle adenine (Tocris Biosciences), LysoTracker®

Red DND-99 (Invitrogen), DAPI with antifade (Invitrogen), and GFP-LC-III expression kit (Invitrogen) was also procured. Other chemicals used in the study were of analytical grade and arranged from local commercial sources.

3.2 Instruments

Actimot (TSE, Germany), Grip strength meter (TSE, Germany), Y-maze, (TSE, Germany), Rotamex (Columbus Instruments, USA), Shuttle Box (Techno, India), Homogenizer (Kika Labortechnik, Germany), Sorvall RC-5B high speed refrigerated centrifuge (DuPont, USA), Vortex - Spinix (Tarson, India), Hot plate – Spinot (Tarson, India), Balance (Afcoset, India), pH meter - Cyberscan - 510 (Merck, Germany), UV-visible spectrophotometer (Cary 300 Bio, Varian, USA), Multiwell plate reader (Biotek Synergy HT, USA), NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA), Hydride system 60 atomic absorption spectrophotometer (HSAAS, Zeenit 700), High pressure liquid chromatograph (Waters, USA), Robotic liquid handling system – Multi*Probe* II_{EX} (Perkin Elmer, USA), Top Count *NXT* (Perkin Elmer, USA), and β-liquid scintillation counter (Perkin Elmer, USA), High resolution binocular microscope - Eclipse, E600 (Nikon, Japan) with computerized image analysis system - Leica Qwin 500 image analysis software (London, UK), Digital gel image analysis system – Image quant LAS, Cryotome – Microm (HM 520, USA), 7900HT Fast Real Time PCR System (Applied Biosystem, USA) were the major instruments used in the study.

3.3 *In vivo* Studies

3.3.1 Experimental Animals and Housing Conditions

Adult male rats (180 ± 20g) of Wistar strain obtained from the central animal breeding colony of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow were housed in a temperature controlled experimental room (25 ± 2ºC) with a 12-h light/dark cycle under standard hygiene conditions. The animals had free access to pellet diet procured from the national suppliers and water *ad libitum*. The animals were acclimatized for 7 days before starting the experiment. The study was approved by the institutional

animal ethics committee of CSIR-IITR, Lucknow (IITR/IAEC/50/13) and all experiments were carried out in accordance with the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests (Government of India), New Delhi, India.

3.3.2 Treatment Procedure and Sample Preparation

To assess the protective efficacy of quercetin in cadmium induced neurotoxicity, rats were randomly divided into four treatment groups and treated as per following schedule.

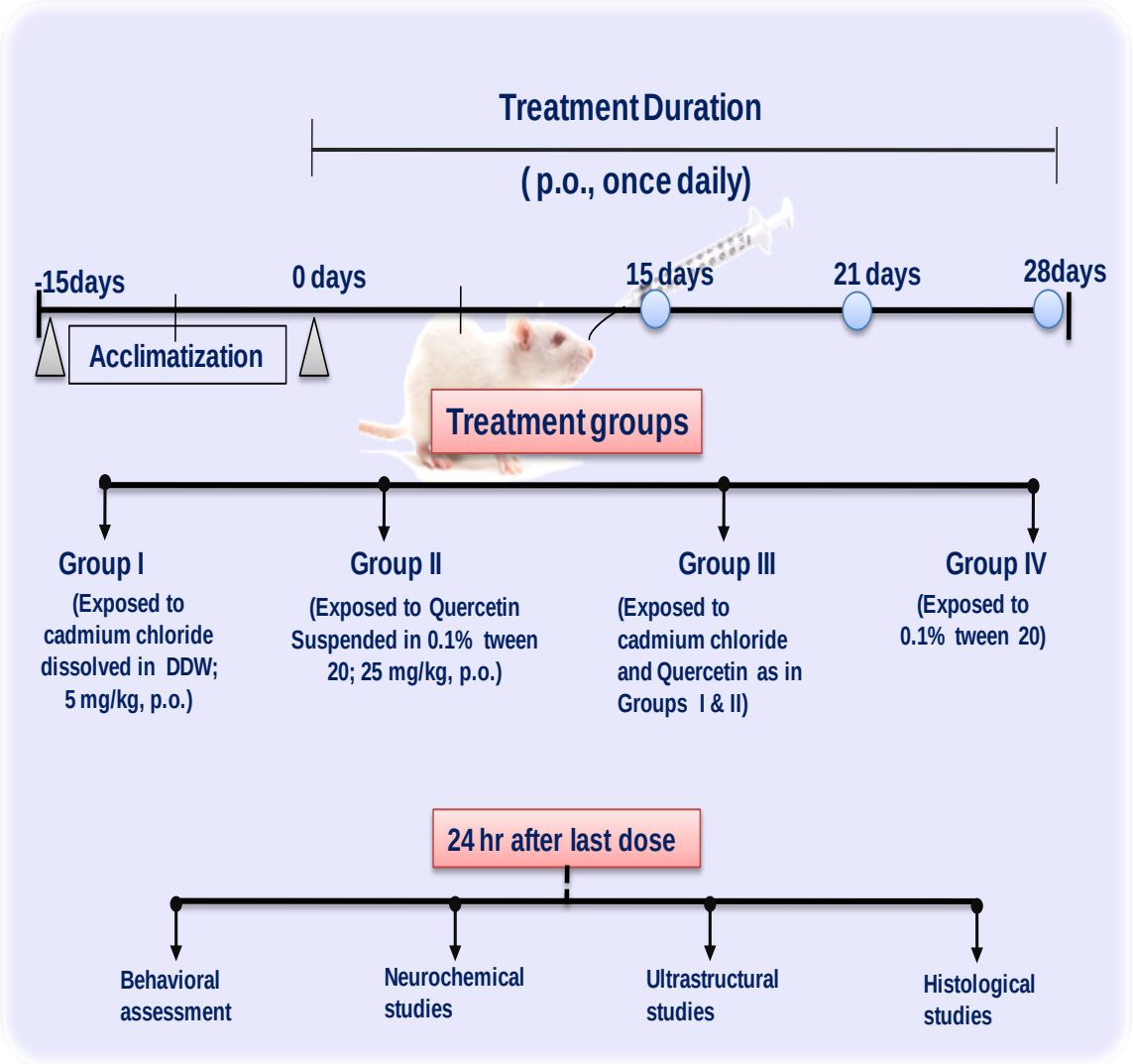


Figure – 3.1. Treatment Schedule

Rats in group I were treated with cadmium as cadmium chloride (5 mg/kg body weight, p.o., once daily for 28 days). In Group II, rats received quercetin (suspended in 0.1% tween 20, 25 mg/kg body weight, p.o., once daily for 28 days). Rats in group III were treated with cadmium and quercetin simultaneously as in treatment groups I and II respectively while rats in group IV received vehicle and served as controls (Figure – 3.1).

24 hours after the last dose of treatment with cadmium and quercetin, a separate set of rats was used for behavioral studies. For neurochemical studies, rats were quickly decapitated and brains were taken out quickly and washed in ice cold saline and dissected into different regions (frontal cortex, hippocampus, corpus striatum) following the standard procedure as described by (Glowinski and Iversen, 1966). For qRT-PCR studies, the brain regions were immediately immersed in Trizol reagent (Qiagen, Germany) and stored at –80˚C until used. For histological studies, a set of rats was perfused in paraformaldehyde (4%) and brains were stored in paraformaldehyde (10%). For ultrastructural studies, rats were perfused with paraformaldehyde (4%) and glutaraldehyde (0.1%).

3.3.3 Behavioral Studies

3.3.3.1 Spontaneous Motor Activity

Alterations in motor activity if any on cadmium exposure in rats was assessed by computerized Actimot (TSE, Germany) following the standard protocol as described by (Yadav et al., 2009). The actimot is fully automated with high density of infrared beams (32 × 32) and measures the activity based on light beam principle. Before starting the experiment, rats were acclimatized for 30min. Rats were placed in the centre of the cage and allowed to freely move for 5 min. The sensor becomes activated and the movement of the animals is tracked automatically. The details of the parameters assessed are as follows,

3.3.3.1.1 Total distance traveled (cm): Distance traveled is the basic feature of animal activity and used in open field studies.

3.3.3.1.2 Resting time (sec): The period during which no movement in the animal occurs and it remains at a fixed place is considered to be resting period.

During this time, no infrared beams are interrupted.

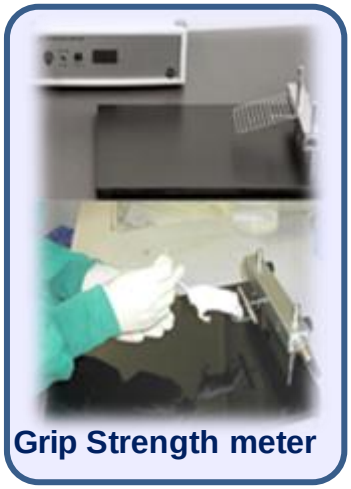

Actimot

3.3.3.1.3 Time moving (sec): The period during which the animal moves in the cage is designated as time moving.

3.3.3.1.4 Number of rearing: The period during which rat stands on its hind limbs away from the wall and with its forelimbs unsupported is referred as rearing.

3.3.3.1.5 Stereotypic count: Scratching, grooming and head swings during which the animal remains in the same location but interrupts light beams is considered as stereotypic movement. The number of these activities is recorded as stereotypic count.

3.3.3.2 Grip Strength


Grip Strength meter

Computerized grip strength meter (TSE, Germany) was used to measure the forelimb grip strength following the method as described earlier (Shukla et al., 2016). Briefly, holding the rat from nape and the base of the tail, the forelimbs were placed on the tension bar. The rat was pulled back gently until it released the bar. Reading was recorded automatically on the computer. Five successive pulls for each animal in the study group were tried by a person unaware of their treatment status. The mean of all the values was taken and processed for statistical analysis. Values are expressed in Pound.

3.3.3.3 Rota-Rod Performance

The motor co-ordination in rats was assessed using Rotamex (Columbus Instruments, USA) following the procedure as described earlier (Yadav et al., 2009). A group of rats from each treatment group received training on the rotating rod of Rotamex (at a constant speed 8 rpm). The rotational speed gradually increased from 4 to 40 rpm for a period of

300 s during the final trial. Further, the final trial was carried out by a person unaware of the treatment status. The time of fall from the rotating rod was scored.


Rotamex

3.3.3.4 Learning and Memory

Effect on learning and memory in rats treated with cadmium or quercetin alone or in combination was assessed by passive avoidance test using shuttle box. However, spatial memory and learning and continuous alteration test were assessed by Y- maze.

3.3.3.4.1 Y-Maze

Spatial memory was assessed through novelty seeking behavior using Y-maze and following the procedure as described by (Wang et al., 2009). Briefly, the test consists of two trials separated by an inter-trial interval of 4hr to assess spatial recognition memory. Out of three arms of Y-maze, one arm was blocked and termed as the novel arm. During the first trial, rats were placed in the start arm and allowed to explore the start arm and other arm for 15 minutes as the third arm (novel arm) was blocked. After an inter-trial interval of 4 hours, rats were placed in the same arm (start arm) and allowed to explore all arms for a period of 5 min. During this time, rats were free to visit all the three arms. Number of entries and time spent in the novel arm versus other arm was recorded on computer. The results are expressed as % time spent and % entries in the novel arm versus other arm.


Y- maze

Further, continuous alternation in rats was assessed using Y- maze as described by (Yamada et al., 1996). Briefly, rats were placed at the end of one arm of Y-maze and allowed to move freely for 5 minutes. The series and sequence of entry in to each arm of the Y-maze was recorded automatically.

the alternation percentage was calculated as the ratio of actual to possible alternation multiplied by 100.

3.3.3.4.2 Passive Avoidance Response

Passive Avoidance Response

The passive avoidance response was monitored using the shuttle box (Techno, India) following the procedure as described earlier (Yadav et al., 2011). Briefly, the shuttle box consists of two chambers – a lighted and a dark chamber separated by a guillotine door. Rats were placed in the lighted chamber of the shuttle box and after acclimatization for 30 sec, the guillotine door was opened. As the rats crossed in to the dark chamber, the guillitone door was closed and a low-intensity foot shock (0.5mA; 10 sec) was given. The 1st trial was for the acquisition and retention was assessed in subsequent trials carried out 24 hr after the 1st trial. The transfer of rat from light to dark compartment was recorded as transfer latency time (TLT) in seconds. The criterion for improved cognitive activity was considered as increase in the TLT on retention trial (2nd trial and more) as compared to the acquisition trial (1st trial). The shock was not given to the rats in the retention trials to avoid reacquisition.

Multiprobe IIEx

3.3.4 Neurochemical Studies

3.3.4.1 Assay of Neurotransmitter Receptors

Radioligand receptor binding technique was employed to assay the muscarinic-cholinergic receptors in frontal cortex and hippocampus and dopamine receptors in corpus striatum following the standard procedure as described earlier by (Khanna et al.,

1994). The method for preparation of crude synaptic membrane and binding assay is briefly described.

3.3.4.1.1 Preparation of Crude Synaptic Membrane

The isolated brain region (frontal cortex, hippocampus, corpus striatum) was homogenized in 19 volumes of Tris-HCl buffer (5mM, pH 7.4). The homogenate was centrifuged (40,000x g) for 15 min at 4ºC. The sedimented pellet was suspended in homogenization buffer (5 mM Tris-HCl, pH 7.4) and recentrifuged (40,000x g) for 15 min at 4ºC. The pellet thus obtained was finally suspended in Tris-HCl buffer (40 mM, pH 7.4) and stored at -20ºC.

3.3.4.1.2 Radioligand Binding Assay

Briefly, the reaction mixture in a final volume of 1 ml containing Tris-HCl buffer (40mM, pH 7.4) together with appropriate radioligand and membrane protein (~300-400 µg) was incubated (15min, 37^{o} c). To determine the extent of nonspecific binding, a set of tubes containing unlabelled competing agent, specific in each case, were run in parallel. Briefly, the assay of DA-D2 receptor was performed using ^{3}H-Spiperone (18.5 Ci/mmol, 1 X 10^{-9} M) as the radioligand and haloperidol (1 X 10^{-6} M) as competitor. For the assay of cholinergic - muscarinic receptors, ^{3}H-Quinuclidinyl benzilate (42 Ci/mmol, 1 X 10^{-9} M) was used as a radioligand and atropine sulphate (1 x 10^{-6} M) as competitor (Table – 3.1).

At the end of the incubation, the samples were rapidly filtered on glass fiber discs (25 mm diameter, 0.3 µm pore size, Whatman (GF/B) and washed twice with 5 ml cold Tris-HCl buffer (40mM, pH 7.4). Filter discs were dried and counted in 5 ml of scintillation mixture containing 2,5-diphenyloxazole, 1,4-bis(5-phenyloxazolezyl) benzene, naphthalene, toluene, 1,4-dioxan and methanol. To determine the membrane bound radioactivity, samples were counted using β-scintillation counter (Packard, USA) at an efficiency of 30-40% for ^{3}H. Specific binding has been calculated by subtracting the nonspecific binding (in the presence of competitor) from the total binding (in the absence of competitor)

Table – 3.1. Radioligands and competitors used for the assay of neurotransmitter receptors

Receptor	Brain Regions	Radioligand (Concentration)	Competitor (Concentration)
Cholinergic - muscarinic	Frontal cortex Hippocampus	3H-Quinuclidinyl benzilate ($1x10^{-9}$ M)	Atropine sulphate ($1X10^{-6}$ M)
DA – D_2	Corpus striatum	3H-Spiperone ($1x10^{-9}$ M)	Haloperidol ($1X10^{-6}$ M)

Concentration of radioligands and competitors used indicated in the parentheses

and results have been expressed as pmoles ligand bound/g protein. Scatchard analysis was carried out using different concentrations of radioligands (normally 1/10 to 10 times of the affinity of radioligand) to determine whether change in the binding is due to alteration in the affinity (Kd) or number of receptor binding sites (Bmax). The method used was essentially similar to other filtration binding method (Khanna et al., 1994). Saturability, specificity, reversibility and regional distribution of receptors were standardized prior to the experiments. The amount of membrane protein was determined following the standard procedure (Lowry et al., 1951) using bovine serum albumin as a standard.

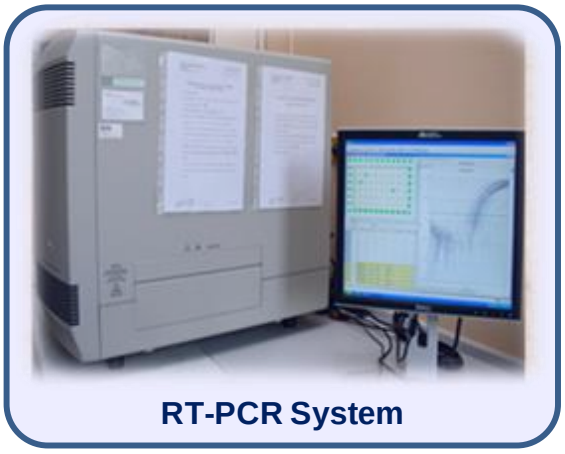
3.3.4.2 Expression of Neurotransmitter Receptor Gene

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to assess the expression of various genes for receptors and enzymes following the standard procedure.

3.3.4.2.1 RNA Extraction and cDNA Synthesis

The selected brain regions (frontal cortex, corpus striatum and hippocampus) were homogenized in 1 ml of ice-cold Trizol (Invitrogen, Carlsbad, CA, USA) followed by chloroform extraction and isopropyl alcohol precipitation. The isolated RNA was dissolved in RNAse free water (50 µl). RNA (0.5 µg) was loaded onto agarose gel (1%)

subjected to electrophoresis, stained with ethidium bromide and visualized by UV transillumination. The degradation of RNA was not observed as indicated by intact ribosomal bands 28S and 18S in all samples. The amount of RNA samples was assessed spectrophotometrically by NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) at OD 260/280. The ratio of the OD 260 / 280 of all extracted RNA samples was between 1.8 and 2.0. RNA was transcribed into single strand cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, USA).



RT-PCR System

The 2X transcription master mix was prepared and for 10µl reaction, 10X RT buffer, 25X DNTP mix (100mM), 10X RT random primer, multiscribe reverse transcriptase and nuclease free water were added and kept in ice. 2 X RT master mix (10µl) was pipetted into each individual tube. 10µl of RNA sample was added into each tube and the tubes were sealed. The tube was placed into thermal cycler using following conditions,

Step I - 25°C for 10 min; Step II - 37°C for 120 min; Step III - 85°C for 5 min;
Step IV - ∞

Table – 3.2. Primer sequences used for real time polymerase chain reaction (RT-PCR)

	CHRM1
	Forward 5'-3 CTGGTTTCCTTCGTTCTCTG
	Reverse 5'-3' GCTGCCTTCTTCTCCTTGAC
	CHRM2
	Forward 5'-3' GGCAAGCAAGAGTAGAATAAA
	Reverse 5'-3' GCCAACAGGATAGCCAAGTG
	CHRM3
	Forward 5'-3 GTGGTGTGATGGATTGGTCTG

	Reverse 5'-3'	TCTGCCGAGGAGTTGGTGTC
CHRM4		
	Forward 5'-3	AGTGCTTCATCCAGTTCTTGTCCA
	Reverse 5'-3'	CACATTCATTGCCTGTCTGCTTTG
CHRM5		
	Forward 5'-3	CTCATCATTGGCATCTTCTCCA
	Reverse 5'-3'	GGTCCTTGGTTCGCTTCTCTGT
ChAT		
	Forward 5'-3	CGGGATCCTGCCTCATCTTCTCTGGTGT
	Reverse 5'-3'	GGCGGAATTCAATCACAACATC
AChE		
	Forward 5'-3	GCTCACGTAGATTTATGCCACCAGA
	Reverse 5'-3'	TTGATCCAGCAGGCCTACATTG
DA-D1		
	Forward 5'-3	GTGGACCTCATGGCCTACAT
	Reverse 5'-3'	TGTGAGGGAGATGCTCAGTG
DA-D2		
	Forward 5'-3	TGGATCCACTGAACCTGTC
	Reverse 5'-3'	TCTCCTCCGACACCTACCCCGA
DA-D3		
	Forward 5'-3	TTAGCCCACATTGCTGTCTG
	Reverse 5'-3'	GGAGTTGAGGTGGGTGCTTA
DA-D4		
	Forward 5'-3	ATGGCCCCTGACTGCAAATC
	Reverse 5'-3'	AGTCCGGTGCCAGTACCTAA
DA-D5		
	Forward 5'-3	AGCATGCTCAGAGTTGCCGG
	Reverse 5'-3'	ACAAGGGAAGCCAGTCTTTGG
TH		
	Forward 5'-3	CCACGGTGTACTGGTTCACT
	Reverse 5'-3'	GGCATAGTTCCTGAGCTTGT

	β-actin	
	Forward 5'-3	CGTGGGCCGCCCTAGGCACCA
	Reverse 5'-3'	GGGGGGACTTGGGATTCCGGTT

3.3.4.2.2 Quantitative RT-PCR Analysis

For quantitative RT-PCR, the PCR reaction mixture in 20 µl contained 1XTaq Man Universal PCR Master Mix (Applied Biosystems), 10pM of each gene primers, 2 µl cDNA and nuclease-free water using the cDNA synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was done. The sequence of primers used has been described in Table – 3.2. RT-PCR assay was performed in triplicate on cDNA samples in 96-well optical plates on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). PCR conditions were set – 50°C for 2 min, 90°C for 10 min, 95°C for 0.15 s and 60°C for 1 min. The results have been analyzed by the $2^{-\Delta\Delta CT}$ method using β-actin as a reference gene (Singh et al., 2015).

3.3.4.3 Nitric Oxide Levels

Levels of nitric oxide in frontal cortex, corpus striatum and hippocampus were estimated using the assay kit procured commercially (Calbiochem, USA). The assay principle involves the conversion of nitrate to nitrite by the enzymatic action of nitrate reductase. Addition of 2,3 - diaminonaphthalene (DAN) and sodium hydroxide converts nitrite to a fluorescent compound 1(H)-naphthotriazole which is measured spectroflurometrically (excitation 430 nm / emission 450 nm) using multiwell plate reader (Misko et al., 1993). The results are expressed as fold changes.

3.3.4.4 Multiplex Bead Assay

Levels of pro and anti-inflammatory cytokines in brain regions were assessed involving specific Magnetic Bead Based Multiplex assay (Hulse et al., 2004).

3.3.4.4.1 Preparation of Membranes from Brain Tissues

The isolated brain regions were homogenized in cell lysis buffer containing PMSF and centrifuged at (12,500xg) 4^{o} C for 15min. The Supernatant was collected and used for further assay. The protein concentration was determined following the method as described earlier (Lowry et al., 1951).


Multiplex Reader

3.3.4.4.2 Bead Assay

To assess the expression of cytokines, a bead based Multiplex assay platform (Bio-Plex MAGPIX Multiplex Reader, Bio-Rad Laboratories, Hercules, CA) and Rat Cytokine /Chemokine Magnetic Bead Panel (RECYTMAG-65K, Millipore) was used. Bio-Plex MAGPIX system is based on the principle of sandwich ELISA which uses magnetic bead coupled antibodies in suspension phase kinetics. Levels of cytokines in samples were calculated after plotting the standard curves and expressed as pg/ml.

3.3.4.5 Western Blotting

Immunoexpression of selected proteins was assessed by Western Blotting following the procedure as described by (Jamal et al., 2007). Briefly, frontal cortex / hippocampus was lysed and equal amount of protein (30 µg protein / lane) was electrophoresed on SDS–PAGE (12%) and transferred on to nitrocellulose membrane followed by blocking with buffer containing BSA (5%). The membrane was incubated overnight at 4^{o}C with either of the monoclonal antibody [AChE (1:1000), ChAT (1:1000), TH (1:1000), DAT (1:1000), VMAT-2 (1: 1000), DA-D1 and DA-D2 receptor (1: 1000), PKA (1:1000), phospho PKA


Western Blotting

(1:1000), phospho DARPP32 (1 : 1000) (thr34), phospho PP1-α (1:1000) (thr320), Akt (1:1000), phospho Akt (1:1000), GSK-3β (1:1000), phospho GSK-3β (1:1000), CREB (1:1000), phospho CREB (1:1000)(ser133), PKC β-1(Sigma, 1:1000), GFAP (1:1000), Iba1 (1:1000), nNOS (1:1000), iNOS (1:1000), COX-2 (1 : 1000), STAT 3 (1 : 1000), CamkIIα (1:1000), MT3 (1:1000 dilution), Bcl-2 (1 : 1000), Bax (1 : 1000), Caspase-3 (1 : 1000), pJNK1/2, phospho JNK3 (1: 1000), phospho p38 (1:1000), AP1 (1:1000), Cyt C (1:1000), ERK1/2 (1:1000), phospho ERK1/2 (1:1000) (thr202/tyr204), LC3-II (1:1000), P62 (1:1000), Beclin1 (1:1000), Atg 3, 5, 7, 12, 16 (1:1000), Lamp 2a (1:1000) and β- Actin (CST, 1:1000) followed by incubation with horseradish peroxidase-linked secondary antibody (anti-mouse IgG, 1:4000; anti-rabbit IgG 1:4000) at room temperature for 60 min and detected by chemiluminescent method. Densitometric measurements of bands in the immunoblots were carried out using digital gel image analysis system (Image Quant LAS 500) and normalized by β- actin to correct variations, if any in protein loading.

3.3.4.6 Assessment of Mitochondrial Integrity

3.3.4.6.1 Assay of Mitochondrial Complexes

To assess the complex I (NADH-ferricyanide reductase) activity, ferricyanide was used as electron acceptor and method of (Hatefi, 1978) was followed. Briefly, the reaction mixture (final volume 1 ml) contained phosphate buffer (50 mM, pH 7.4), NADH (0.17 mM), ferricyanide (0.6 mM), and Triton X-100 (0.1% v/v). Mitochondrial suspension (~ 10 - 30 µg protein) was added to the reaction mixture in the cuvette to start the reaction at 30 °C. The rate of oxidation of NADH was assessed using a spectrophotometer at 340 nm. The activity of complex I has been expressed as nmoles of NADH oxidized/min/mg protein.

For the assay of complex II-III (succinate - cytochrome c reductase) activity, reduction of ferricytochrome c to ferrocytochrome c was monitored in the presence of succinate following the method as described by (Clark et al., 1997). The assay mixture in a total volume of 1 ml contained phosphate buffer (100 mM), succinate (2 mM), KCN (1 mM), EDTA (0.3 mM) and cytochrome c (1.2 mg/ml). Mitochondrial suspension (~ 10 - 30

µg) was added to initiate the reaction and reduction of ferricytochrome c was monitored at 550 nm. The activity of complex II-III has been expressed as nmoles oxidized cytochrome c reduced/min/mg protein.

The activity of complex IV (cytochrome c-oxidase) was assessed by monitoring the oxidation of reduced cytochrome c (ferrocytochrome c) following the standard procedure (Wharton, 1967). Briefly, ferricyanide (1 mM) in phosphate buffer (10 mM, pH 7.4) was added to oxidized ferrocytochrome c in a final volume of 1 ml at room temperature. The reaction was initiated by adding mitochondrial suspension (~ 10 - 30 µg protein) and rate of oxidation was recorded at 550 nm. The activity of complex IV has been expressed as nmoles reduced cytochrome c oxidized/min/mg protein.

3.3.4.6.2 Estimation of Reactive Oxygen Species

The generation of reactive oxygen species (ROS) in brain regions was assessed following the method of (Rush et al., 2007). Briefly, mitochondrial suspension was incubated with DCFH-DA dye (10µl, 100µM final concentration) at room temperature for 30 min. The DCFH-DA is oxidized to fluorescent DCF by intracellular ROS during the course of reaction. The generation of ROS was measured using fluorescence reader at excitation 485 nm / emission 520 nm and results have been expressed as % of control.

3.3.4.6.3 Assessment of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) in selected brain regions was estimated using JC-1 dye as it is a sensitive indicator to assess the change in fluorescence from red to green following the standard procedure (Kane et al., 2008). Briefly, the mitochondrial cell suspension was incubated with JC-1 dye (10 µM) at 37^{o}C for 15 min. and washed with PBS. The cells were finally suspended in 0.5ml PBS (10^{6} cells/ml) and analyzed by flow cytometer (FACS CantoTM II, BD Bio- Sciences, San Jose, CA, USA).

3.3.4.7 Estimation of Cadmium Levels

Estimation of cadmium levels in brain regions - frontal cortex, hippocampus and corpus

striatum was carried out using atomic absorption spectrometer (AAS). Briefly, samples were prepared by digestion of samples in the acid mixture following the procedure as described in Bulat et al., 2012. In short, brain samples were exposed to acid mineralizing process with concentrated HNO3 and HClO4 in a ratio 4:1 at 130°C for 3 hr or overnight. The solution was digested over a sand bath until it became yellow in color. If the color of the digest tissue was brown, more acid mixture added and the process of oxidation repeated. After mineralization process, samples were diluted with 0.1mol/L HNO3 and used for the estimation of cadmium levels in different brain regions using atomic absorption spectrometer. Detection limit of the instrument was 1 ppb.

atomic absorption Spectrometer

3.3.4.8 Estimation of Neurotransmitter Levels and Their Metabolites in Brain Regions

Levels of dopamine (DA), norepinephrine (NE), epinephrine (EPN), serotonin (5HT) and metabolites of dopamine - 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were estimated in brain regions (frontal cortex, corpus striatum and hippocampus) of rats treated with cadmium or quercetin alone or on their simultaneous treatment for 28 days using reversed phase high performance liquid chromatography with electrochemical detector (HPLC-ECD) following the standard procedure of (Kim et al., 1987) with minor modifications.

High Performance Liquid Chromatography

Briefly, brain regions were homogenized in perchloric acid (0.1M) containing 3,4 - dihydroxybenzylamine, an internal standard at a final concentration of 25 ng/ml followed by centrifugation at 36,000×g for 10 min at 4°C. The supernatant obtained was then filtered through 0.25 µm nylon filters and used for the determination of neurotransmitter levels. Sample (20 µl) was injected in the injector port. The mobile phase (pH 4.2) containing sodium dihydrogen phosphate (0.15 M), ethylenediaminetetra acetic acid (0.25 mM), sodium octyl sulphate (1.75 mM) and methanol (4%) was used to separate peaks in the samples at a flow rate of 1.5 ml/min. Electrochemical detector with glassy carbon and silver nitrate electrode was operated at a potential of +0.800V with sensitivity range 2nA at ambient temperature. Data were recorded and analyzed with the help of Empower2 software and results are expressed as ng/g tissue weight.

3.3.5 Histological Studies

Histological studies were carried out using Nissl staining as described by (Veena et al., 2011). Briefly, thin sections from the brain were cut using Cryotome (Microm, HM520 USA.). The sections were stained with cresyl violet (0.1%) and dehydrated through graded series of alcohol. Finally, the sections were cover slipped with DPX mounting media and the intensity of Nissl stained neurons was determined using a computerized image analysis system (Image J).

3.3.6 Ultrastructural studies

For ultrastructural studies, a separate set of rats was used. Rats were anesthetized with ketamine (30 mg/kg) and perfused with paraformaldehyde (4%) and glutaraldehyde (0.1%). The perfused brain was dissected into frontal cortex and hippocampus and cut into fine pieces (2mm approx). Primary fixation of sections was carried out for 2 hr in glutaraldehyde (2.5%) prepared in sodium cacodylate buffer (0.1 M, pH 7.2). Subsequently, post fixation was carried out in osmium tetraoxide (1%) for 1 – 2 h followed by dehydration and embedding in araldite and DDSA medium. The tissues were baked at 65 °C for 48 h and cut into thin sections (60–90 nm) using ultramicrotome (Leica EM UC 67). The thin sections on copper mesh grids were stained with uranyl

acetate and lead citrate (2%) for contrast. Examination of the brain sections was carried out through transmission electron microscope (Tecnai G2 spirit transmission electron microscope equipped with Gatan CCD/Orius camera at 60 KV).

3.4 *In vitro* Studies

3.4.1 Characterization and Preparation of PLGA Quercetin-NPs

Poly (D, L-lactide-co-glycolide) (PLGA) quercetin-NPs were prepared following the emulsion solvent evaporation method with minor modifications. Briefly, 100 mg of quercetin was dissolved in 3.5 mL of dimethyl sulfoxide in dark. PLGA (1.0 g) solution prepared in 10 mL of DCM was added and the solution was stirred at 1,600 rpm for 15 min. Following this, 1% polyvinyl alcohol (PVA) solution (50 mL) was added slowly into organic solution and stirred for 2 h at 25ºC. The reaction mixture was kept in vented position at 25ºC for 12 h with stirring at 1,600 rpm to remove the organic solvent. The yellow colored emulsion was centrifuged at 12,000 rpm for 30 min at 4ºC. The yellow pellet was re-suspended in water and centrifuged and the process was repeated thrice. The yellow colored nanoparticles received were freeze dried to obtain dry powder. Nanoparticles were stored at 4ºC under anhydrous conditions in dark till use.

3.4.2 Characterization of PLGA-Quercetin-NPs

3.4.2.1 Percent Yield

After achieving the constant weight, yield (%) of NPs was calculated by following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of nanoparticle}}{\text{Weight of (drug + polymer)}} \times 100$$

3.4.2.2. Particle Size Measurement

The mean particle size and the polydispersity index (PDI) of PLGA-quercetin-NPs were determined by dynamic light scattering (DLS) technique employing a nominal 5mW He-Ne laser operating at 633 nm wavelength. The freeze dried nanoparticles were dispersed

in aqueous buffer and the size was measured. The measurement was carried out at 25 ºC with the following settings: 10 measurements per sample; refractive indices of water, 1.33; viscosity of water, 0.89 cP. The particle size was measured in triplicate.

3.4.2.3 Drug Loading and Entrapment Efficiency

The drug loading and encapsulation efficiency were determined by analyzing the NPs spectrophotometrically using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA). The amount of quercetin present in the nanoparticles was estimated as per following procedure.

Known amount of NPs (1.0 mg, dry powder) was dispersed in 1 mL double distilled water by stirring the sample vigorously and the absorbance of the solution was measured at 373 nm and the amount of drug present was calculated from a previously drawn calibration curve of concentration vs. absorbance with different concentrations of known drugs. All the measurements were performed in triplicate. The percent drug loading (%DL) and entrapment efficiency (%EE) were calculated using following formula.

$$\%DL = \frac{\text{Weight of drug in NPs x100}}{\text{Weight of NPs}}$$

$$\%EE = \frac{\text{Amount of drug present in the polymeric NPs x100}}{\text{Amount of drug used}}$$

3.4.2.4 Nanoparticle Surface Morphology

The surface morphology of the NPs was characterized using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

3.4.2.4.1 Transmission Electron Microscopy (TEM)

Briefly, a drop of aqueous solution of lyophilized powder (1 mg/mL) was placed on a TEM grid surface and a drop of 1% uranyl acetate was added to the surface of the

Formvar-coated grid. After 1 min of incubation, excess fluid was removed and the grid surface was air dried at 25 ± 2 ºC before being loaded into the microscope. The NPs were visualized under the transmission electron microscope (FEI Company, OR, USA) operated at 80 kV, attached to a Gatan Digital Micrograph (PA, USA).

Transmission Electron Microscopy

3.4.2.4.2 Scanning Electron Microscopy (SEM)

The NPs were characterized for their shape, surface morphology, and particle size distribution by high-resolution field emission SEM (Quanta FEG 450, FEI, Netherlands). The sample was placed on a double-stick conducting carbon tape over an aluminum stub and coated with gold under an argon atmosphere by means of a sputter coater (SC 7620, mini sputter coater, Quorum Technology Ltd., UK). Samples were analyzed at an accelerating voltage of 10 kV and a working distance of 10 mm in a high-vacuum mode.

SEM

3.4.2.4.3 Evaluation of Quercetin Released From Nps

To determine the release profile of PLGA-quercetin-NPs, known quantity of the particles (~5 mg) was dispersed in 1 mL of phosphate buffered saline (PBS), pH 7.4, and kept in the dialysis tube, which was suspended in 20 mL of PBS in a glass vial and the solution was stirred at 1600 rpm at 37 ºC. At pre-determined intervals of time, samples were collected (ca. 200 mL) from the glass vial followed by spectroscopic analysis at 373 nm using UV/VIS spectrophotometer. The same amount of fresh buffer was added to the

glass vial and the release study was continued. The quantity of the released drug was then calculated using a previously drawn standard curve of the pure drugs in PBS.

3.4.3 Cell Culture and NGF Induced Neuronal Differentiation

PC12, a cell line derived from the pheochromocytoma of the rat adrenal medulla and SHSY5Y, a human neuroblastoma originally procured from National Centre for Cell Science (NCCS), Pune has been maintained at *In vitro* Toxicology Laboratory at CSIR-IITR and used for the present study. In brief, PC12 cells were cultured in standard conditions in RPMI cell culture medium supplemented with FBS (5%), HS (10%), sodium bicarbonate (0.2%) and antibiotic/antimycotic cocktail (1%) under CO2 (5%), and high atmospheric humidity at 37°C. SH-SY5Y cells were cultured in the following conditions:5 % CO2, 95 % atmosphere of high humidity at 37 °C in DMEM/F-12 Ham's cell culture medium supplemented with 10 % FBS, 0.2 % sodium bicarbonate and antibiotic/ antimycotic cocktail (1×). For all studies, cells at passage 6–12 were used. Viability of cells was measured by trypan blue dye exclusion and batches of cells having more than 95% viability were used in the study.

After passage # 6, cells were plated on poly-L Lysine (PLL) coated flasks. For inducing neuronal differentiation, cells were incubated in medium containing NGF (100 ng/ml). Differentiation medium was changed on alternate day. For experimental purpose, confluent growing cells were sub-cultured in PLL pre-coated six-well culture plate and 75-Cm^2 culture flask.

3.4.4 Exposure Schedule

After identification of noncytotoxic and cytoprotective doses of cadmium, quercetin and nanoquercetin respectively at time point 24, 48, 72 and 96 hr using standard endpoints - tetrazolium bromide MTT assay as described by Agrawal et al. 2012, differentiated cells were exposed to Cadmium (10 μM) and quercetin (100 μM) and coexposed with cadmium and quercetin for 24 hr. To assess the comparative profiling of bulk vs nano quercetin, another set was also run where the differentiated cells were exposed to

cadmium (10 μM for 24 hr), quercetin (100 μM for 48hr) and nanoquercetin (100 μM for 48 hr) individually and in combination with cadmium, quercetin and nanoquercetin. Combined exposure was further devised in three major groups - pre-exposure, post-exposure and co-exposure for quercetin and nanoquercetin both. Unexposed sets were also run under identical conditions and served as basal control. Following respective exposures, cell viability was assessed. Further, markers associated with dopamine receptor mediated signaling, autophagy and apoptosis were also assessed.

3.4.5 Cell Viability Assay

Cell viability was ascertained by MTT assay following the standard protocol (Agrawal et al., 2012). In brief, cells (1x 10^4 cells/ml) were seeded in 96-well plates for 24 hr under high humid environment with CO2 (5%) and atmospheric air (95%) at 37^{o}C. The medium was aspirated and cells were exposed to variable concentrations of cadmium (0.1-1000 μM), quercetin (0.1-1000 μM) and naoquercetin (0.1-1000 μM) for 24 - 96 hr. Tetrazolium bromide salt (10 μl/well; 5 mg/ml of stock in PBS) was added 4 h prior to the completion of incubation in respective case. Plates were incubated at 37°C for 4 hr, MTT solution removed and cells were lysed using a culture grade DMSO by pipetting up and down several times until the content was homogenized. After 10 min incubation, the color was read at 550 nm using multi-well microplate reader (Synergy HT, Bio-Tek, USA). The unexposed sets were also run simultaneously under identical conditions which served as control.

3.4.6 Western Blotting (*In vitro*)

Western blotting was conducted following the protocol as described earlier (Kumar et al., 2015). After respective exposure with cadmium or quercetin alone or in combination, cells were scraped, pelleted, and lysed using CelLytic M Cell Lysis Reagent (Sigma) in the presence of protein inhibitor cocktail (Sigma). Equal amount (40 μg/ well) of denatured protein (determined by Bradford method) was loaded on SDS-PAGE gel (10-15%) and blotted onto a nitrocellulose membrane by wet transfer method. Nonspecific binding was blocked with BSA (5%) in TBST for 1 hr at 37°C. After blocking, the

membrane was incubated overnight at 4°C with primary antibodies specific for TH (1;1000), DAT (1:1000), VMAT2 (1 : 1000), DA-D1 and DA-D2 receptor (1 : 1000), PKA (1:000), phospho-PKA (1:1000), Akt antibody (1:1000), phospho Akt antibody (1:1000), phospho DARPP32 (1:1000), phospho PP1α (1:1000), GSK-3β (1:1000) CREB (1:000), phospho CREB (1:000), Bax (1:1000), Bcl2 (1:1000), caspase (1:1000) and β-actin (1;2000) in blocking buffer (pH 7.5). The membrane was incubated for 2 hr at room temperature with secondary anti-primary immunoglobulin G (IgG)–conjugated horseradish peroxidase. The blots were developed using Super Signal West Femto Chemiluminescent Substrate (ThermoFisher Scientific). The densitometry for protein-specific bands was conducted in Gel Documentation System (Alpha Innotech) with the help of Alpha Ease FC Stand Alone V.4.0 software. The marker proteins analyzed to study the altered expression were same as studied in the *in vivo* studies.

3.4.7 Pharmacological Inhibitor Studies

Role of DA-D2 and its downstream pathway molecules was confirmed using specific pharmacological inhibitors. The cells were seeded in PLL pre-coated 96 well culture plates and allowed to adhere for 24 h prior to the experimental exposure. Prior to exposure to cadmium (10μM) and quercetin (100μM) for 24 h, cells were exposed to pharmacological inhibitors of Akt (A6730: 5 μ M), PKA (H-89: 10 μM) for 1 hr respectively.

3.5 *In silico* Studies

To study the molecular level interaction of cadmium chloride (CdCl2), Quercetin with D1 and D2-Dopamine receptor, computational studies were done.

3.5.1 Homology Modeling of DA-D1 and DA-D2 Receptor

Protein sequence of the Dopamine D1 and D2 receptor in animal model *Rattus norvegicus* was retrieved from UniProt database (P18901, P61169). Homology based model for DA –D1 and DA- D2 receptor was built by homology modeling approach using Modeller version 9.15 (Martí-Renom et al., 2000). Templates for modeling protein

sequence were identified using blastP tool against Protein data Bank (Bernstein et al., 1977). Homolog having good structural similarity was used to build 200 protein models and top 20 models selected on basis of their lowest DOPE score were analyzed for structural stability using RAMACHANDRAN PLOT analysis feature of PROCHECK server (Laskowski et al., 1993).

3.5.2 Ligand Preparation

Structure of CdCl2, Quercetin and dopamine were built and minimized using MarvinSketch version 6.1.2 from ChemAxon and then clean in 3D using Steepest Descent method.

3.5.3 Molecular Docking

Ligand binding pockets on DA-D1 and DA- D2 protein were identified using binding site identification module of Discovery Studio 4.1(Wu et al., 2003). Top 3 sites identified in D2 receptor were docked with all 3 compounds (Quercetin, Dopamine and CdCl2) using CDOCKER module of DS version 4.1. UCSF Chimera was utilized for image generation (Pettersen et al., 2004).

3.5.4 DFT Studies

For predicting the structure of Cd-Quercetin complex, DFT calculations were performed with the Gaussian09 package (Frisch et al., 2009). DFT studies were carried out using Ground State, Default Spin and RB3LYP method. Basic set used was LanL2DZ. Guess method used was Default and solvation was performed without any constraint using the SMD model in water. At 298 K and1 atm Pressure, frequency analysis was carried out to confirm that each structure is a local minimum with no imaginary frequency, or either a transition state with only one imaginary frequency. The 3D images of the calculated structure were prepared using Gauss-View 5.0 (Dennington et al., 2009).

3.6 Statistical Analysis

Results are expressed as mean ± standard error of mean (SEM) for the values. One way analysis of variance (ANOVA) using the GraphPad prism3 software was used to analyze the data. To assess the levels of significance comparing all the pair of columns, Newman–Keuls test was employed and value up to $p < 0.05$ considered significant.

Chapter IV

Results

4.1 Module 1- Effect on Brain Cholinergic Modulations Following Exposure of Rats to Cadmium, Quercetin and their Co-Exposure for 28 Days

4.1.1 Effect on Learning and Memory

As cholinergic dysfunctions may cause impairment in learning and memory, effect of cadmium on spatial memory and learning and protective effect of quercetin was assessed using shuttle box and Y-maze respectively.

4.1.1.1 Effect on Spatial Memory and Continuous Alternation

To assess the effect of cadmium on spatial memory, percentage of alternation was monitored using Y- maze. A significant impairment ($F_{(3, 16)} = 4.505$, 43%, $p<0.05$) in alternation was observed in rats on cadmium exposure as compared to those in the control group. Simultaneous exposure with quercetin in cadmium exposed rats resulted to cause improvement in alternation as compared to rats treated with cadmium ($F_{(3, 16)} = 4.505$, 50%, $p<0.05$) alone (Figure – 4.1A).

Further, effect on the novelty seeking behavior on cadmium exposure and protective effect of quercetin was also assessed by Y maze. Percent entries and time spent in the novel arm and other arm was found to be similar in rats on cadmium exposure ($p>0.05$). Rats simultaneously exposed to cadmium and quercetin was found to spend more time in

the novel arm with increase in percentage of entries ($p<0.05$) as compared to those treated with cadmium alone (Figure – 4.1B, C). Further, frequency of visits and time spent in novel arm was found to be higher ($p<0.01$) as compared to other arm in rats in control group and those exposed to quercetin.

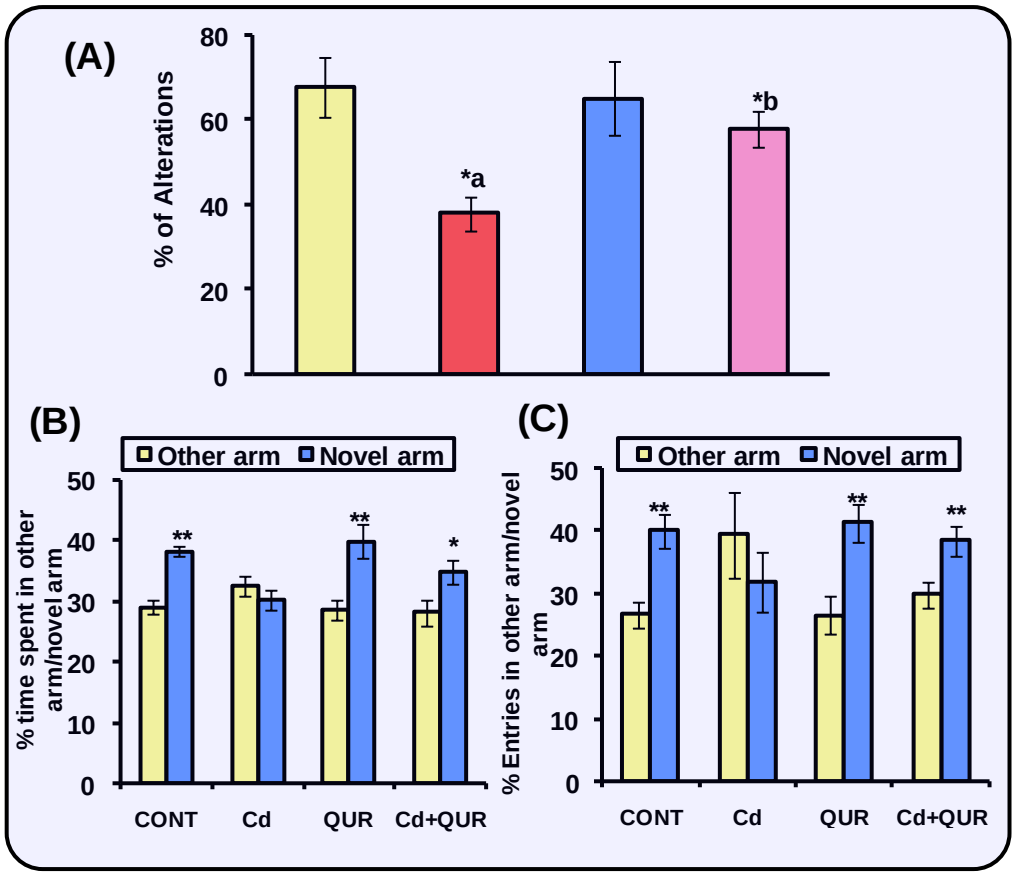


Figure – 4.1. Effect on spatial learning and memory assessed by Y-maze (A) Continuous alternation test (B) % entries in other/novel arms (C) % time spent in other/novel arms.

Values are mean ± SEM of five animals in each group; significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

4.1.1.2 Effect on Passive Avoidance Response

Exposure of rats to cadmium resulted to decrease transfer latency time (TLT) in the retention trials ($p<0.05$) as compared to the acquisition trial. These rats crossed into dark chamber of the shuttle box early and thus indicate impairment in learning and memory.

Simultaneous treatment with quercetin in cadmium treated rats was found to protect these changes as evident by increase in the transfer latency time ($p<0.01$) in retention trials as compared to cadmium treated rats. Interestingly, the transfer latency time was found to be increased in rats in control ($p<0.001$) and quercetin ($p<0.001$) exposed groups suggesting that there was no impairment in learning and memory. Further, no significant difference in the transfer latency time on acquisition trial was observed in rats in any of the treatment groups (Figure - 4.2).

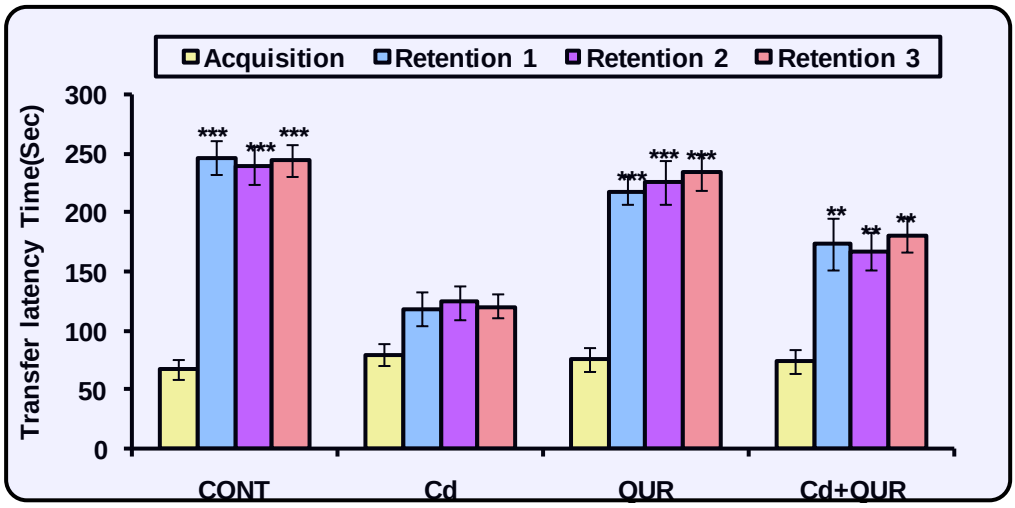


Figure – 4.2. Effect on learning and memory assessed by shuttle box

Values are mean ± SEM of five animals in each group; significantly differs (**p < 0.05, ***p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

4.1.2 Neurochemical Studies

4.1.2.1 Effect on the Binding of Cholinergic Muscarinic Receptors and Expression of Cholinergic Muscarinic Receptor Genes in Frontal Cortex and Hippocampus

A significant decrease in the binding of ^{3}H-QNB was observed both in frontocortical ($F_{(3,16)}$ = 4.963, 40%, $p<0.05$) and hippocampal ($F_{(3,16)}$ = 17.25, 56%, $p<0.001$) membranes of rats on cadmium exposure as compared to controls. Alteration in the binding was found due to decreased number of binding sites (Bmax) with no significant effect on the affinity (Kd) in both brain regions as revealed by Scatchard analysis.

Interestingly, simultaneous exposure to quercetin was found to protect cadmium induced decrease in the binding of cholinergic-muscarinic receptors both in frontal cortex ($F_{(3,16)}$ = 4.963, 42%, p<0.05) and hippocampus ($F_{(3,16)}$ = 17.25, 56%, p<0.05). No significant effect on the binding of cholinergic-muscarinic receptors was observed in either of the brain region of rats exposed to quercetin alone as compared to rats in the control group (Figure - 4.3).

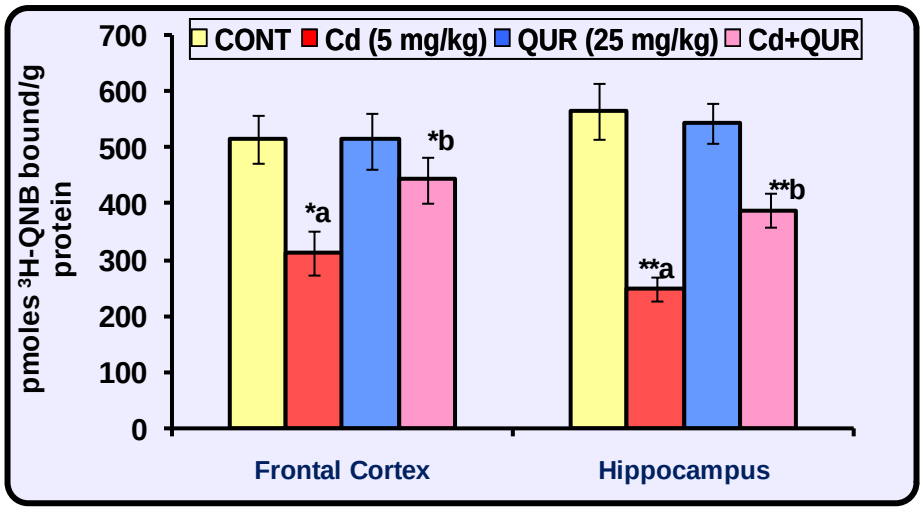


Figure – 4.3. Effect on ^{3}H-QNB binding in frontocortical and hippocampal membranes

Values are mean ± SEM of five animals in each group; significantly differs (*p < 0.05, **p < 0.01); a - compared to control group, b - compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

Impact of cadmium exposure on the mRNA expression of cholinergic receptor gene in frontal cortex and hippocampus was assessed by qRT-PCR. Cadmium exposure for 28days in rats caused decrease in the expression of CHRM1($F_{(3,8)}$ = 6.471, 55%, p<0.01; $F_{(3,8)}$ = 11.45, 54%, p<0.01), CHRM2($F_{(3,8)}$ = 9.107, 45%, p<0.01; $F_{(3,8)}$ = 10.98, 58%, p<0.01), CHRM3($F_{(3,8)}$=0.7009,15%, p>0.05; $F_{(3,8)}$ = 0.8996, 18%, p>0.05) CHRM4($F_{(3,8)}$ = 6.839, 45%, p<0.05; $F_{(3,8)}$ = 5.606, 46%, p<0.05) and CHRM5($F_{(3,8)}$ = 1.296, 16%, p>0.05; $F_{(3,8)}$ = 1.125, 20%, p>0.05) receptor genes both in frontal cortex and hippocampus respectively as compared to controls. Interestingly, changes in the mRNA expression of CHRM1($F_{(3,8)}$ = 6.471 $F_{(3,8)}$ = 11.45, 95%, p<0.05), CHRM2($F_{(3,8)}$ = 9.107, 93%, p<0.01; $F_{(3,8)}$ = 10.98, 92% p<0.01;), CHRM3($F_{(3,8)}$ = 0.7009, 78% p<0.05; 21%),

CHRM4($F_{(3,8)}$ = 6.83970%, p<0.05,) and CHRM5($F_{(3,8)}$ = 1.296, 82% p<0.0131%, p>0.05)

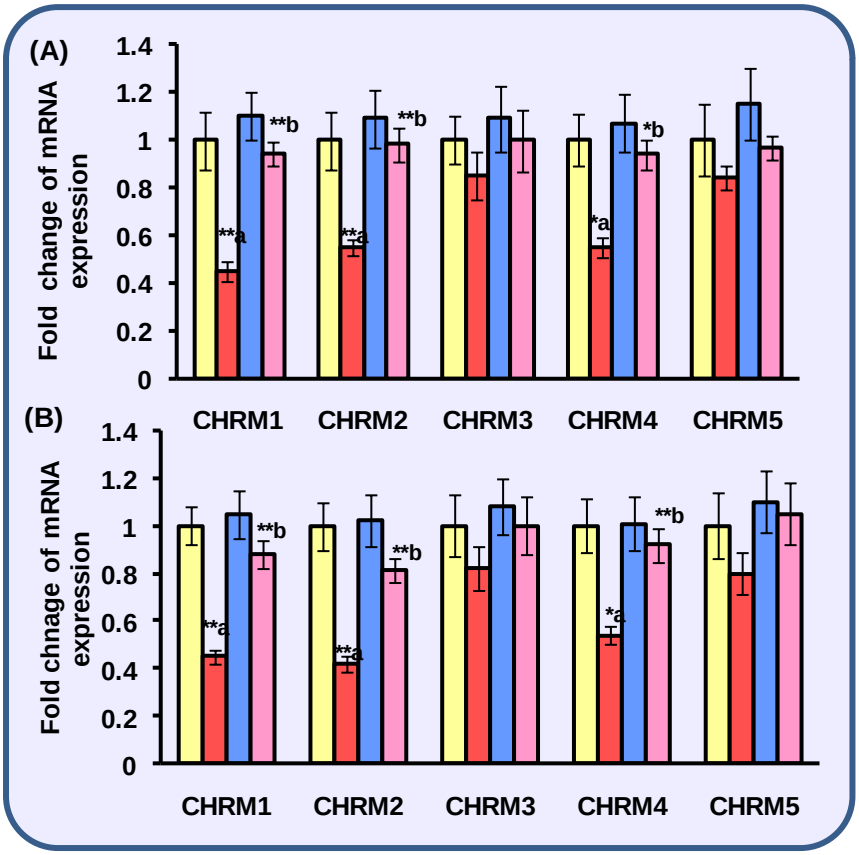


Figure – 4.4. Effect on the expression of muscarinic-cholinergic receptor gene in frontal cortex (A) and hippocampus (B)

Values are mean ± SEM of three animals in each group; significantly differs (*p < 0.05, **p < 0.01); a - compared to control group, b - compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

genes on cadmium exposure were protected in rats on simultaneous treatment with quercetin both in frontal cortex and hippocampus respectively (Figure –4.4A, B).

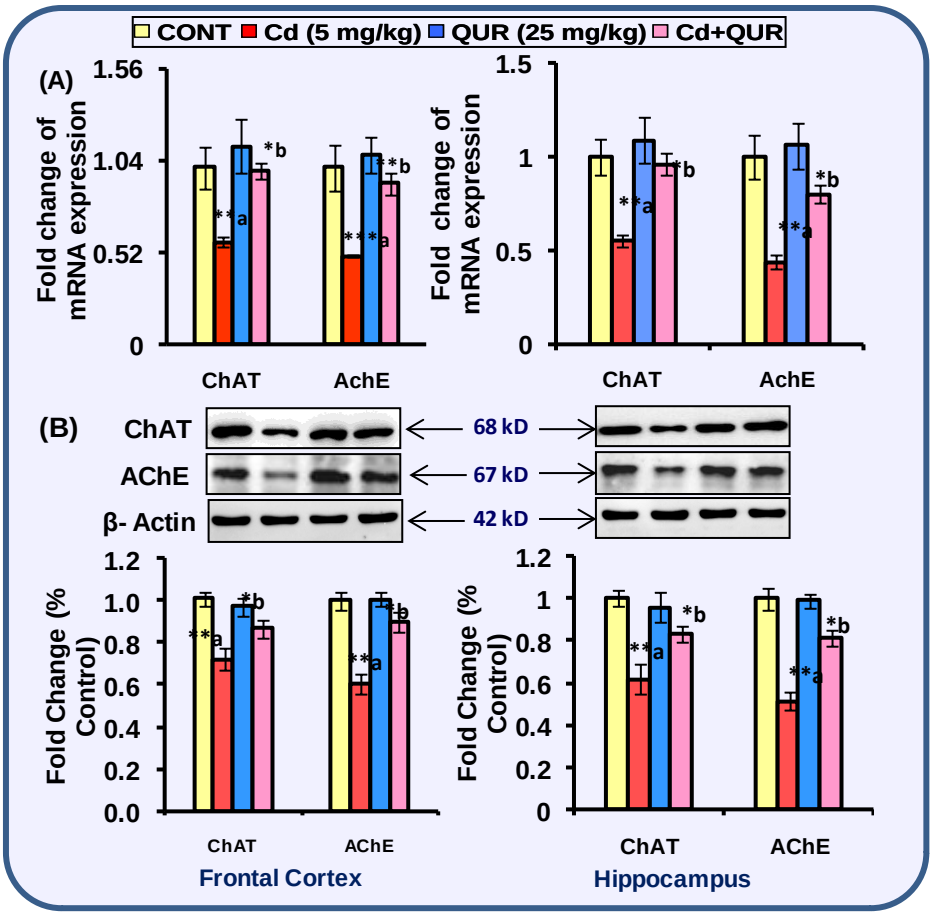
4.1.2.2 Effect on the Expression of Choline Acetyltransferase and Acetylcholinesterase in Frontal Cortex and Hippocampus

Effect of cadmium on the expression of ChAT, a marker for the integrity of cholinergic neurons and AChE, an enzyme involved in the metabolism of acetylcholine and protective efficacy of quercetin was assessed both in frontal cortex and hippocampus. Exposure of rats to cadmium resulted to decrease the mRNA expression of ChAT and AChE in frontal cortex ($F_{(3,8)}$ = 5.479, 42%, $p<0.05$; $F_{(3,8)}$ = 8.528, 50%, $p<0.01$) and hippocampus ($F_{(3,8)}$ = 11.25, 55%, $p<0.05$; $F_{(3,8)}$ = 10.91, 60%, $p<0.01$) as compared to controls indicating alterations in the cholinergic system. Simultaneous exposure with quercetin in cadmium exposed rats caused a significant protection in the mRNA expression of ChAT and AChE both in frontal cortex ($F_{(3,8)}$ = 5.479, 82% $p<0.01$; $F_{(3,8)}$ = 8.528, 68% $p<0.05$) and hippocampus ($F_{(3,8)}$ = 11.25, 56% , $p<0.05$; $F_{(3,8)}$ = 10.91, 74% $p<0.01$) respectively as compared to those exposed to cadmium alone (Figure –4.5A).

Decrease in the protein expression of ChAT and AChE in frontal cortex ($F_{(3,8)}$ = 8.594, 1.74Fold, $p<0.01$; $F_{(3,8)}$ = 19.65, 1.65Fold, $p<0.01$) and hippocampus ($F_{(3,8)}$ = 8.740, 1.61Fold, $p<0.01$; $F_{(3,8)}$ = 28.69, 1.58Fold, $p<0.001$) was observed in rats on cadmium exposure as compared to controls. Simultaneous exposure to cadmium and quercetin was found to cause an increase in the expression of ChAT and AChE proteins both in frontal cortex ($F_{(3,8)}$ = 8.594, 1.38 Fold, $p<0.05$; $F_{(3,8)}$ = 19.65, 1.42Fold, $p<0.05$) and hippocampus ($F_{(3,8)}$ = 8.740, 1.20Fold, $p<0.05$; $F_{(3,8)}$ = 28.69, 1.54Fold, $p<0.01$) respectively as compared to rats exposed to cadmium alone. No significant change in the mRNA and protein expression of ChAT and AChE was observed either in frontal cortex or hippocampus of rats exposed to quercetin alone as compared to controls (Figure – 4.5B).

4.1.2.3 Effect on the Expression of PKC β-1

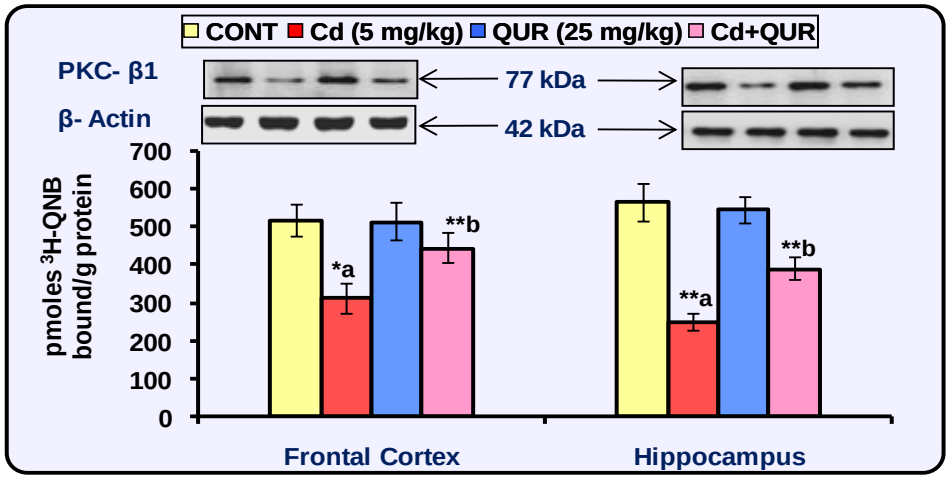
Exposure to cadmium in rats significantly decreased the expression of PKC β-1, an important protein involved in synaptic signaling both in frontal cortex ($F_{(3,8)}$ = 18.83, 1.55Fold, $p<.001$) and hippocampus ($F_{(3,8)}$ = 28.72, 1.40Fold, $p<0.001$) as compared to



Figure–4.5. Effect on the expression of ChAT and AChE gene (A) and their proteins (B)

Values are mean ± SEM of three animals in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin, ChAT – Choline acetyltransferase, AChE – Acetylcholinestrase.

rats in the control group. Simultaneous exposure with quercetin in cadmium exposed rats was found to protect the change on the expression of PKCβ1both in frontal cortex ($F_{(3,8)}$ = 18.83, 1.28Fold, p<0.05) and hippocampus ($F_{(3,8)}$ = 28.72, 1.70Fold, p<0.01) as compared to cadmium exposed rats. No significant change in the expression of PKCβ1 was observed either in frontal cortex or hippocampus of rats exposed to quercetin alone, compared to controls (Figure –4.6).



Figure–4.6. Effect on the expression of PKC-β1

Values are mean ± SEM of three animals in each group; significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin.

4.1.2.4 Effect on Mitochondrial Dysfunctions

To understand the molecular mechanism underlying cadmium induced cholinergic deficits and protective potential of quercetin in cholinergic neurons, the focus was to assess the mitochondrial integrity and associated activation of caspase cascade which leads to neuronal cell death.

4.1.2.4.1 Effect on the Activity of Mitochondrial Complexes

Effect of cadmium on the activity of enzyme complexes involved in electron transport chain and cellular bioenergetics and protective efficacy of quercetin was assessed in frontal cortex and hippocampus, the cholinergic rich areas of brain. Exposure to cadmium in rats for 28 days resulted to decrease the activity of complex I ($F_{(3,16)}$ = 4.697, 30%, $p<0.05$; $F_{(3,16)}$ = 4.611, 28%, $p<0.05$), complex II-III ($F_{(3,16)}$ = 27.87, 58%, $p<0.001$; $F_{(3,16)}$ = 12.25, 53%, $p<0.01$) and complex IV (($F_{(3,16)}$ = 1.935, 22%, $p<0.05$; $F_{(3,16)}$ = 4.892, 26%, $p<0.05$) both in frontal cortex and hippocampus respectively as compared to controls. Interestingly, cadmium induced decrease in the activity of mitochondrial complexes was found to be protected as evident by increase in the activity of complex I

($F_{(3,16)}$ = 4.697, 28%, p<0.05; $F_{(3,16)}$ = 4.611, 18%, p< 0.05), complex II-III ($F_{(3,16)}$ = 27.87, 71%, p<0.01; $F_{(3,16)}$ = 12.25, 55%, p<0.05) and complex IV ($F_{(3,16)}$ = 1.935, 17%, p<0.05; $F_{(3,16)}$ = 4.892, 28%, p<0.05) in rats simultaneously treated with quercetin. No significant change in the activity of any of the complexes was observed either in frontal cortex or hippocampus treated with quercetin as compared to controls (Figure – 4.7).

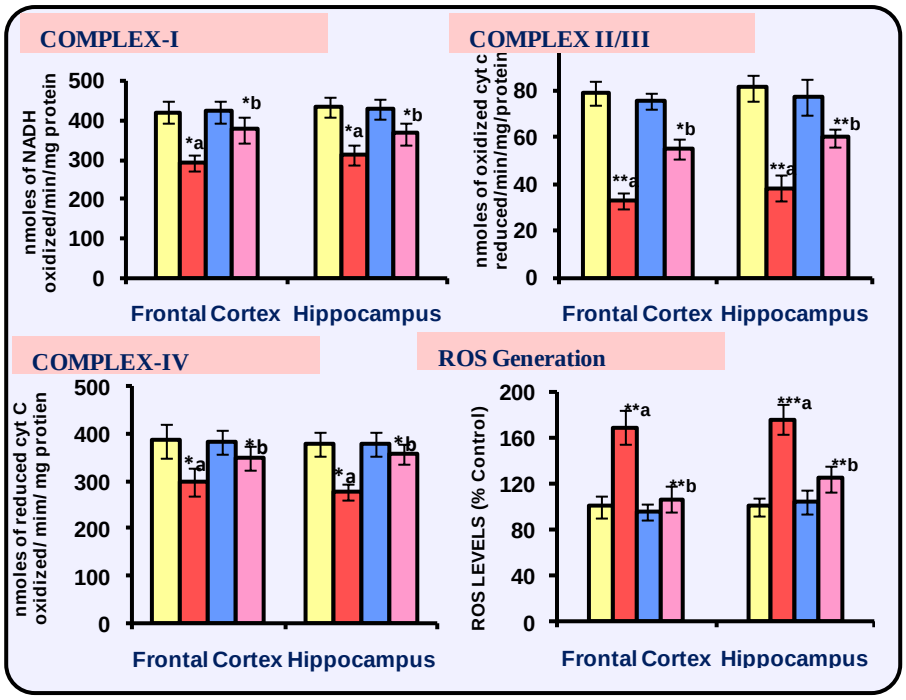


Figure – 4.7. Effect on the activity of mitochondrial complexes and ROS generation in frontal cortex and hippocampus

Values are mean ± SEM of five animals in each group; significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin

4.1.2.4.2 Effect on the Generation of Reactive Oxygen Species

Exposure of rats to cadmium resulted to increase the generation of ROS in frontal cortex ($F_{(3,16)}$ = 9.577, 69%, p<0.01) and hippocampus ($F_{(3,16)}$ = 10.36, 75%, p<0.01) as evident by enhanced fluorescence of DCHF dye as compared to rats in control group (Figure - 13). Simultaneous exposure to quercetin in cadmium exposed rats was found to protect the ROS generation both in frontal cortex ($F_{(3,16)}$ = 9.577, 37%, p<0.01) and hippocampus

($F_{(3,16)}$ = 10.36, 28%, p<0.01) as compared to rats treated with cadmium alone. No significant change in ROS levels was observed both in frontal cortex and hippocampus of rats treated with quercetin alone as compared to control rats (Figure – 4.7).

4.1.2.4.3 Effect on Mitochondrial Membrane Potential

Cadmium exposure for 28 days resulted to cause significant impairment in the mitochondria containing green JC-1 dye detected at the FL1 channel. Functional

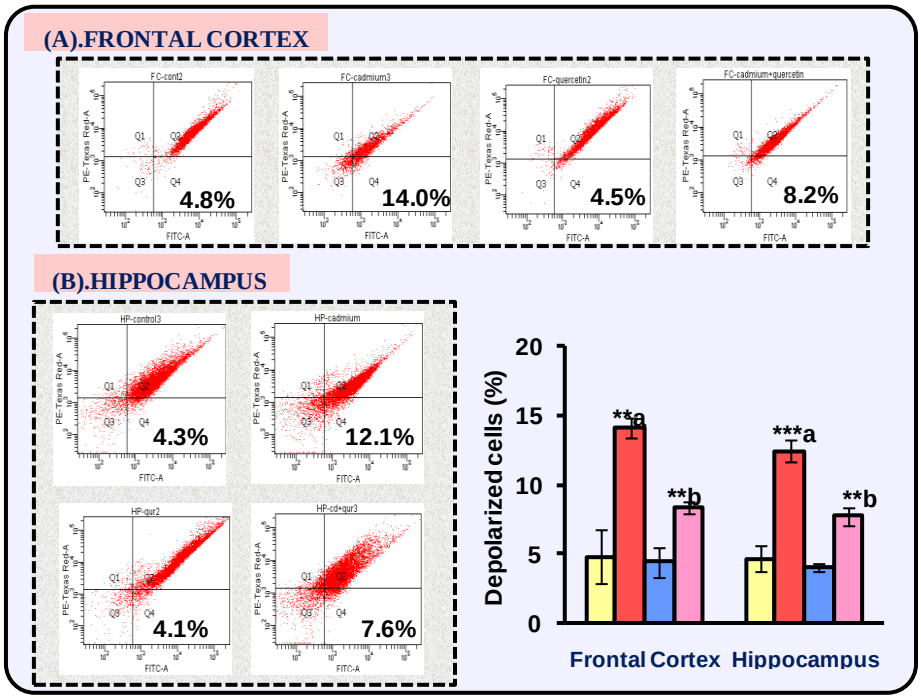


Figure – 4.8. Effect on the mitochondrial membrane potential

Values are mean ± SEM of three animals in each group; Significantly differs (**p < 0.05, ***p < 0.01); a - compared to control group, b - compared to cadmium exposed group; Representative dot plot of individual experiment in frontal cortex (A) in hippocampus (B).

mitochondria displayed red JC-1-aggregates and were detected at FL2 channel. Exposure of rats to cadmium increased the percentage of depolarized cells suggesting decrease in the mitochondrial membrane potential in frontal cortex ($F_{(3,8)}$ = 14.52, 14.10%, p<0.01) and hippocampus ($F_{(3,8)}$ = 30.28, 12.47%, p<0.001) as compared to controls. Simultaneous exposure to cadmium and quercetin was found to attenuate the percentage

of depolarized cells in frontal cortex ($F_{(3,8)}$ = 14.52, 8.4%, p<0.01) and hippocampus ($F_{(3,8)}$ = 30.28, 7.7%, p<0.01) suggesting increase in the mitochondrial membrane potential as compared to rats exposed to cadmium alone (Figure – 4.8). No significant change in the mitochondrial membrane potential was observed in frontal cortex and hippocampus of rats exposed to quercetin as compared to controls.

4.1.2.5 Effect on the Expression of Pro- And Anti- Apoptotic Proteins

Exposure of rats to cadmium resulted to increase the expression of Bax, a pro-apoptotic protein in frontal cortex ($F_{(3,8)}$ = 4.967, 1.34Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 19.58, 2.08Fold, p<0.001) associated with decrease in the expression of Bcl2, an anti-apoptotic protein both in frontal cortex ($F_{(3,8)}$ = 13.97, 1.54Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 32.42, 1.49Fold, p<0.001) respectively as compared to control rats. Increase in the ratio of Bax / Bcl2 was distinct both in frontal cortex and hippocampus suggesting enhanced apoptosis following cadmium exposure. An increase in the expression of caspase-3, an executer protein was also evident in frontal cortex ($F_{(3,8)}$ = 5.671, 1.93Fold, p<0.05) and hippocampus ($F_{(3,8)}$ = 7.811, 2.40Fold, p<0.01) respectively on cadmium exposure in rats. Simultaneous exposure of rats to cadmium and quercetin resulted to decrease the expression of Bax in frontal cortex ($F_{(3,8)}$ = 4.967, 1.84Fold, p<0.05) and hippocampus ($F_{(3,8)}$ = 19.58, 1.71Fold, p<0.01) and increase the expression of Bcl-2 in frontal cortex ($F_{(3,8)}$ = 13.97, 1.44Fold, p<0.05) and hippocampus ($F_{(3,8)}$ = 32.42, 1.59Fold, p<0.01) as compared to cadmium exposed rats. Decrease in the expression of caspase-3 in frontal cortex ($F_{(3,8)}$ = 5.671, 1.82Fold, p<0.05) and hippocampus ($F_{(3,8)}$ = 7.811, 1.76Fold, p<0.05) was also evident in cadmium treated rats simultaneously exposed to quercetin. No significant change in the expression of any of these proteins was observed either in frontal cortex or hippocampus of rats exposed to quercetin as compared to rats in the control group (Figure - 4.9).

4.1.2.6 Effect on the Expression of Cytochrome C

Release of Cyto C from the inner mitochondrial space is the main key event in the

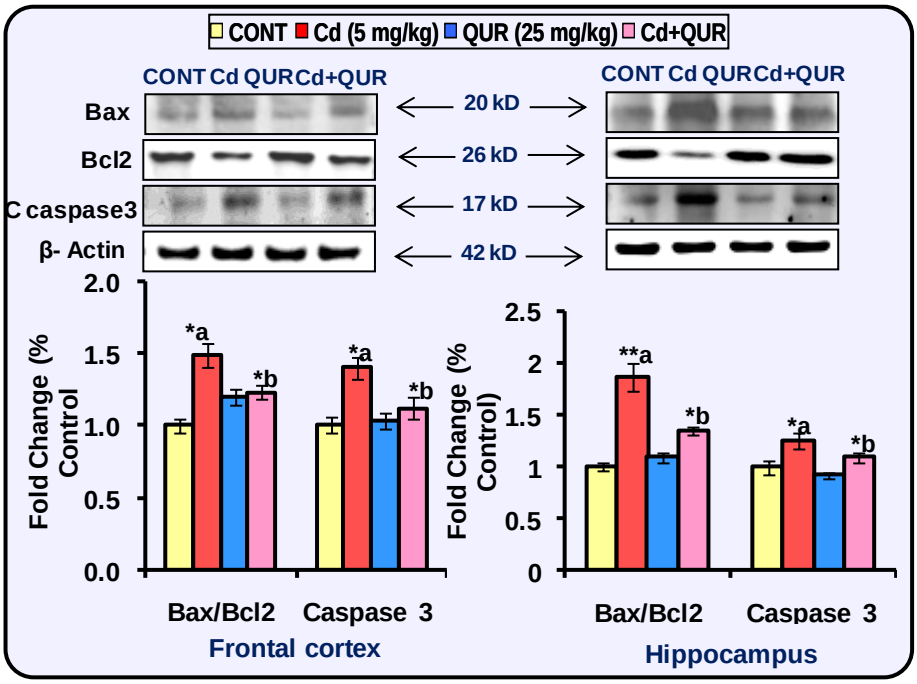


Figure – 4.9. Effect on the expression of pro-, anti- apoptotic and executer proteins in frontal cortex and hippocampus

Values are mean ± SEM of three animals in each group; Significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin; Ratio of Bax/Bcl2 was determined to quantitate apoptosis

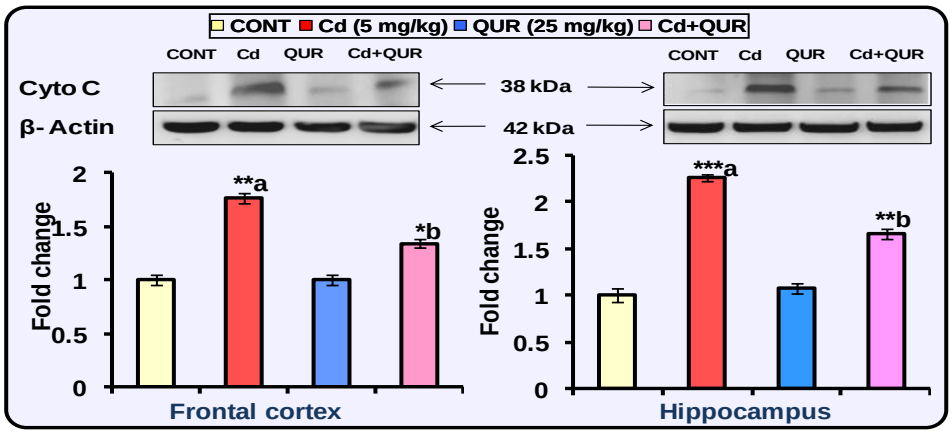


Figure – 4.10. Effect on the expression of Cyto C in frontal cortex and hippocampus

Relative protein levels were quantified after normalization with β-actin; Values are mean ± SEM of three animals in each group; Significantly differs (*p < 0.05, **p < 0.01, ***p <0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin, CytoC – cytochrome C.

caspase mediated apoptotic cell death. Effect of cadmium on Cyto C release from the mitochondria was assessed through protein expression using western blotting techniques. Cadmium exposure significantly increased the expression of Cyto C protein in frontal cortex ($F_{(3, 8)}$ = 41.13, 1.70Fold, p<.001) and hippocampus ($F_{(3, 8)}$ = 54.62, 2.25Fold, p<0.001) respectively as compared to controls. Simultaneous exposure to cadmium and quercetin was found to decrease the expression of Cyto C protein both in frontal cortex ($F_{(3, 8)}$ = 41.13, 1.75Fold, p<0.01) and hippocampus ($F_{(3, 8)}$ = 54.62, 1.73Fold, p<0.001) as compared to rats treated with cadmium alone. No significant change in the Cyto C expression was observed both in frontal cortex and hippocampus of rats exposed to quercetin alone as compared to controls (Figure – 4.10).

4.1.2.7 Effect on the Expression of MAPK Proteins

To understand the mechanism of cadmium induced apoptotic cell death, effect on the expression of MAPkinase proteins was assessed in frontal cortex and hippocampus. Exposure to cadmium resulted to increase the expression of AP1, p-p38, pJNK1/2 and pJNK3 both in frontal cortex ($F_{(3,8)}$ = 9.465, 1.59Fold, p<0.01; $F_{(3,8)}$ = 4.430, 1.25Fold, p<0.05, $F_{(3,8)}$ = 3.962, 1.82Fold, p<0.05, $F_{(3,8)}$ = 25.73, 1.80Fold, p<0.001, $F_{(3,8)}$ = 10.43, 1.38Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 18.71, 1.85Fold, p<0.001; $F_{(3,8)}$ = 15.88,1.49Fold, p<0.01, $F_{(3,8)}$ = 17.82,1.94 Fold, p<0.01; $F_{(3,8)}$ = 25.83, 1.6Fold, p<0.001; $F_{(3,8)}$ = 8.412, 1.52Fold, p<0.01) respectively as compared to controls. Simultaneous exposure to cadmium and quercetin was found to decrease the expression of AP1, p-p38 JNK1/2 and JNK3 proteins in frontal cortex ($F_{(3,8)}$ = 9.465, 1.79Fold, p<0.05; $F_{(3,8)}$ = 4.430,1.90Fold, p<0.05; $F_{(3,8)}$ = 3.962,1.70Fold, p<0.05; $F_{(3,8)}$ = 25.73, 1.84Fold, p<0.01; $F_{(3,8)}$ = 10.43, 1. 81Fold, p<0.05) and hippocampus ($F_{(3,8)}$ = 18.71, 1.75Fold, p<0.01; $F_{(3,8)}$ = 15.88, 1.87Fold, p<0.05; $F_{(3,8)}$ = 17.82¸1.64Fold, p<0.05; $F_{(3,8)}$ = 25.83, 1.71Fold, p<0.01; $F_{(3,8)}$ = 8.412, 1.80Fold, p<0.01) in comparison to rats exposed to cadmium alone. No significant change in the expression of any of these proteins was observed either in frontal cortex or hippocampus of rats exposed to quercetin as compared to rats in the control group (Figure – 4.11).

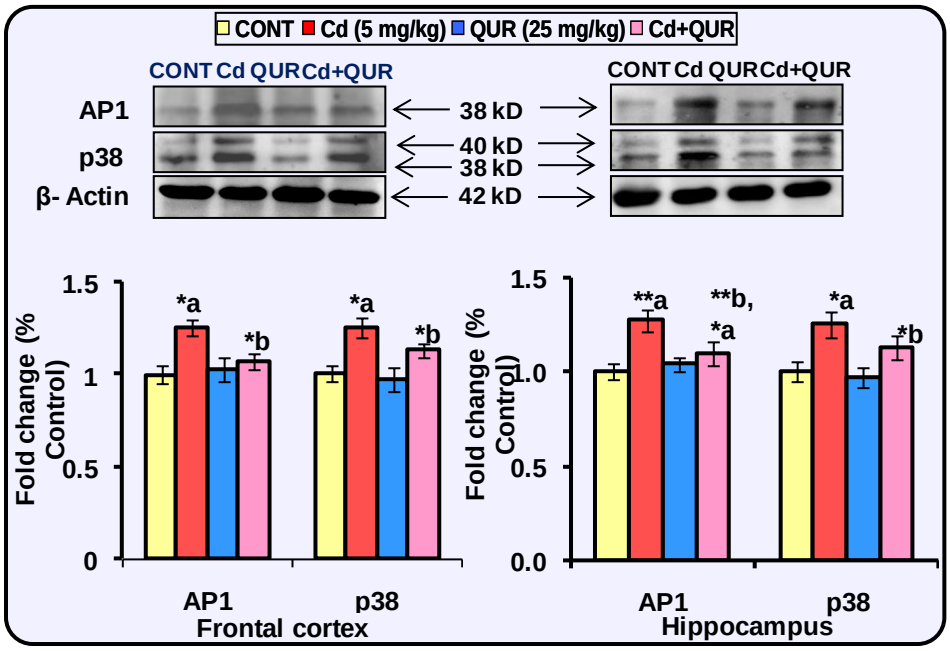


Figure – 4.11. Effect on the expression of p-p38 and AP1 in frontal cortex and hippocampus

Relative protein levels were quantified after normalization with β-actin; Values are mean ± SEM of three animals in each group; significantly differs (*p < 0.05, **p < 0.01); a - compared to control group, b - compared to cadmium exposed group; CONT – Control, Cd– Cadmium,

4.1.2.8 Histological Studies

Severe degeneration of neurons in frontal cortex ($F_{(3,8)}$ = 63.01, 1.72 fold, p<0.01) and dentate gyrus area of hippocampus ($F_{(3,8)}$ = 87.55, 1.65 fold, p<0.001) was clearly visible in rats on exposure to cadmium for 28 days in comparison to controls. Loss of synapse or the loss of neuron in cholinergic rich area is the major event of any cholinergic dysfunction and associated functional changes. A trend of recovery in frontal cortex ($F_{(3,8)}$ = 63.01, 1.49 fold, p<0.01) and dentate gyrus ($F_{(3,8)}$ = 87.55, 1.32 fold, p<0.001) was evident in cadmium exposed rats on simultaneous exposure with quercetin as compared to rats treated with cadmium alone. There was no significant change in the neuron density in any of the brain regions of rats exposed to quercetin alone as compared to rats in the control group (Figure –4.12).

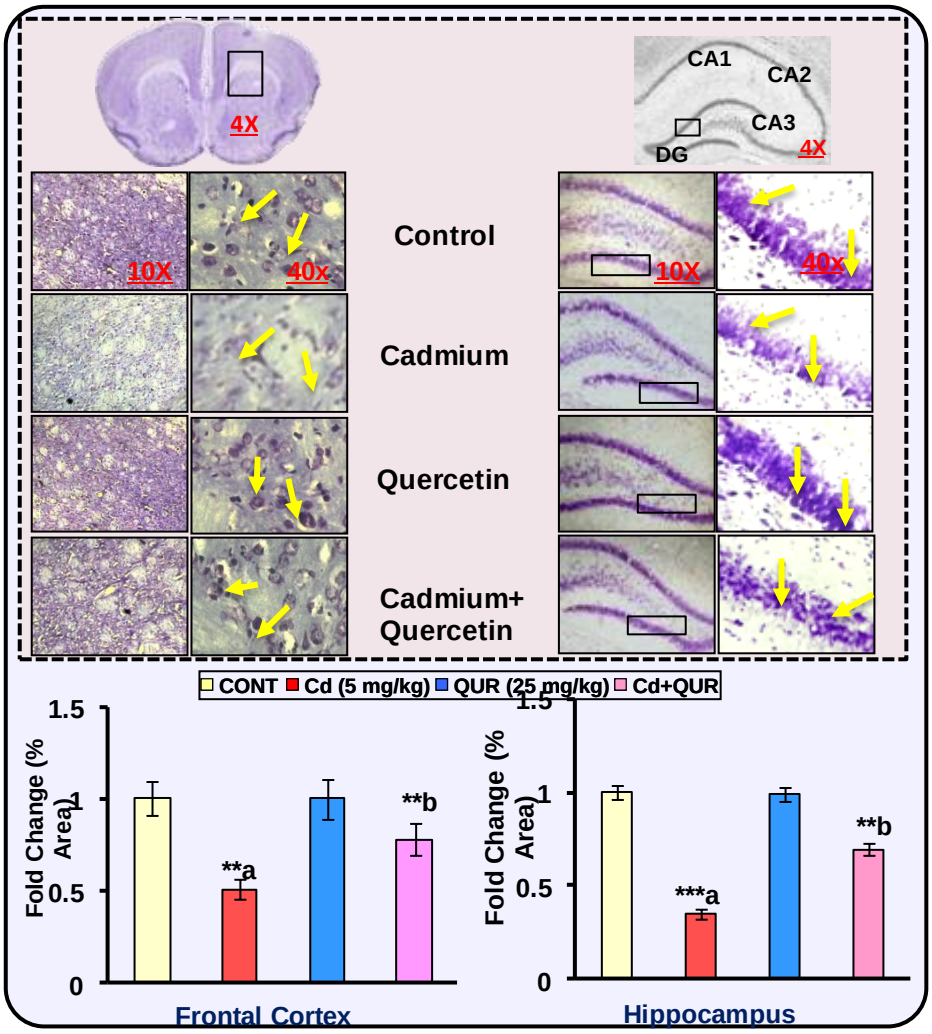
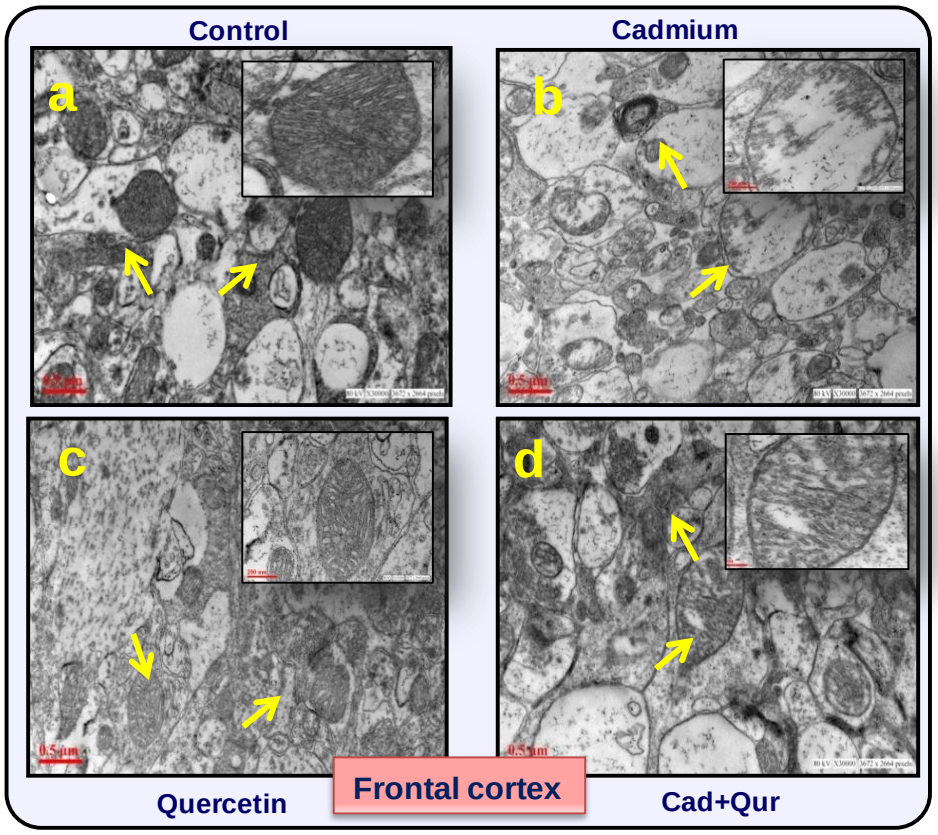


Figure – 4.12. Photomicrographs of frontocortical (A) and hippocampal sections (B) illustrating Nissl's staining

Fold change exhibit % area of degenerated neurons. Values are mean ± SEM of three sections from each group; significantly differs (**p < 0.05, ***p<.001); a - compared to control group, b-compared to cadmium exposed group; Scale bar = 100 μm, CONT – Control, Cd–

4.1.2.9 Ultrastructural Changes

Cadmium exposure in rats resulted to disrupt the ultrastructures both in frontal cortex and hippocampus as compared to rats in the control group. A marked deterioration in



hippocampus and frontal cortex as visualized by the loss in cell organelles, vacuole formation in cytoplasm, mitochondrial damage, swollen mitochondria with loss in cristae (cracked or missing cristae) along with disruption in mitochondrial membrane in unmyelinated axons was observed on cadmium exposure. Further, loss of myelin sheath was also clearly visible both in frontal cortex and in hippocampus on cadmium exposure as compared to control rats. Interestingly, simultaneous exposure with quercetin was found to protect cadmium induced ultrastructural changes and preserves the mitochondrial integrity. Restoration in mitochondria with improvement in electron density and cristae structure was clearly evident. Further, mitochondria with complete mitochondrial membrane and cristae were observed in rats simultaneously treated with cadmium and quercetin (Figure –4.13).

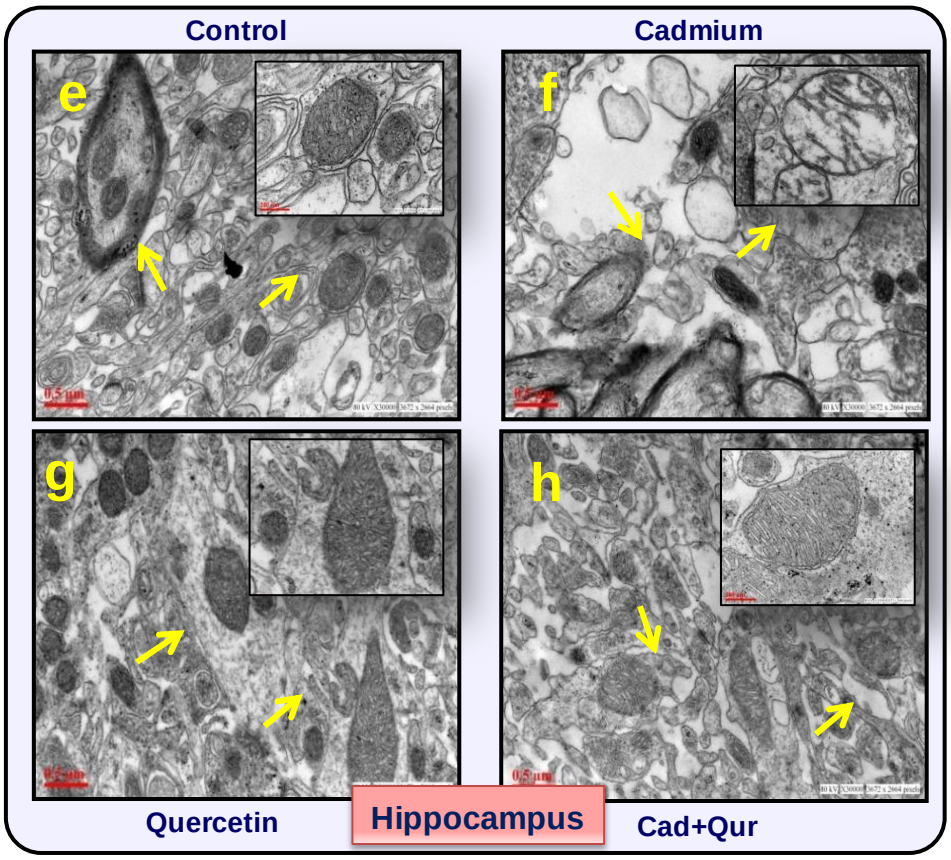


Figure – 4.13. Effect on ultrastructural changes following in frontal cortex and hippocampus

Focus is on mitochondria and electron micrographs (30000X) and inset view (110000X) have been presented. Figure – a, e control group of frontal cortex and hippocampus showing normal anatomy of unmyelinated and myelinated neurons with well developed mitochondria; Figure – b, f cadmium exposed group exhibits loss in cell organelles and mitochondrial damage; Figure – c, g quercetin exposed group shows normal anatomy like controls; Figure – d, h simultaneous exposure to cadmium and quercetin exhibit protection.

4.2 Module II - Effect on Brain Dopaminergic Modulations Following Exposure to Cadmium, Quercetin and their Co-Exposure

4.2.1 *In vivo* Studies

4.2.1.1 Effect on the Expression of Key Proteins Involved in Presynaptic Dopamine Signaling

Exposure of rats to cadmium for 28 days resulted to decrease the expression of TH, a rate limiting enzyme in dopamine synthesis in corpus striatum ($F_{(3,8)}$ = 11.12, 1.62 fold, $p<0.01$) as compared to controls. A significant decrease in the expression of DAT ($F_{(3,8)}$ = 8.176, 1.69 fold, $p<0.01$) and VMAT 2($F_{(3,8)}$ = 5.183, 1.52 fold, $p<0.05$) proteins was also distinct in rats on cadmium exposure. Simultaneous exposure to quercetin in cadmium

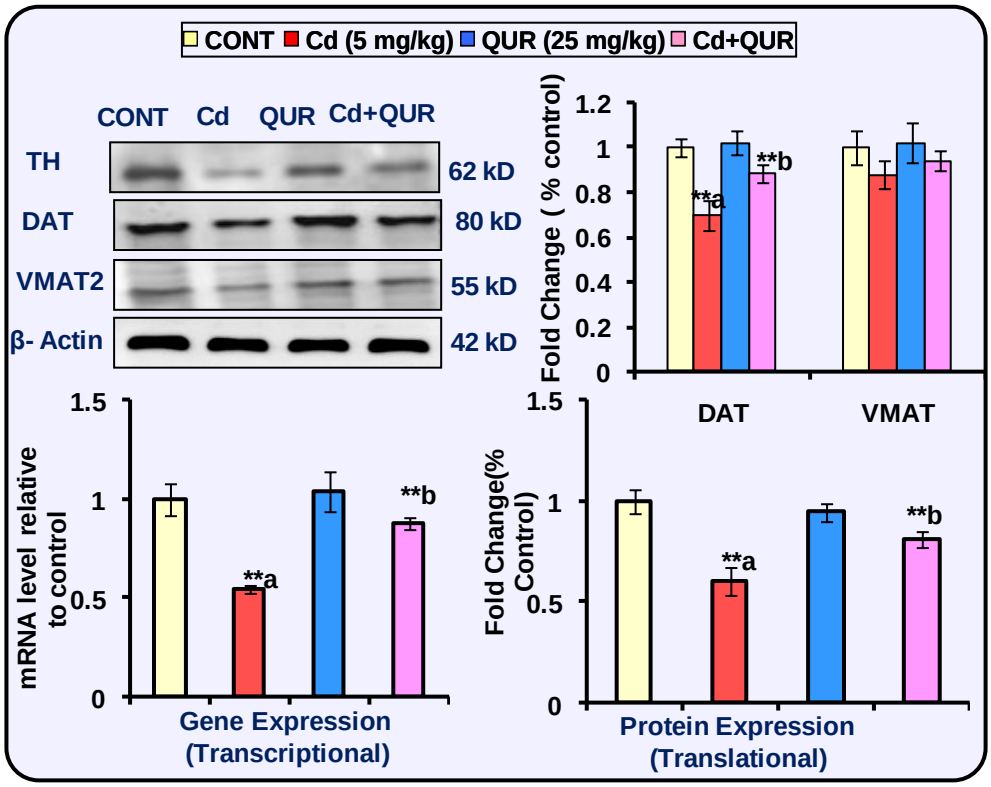


Figure – 4.14. Effect on the expression of tyrosine hydroxylase, dopamine transporter and vesicular monoamine transporter proteins in corpus striatum of rats

Relative protein levels were quantified after normalization with β-actin. Values are expressed as mean ± SEM of three rats in each group; significantly differs (*p<0.05, **p < 0.01; **p < 0.001); a-compared to control, b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin

exposed rats caused a significant up regulation in TH ($F_{(3,8)}$ = 11.12, 1.35 fold, $p<0.05$), DAT ($F_{(3,8)}$ = 8.176, 1.27 fold, $p<0.05$) and VMAT 2 ($F_{(3,8)}$ = 5.183, 1.39 fold, $p<0.05$) as compared to rats exposed to cadmium alone. However, there was no significant change in the expression of any of these proteins in corpus striatum of rats exposed to quercetin alone as compared to controls (Figure –4.14).

4.2.1.2 Transcriptional and Translational Changes in Dopamine Receptors

As dopamine receptors have central role in dopaminergic signaling, transcriptional and translational changes in the expression of DA-D1 and DA-D2 receptor type in corpus striatum were assessed on cadmium exposure in rats. No significant change in the mRNA and protein expression of DA-D1 receptors was observed in corpus striatum of cadmium exposed rats. Exposure to cadmium however, resulted to decrease mRNA ($F_{(3,8)}$ = 7.662, 50%, $p<0.01$) and protein ($F_{(3,8)}$ = 10.07, 1.63 fold, $p<0.01$) expression of DA-D2 receptors in corpus striatum as compared to controls. Interestingly, simultaneous treatment with quercetin in cadmium exposed rats caused a significant protection in the expression of mRNA ($F_{(3,8)}$ = 7.662, 76%, $p<0.05$) and protein ($F_{(3,8)}$ = 10.07, 1.31 fold, $p<0.05$) in DA-D2 receptors on comparing with rats treated with cadmium alone. No transcriptional and translational changes in the expression of DA-D1 and DA-D2 receptor type was observed in corpus striatum of rats exposed to quercetin alone as compared to controls (Figure –4.15).

4.2.1.3Assessment of DA-D2 Receptor Binding

To further confirm cadmium induced changes on the expression of DA-D2 receptors, effect on the binding of DA-D2 receptors was assessed in the corpus striatum. Statistical analysis revealed that there was a significant decrease ($F_{(3,16)}$ = 11.13, 47%, $p<0.001$) in the binding of ^{3}H-spiperone to striatal membranes, known to label DA-D2 receptors on exposure of rats to cadmium as compared to controls. Scatchard analysis revealed that decrease in the binding of DA-D2 receptors in corpus striatum on cadmium exposure was due to alteration in number of receptor binding sites and no change in the binding affinity. Interestingly, treatment with quercetin was found to protect cadmium induced

changes in the binding of DA-D2 receptors in corpus striatum ($F_{(3,16)}$ = 11.13, 51%, $p<0.01$) as compared to rats treated with cadmium alone. Further, no change in the

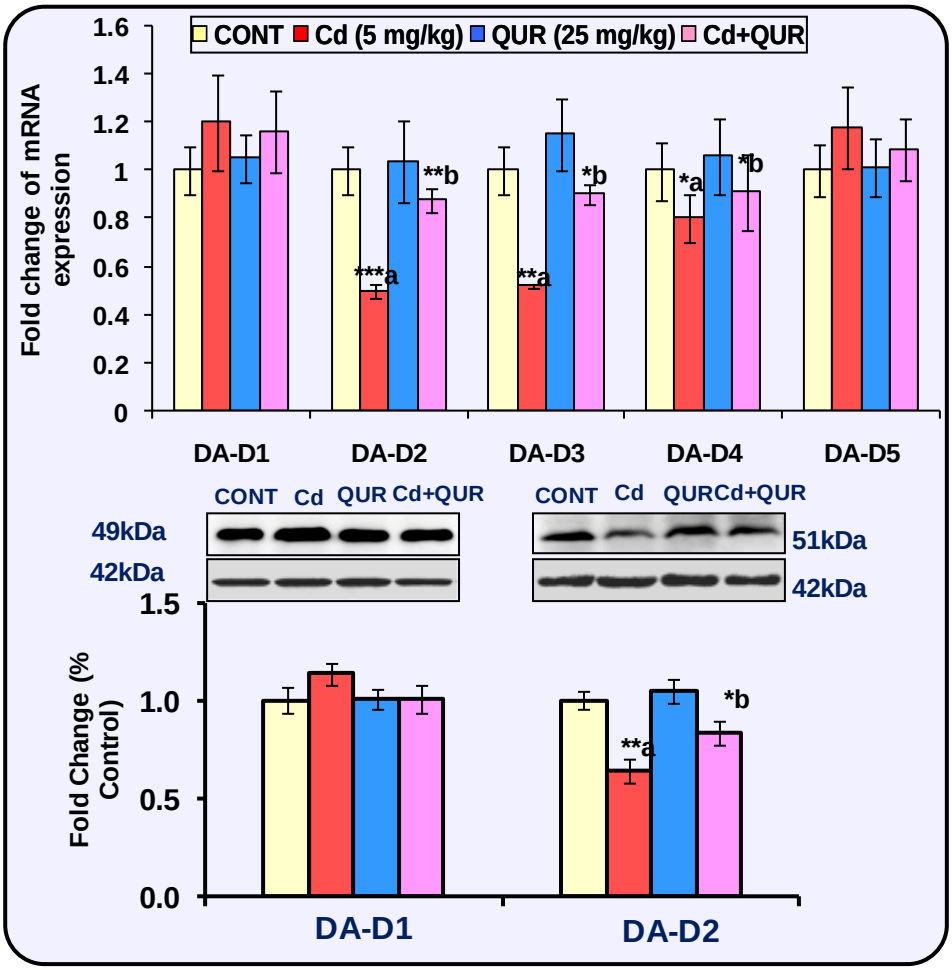


Figure – 4.15. Effect on the expression of dopamine receptors in corpus striatum of rats

β-actin was used as housekeeping gene in qRT-PCR studies and as loading control in Western blotting. Values are mean ± SEM of three rats in each group; Significantly differs (*p < 0.05, **p < 0.01); a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin.

binding of DA-D2 receptors was observed in rats exposed to quercetin alone as compared controls (Figure –4.16).

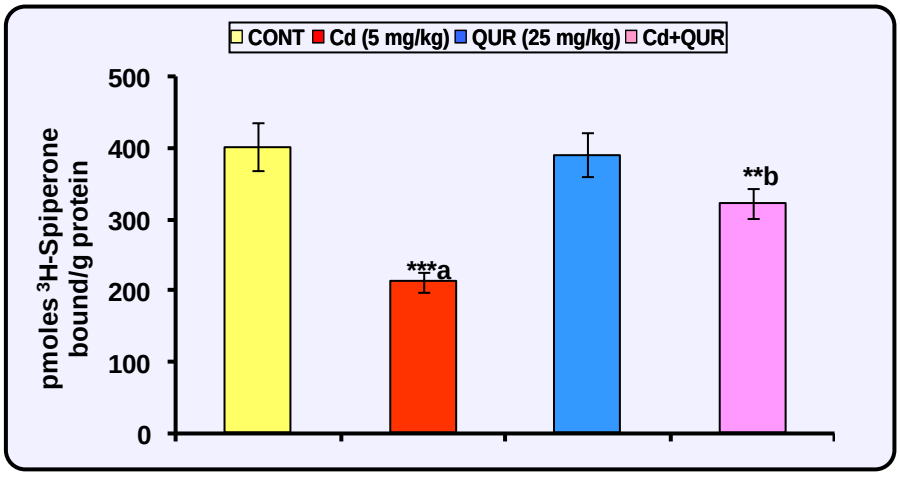


Figure – 4.16. Effect on 3H-spiperone binding in corpus striatum

Values are mean ± SEM of five rats in each group; Significantly differs (**p < 0.01, ***p < 0.001); a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin

Table – 4.1. Scatchard analysis of 3H-Spiperone binding to corpus straital membranes of rats

Brain Region	Treatment group			
	CONT	Cd (5mg/kg)	QUR (25mg/kg)	Cd+QUR
Corpus Striatum				
Bmax	1011±88	696±51*a	964±58	913±60*b
Kd	1.18±.24	1.32±.19	1.23±1.6	1.31±.19

Values are mean ± SEM of five rats in each group; Significantly differs (*p < 0.05); a-compared to control group; b-compared to cadmium exposed group; Kd – Dissociation constant expressed nM, Bmax – Maximum number of binding sites expressed as pmoles on 3H-Spiperone bound/g protein.

4.2.1.4 Effect on DA-D2 Receptor Mediated PKA Signaling

Effect on key targets associated with DA-D2 receptor mediated down streaming signaling in corpus striatum was assessed on cadmium exposure and protective effect of quercetin

was assessed in rats. As phosphorylation of DARPP-32 at thr- 34 by PKA activates inhibitory function of DARPP-32 over the protein phosphates (PP1α) and affects motor functions.

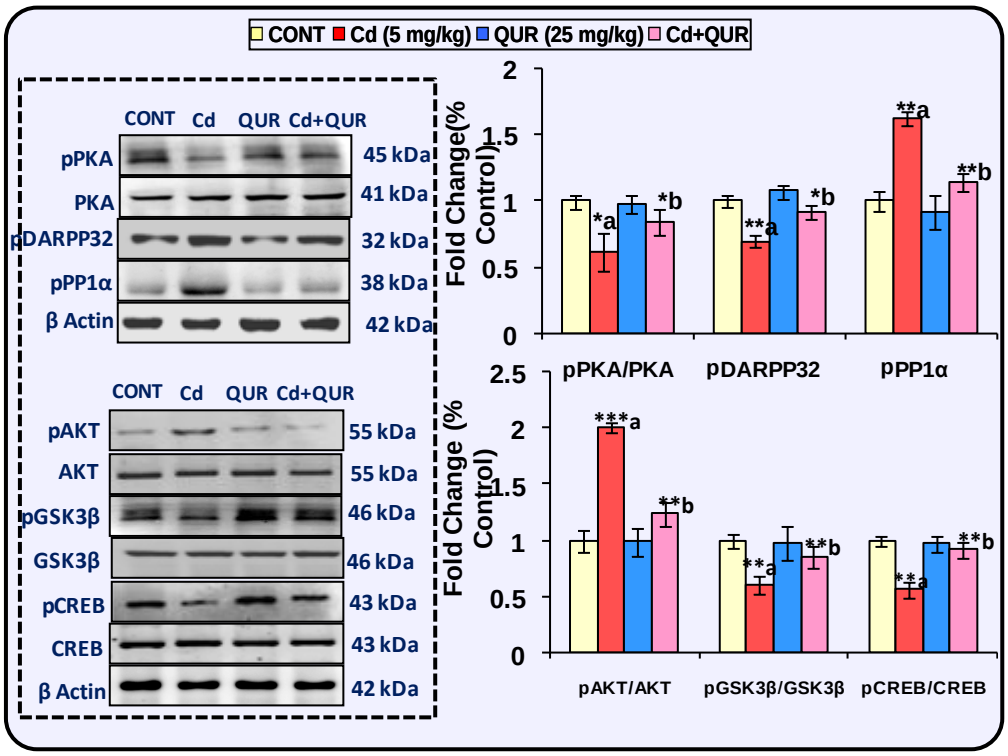


Figure – 4.17. Effect on the expression of DA-D2 receptor mediated targets associated with post synaptic signalling pathway in corpus striatum.

β-actin was used as loading control. Values are mean ± SEM of three rats in each group; significantly differs (**p < 0.01, ***p < 0.001); a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin

Exposure to cadmium in rats for 28 days resulted to decrease the expression of PKA ($F_{(3,8)}$ = 5.921, 1.63 fold, p<0.05), DARPP32 ($F_{(3,8)}$ = 12.38, 1.7 fold, p<0.01) and CREB ($F_{(3,8)}$ = 8.752, 1.66 fold, p<0.01) in corpus striatum as compared to controls. Significant increase in the expression of pPP1 α ($F_{(3,8)}$ = 11.96, 1.62 fold, p<0.01), a negative regulator of dopaminergic functions was observed in corpus striatum of cadmium exposed rats. Simultaneous exposure to quercetin in cadmium exposed rats significantly

increased the phosphorylation of PKA ($F_{(3,8)}$ = 5.921, 1.38 fold, $p<0.05$), DARPP32 ($F_{(3,8)}$ = 12.38, 1.3 fold, $p<0.05$) and CREB ($F_{(3,8)}$ = 8.752, 1.38 fold, $p<0.01$) and decreased the expression of PP1-α ($F_{(3,8)}$ = 11.96, 1.72 fold, $p<0.01$) as compared to rats exposed to cadmium alone. Treatment with quercetin in rats had no significant effect on the expression of any of these proteins in corpus striatum as compared to controls (Figure – 4.17).

4.2.1.5 Effect on Cell Survival Pathway Involving β-Arrestin Mediated Signaling Regulation of Akt by Dopamine

As β-arrestin mediated signaling has important modulatory role in dopamine dependent behavior and moreover is independent of cAMP signaling, effect on the expression of Akt and GSk-3 β was assessed in corpus striatum on cadmium exposure. An increase in the expression of Akt ($F_{(3,8)}$ = 17.90, 2.01 fold, $p<0.001$) associated with decreased expression of GSK-3β ($F_{(3,8)}$ = 9.958, 1.62 fold $p<0.01$) was evident in corpus striatum of cadmium exposed rats as compared to controls. Simultaneous exposure with quercetin in rats was found to protect cadmium induced changes in the expression of Akt ($F_{(3,8)}$ = 7.662, 1.6 fold, $p<0.01$) and GSK-3β ($F_{(3,8)}$ = 9.958, 1.42 fold, $p<0.01$) as compared to rats treated with cadmium alone suggesting that quercetin may modulate the cell survival pathway (Figure –4.17).

4.2.1.6 Effect on Mitochondrial Integrity

4.2.1.6.1 Effect on the Activity of Mitochondrial Complexes and ROS Generation

In view of important role of mitochondria in modulating the bioenergetics, protective effect of quercetin on cadmium induced changes on the activity of enzyme complexes associated with mitochondrial bioenergetics was assessed in the corpus striatum. Exposure to cadmium for 28 days in rats resulted to decrease the activity of complex I ($F_{(3,16)}$ = 6.054, 36%, $p<0.01$), complex II-III ($F_{(3,16)}$ = 29.11, 61%, $p<0.001$) and complex IV ($F_{(3,16)}$ = 6.439, 30%, $p<0.01$) in corpus striatum as compared to controls. Also, increase in the generation of ROS in corpus striatum ($F_{(3,16)}$ = 17.87, 74%, $p<0.001$) exhibit enhanced oxidative stress on cadmium exposure. Interestingly, quercetin exposure

in cadmium treated rats protected these changes as evident by increase in the activity of complex I ($F_{(3,16)}$ = 6.054, 34%, p<0.05), complex II-III ($F_{(3,16)}$ = 29.11, 85%, p<0.01) and complex IV ($F_{(3,16)}$ = 6.439, 36%, p<0.01) as compared to rats treated with cadmium alone. Increased ROS generation on cadmium exposure was also protected on simultaneous treatment with quercetin in corpus striatum ($F_{(3,16)}$ = 17.87, 28%, p<0.01) (Figure –4.18).

4.2.1.6.2 Effect on Mitochondrial Membrane Potential

Percent of depolarized cells in corpus stratum was assessed by FACS using JC-1 dye on cadmium exposure and protective efficacy of quercetin to protect such changes was examined. Exposure to cadmium increased the percentage of depolarized cells in the

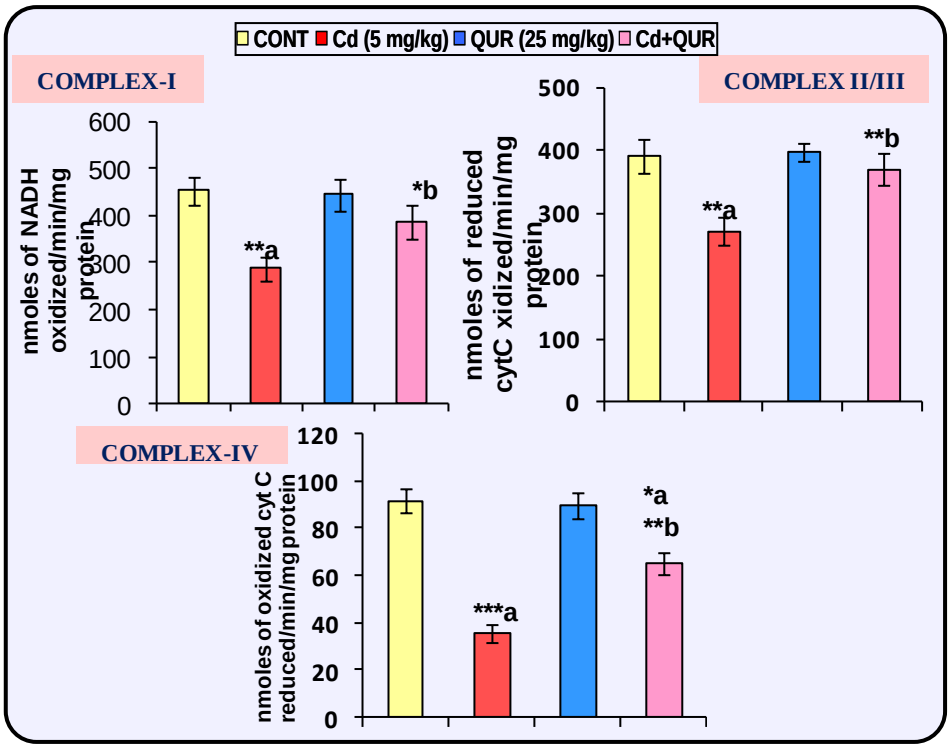


Figure – 4.18. Effect on activity of mitochondrial complexes in corpus striatum

Values are mean ± SEM of five animals in each group; significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin.

Corpus striatum ($F_{(3,8)}$ = 29.81, 80.12%, p<0.001) as evident by enhanced fluorescence of green JC-1 monomers detected at FL1 channel as compared to controls. Simultaneous exposure with quercetin was found to protect cadmium induced changes in the percentage of depolarized cells ($F_{(3,8)}$ = 29.81, 50.12%, p<0.001) exhibiting decrease in the green JC-1 aggregates at FL-1 channel. Further, the JC-1 aggregates detected at FL-2 channel in these samples were found to be more and thus indicating higher number of healthy mitochondria as compared to those treated with cadmium alone. Treatment with quercetin had no significant effect on the JC-1 aggregates in corpus striatum (Figure –4.19).

4.2.1.7 Effect on the Expression of Pro- And Anti- Apoptotic Proteins

In view of disruption in the mitochondrial electron transport chain, effect on the key regulators associated with intrinsic mitochondrial pathway was assessed to understand the extent of apoptosis. Cadmium exposure in rats resulted to increase the expression of Bax,

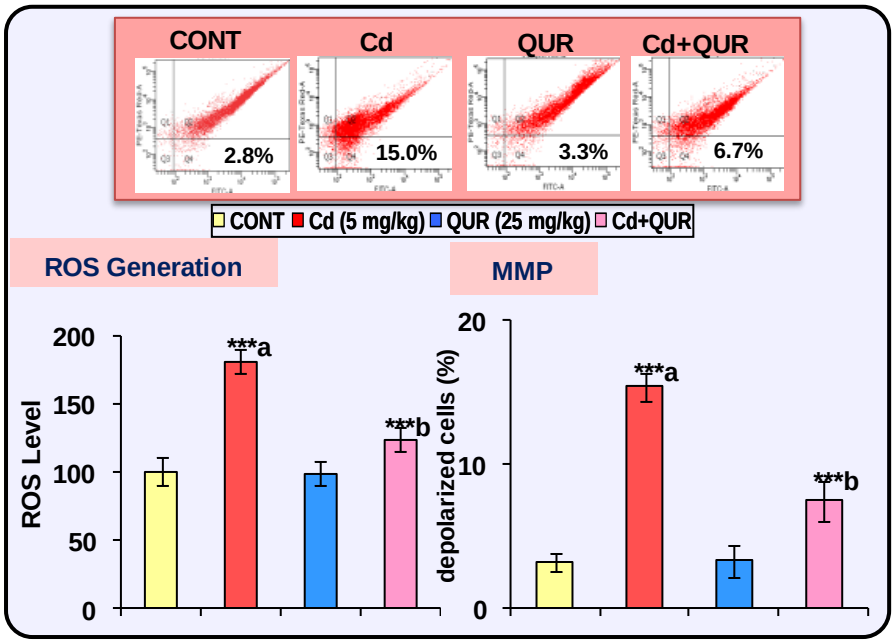


Figure – 4.19. Effect on reactive oxygen species generation and mitochondrial membrane potential in corpus striatum

Values are mean ± SEM of five animals in each group; Significantly differs (**p < 0.01, ***p < 0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

a pro-apoptotic protein ($F_{(3,8)}$ = 36.97, 2.2 fold, p<0.001) and caspase-3, an executer protein ($F_{(3,8)}$ = 55.62, 2.01 fold, p<0.001) and decrease the expression of Bcl2, an anti-apoptotic protein ($F_{(3,8)}$ = 18.17, 0.561 fold, p<0.001) as compared to controls. Interestingly, simultaneous treatment with quercetin in cadmium exposed rats resulted to decrease the expression of Bax ($F_{(3,8)}$ = 36.97, 2.2 fold, p<0.001), caspase-3 ($F_{(3,8)}$ = 55.62, 2.01 fold, p<0.001) and Bcl-2 ($F_{(3,8)}$ = 18.17, 1.97 fold, p<0.01) in corpus striatum as compared to cadmium exposed rats. No significant change in the expression of any of the protein was found in rats treated with quercetin as compared to controls (Figure – 4.20).

4.2.1.8 Expression of MAPK Proteins

Increase in the expression of AP1($F_{(3,8)}$ = 16.49, 1.36 fold, p<0.01), pp38 ($F_{(3,8)}$ = 57.42, 2.45 fold, p<0.001), pJNK1/2 ($F_{(3,8)}$ = 43.21 1.89 fold, p<0.01; $F_{(3,8)}$ = 36.01, 2.80 fold, p<0.001) and pJNK3 ($F_{(3,8)}$ = 27.51, 1.65 fold, p<0.001) was evident in corpus striatum of rats exposed to cadmium for 28 days as compared to controls suggesting the involvement of MAPKinases in mitochondrial mediated cell death. Simultaneous exposure with quercetin was found to decrease the expression of AP1($F_{(3,8)}$ = 16.49, 1.75 fold, p<0.05), p38 ($F_{(3,8)}$ = 57.42, 1.735 fold, p<0.01), JNK1/2 ($F_{(3,8)}$ = 43.21, 1.75 fold, p<0.01, $F_{(3,8)}$ = 36.01, 1.65 fold, p<0.01) and JNK3 proteins ($F_{(3,8)}$ = 27.51, 1.63 fold, p<0.001) in corpus striatum of cadmium treated rats. Treatment with quercetin in rats did not affect the expression of any of these proteins as compared to controls (Figure –4.20).

4.2.1.9 Degeneration of Straital Neurons

Effect on neuronal degeneration on exposure of rats to cadmium and protective efficacy of quercetin was assessed in corpus striatum using Nissl staining. Cadmium exposure resulted to decrease neuronal density ($F_{(3,8)}$ = 9.876, 2.01fold, p<0.001) as evident by reduced number of Nissl bodies in corpus striatum, compared to controls. Interestingly, simultaneous exposure with quercetin protected cadmium induced changes in the neuronal density ($F_{(3,8)}$ = 9.876, 1.89 fold, p<0.001) in corpus striatum as compared to rats treated with cadmium alone. Exposure to quercetin alone had no effect on the Nissl

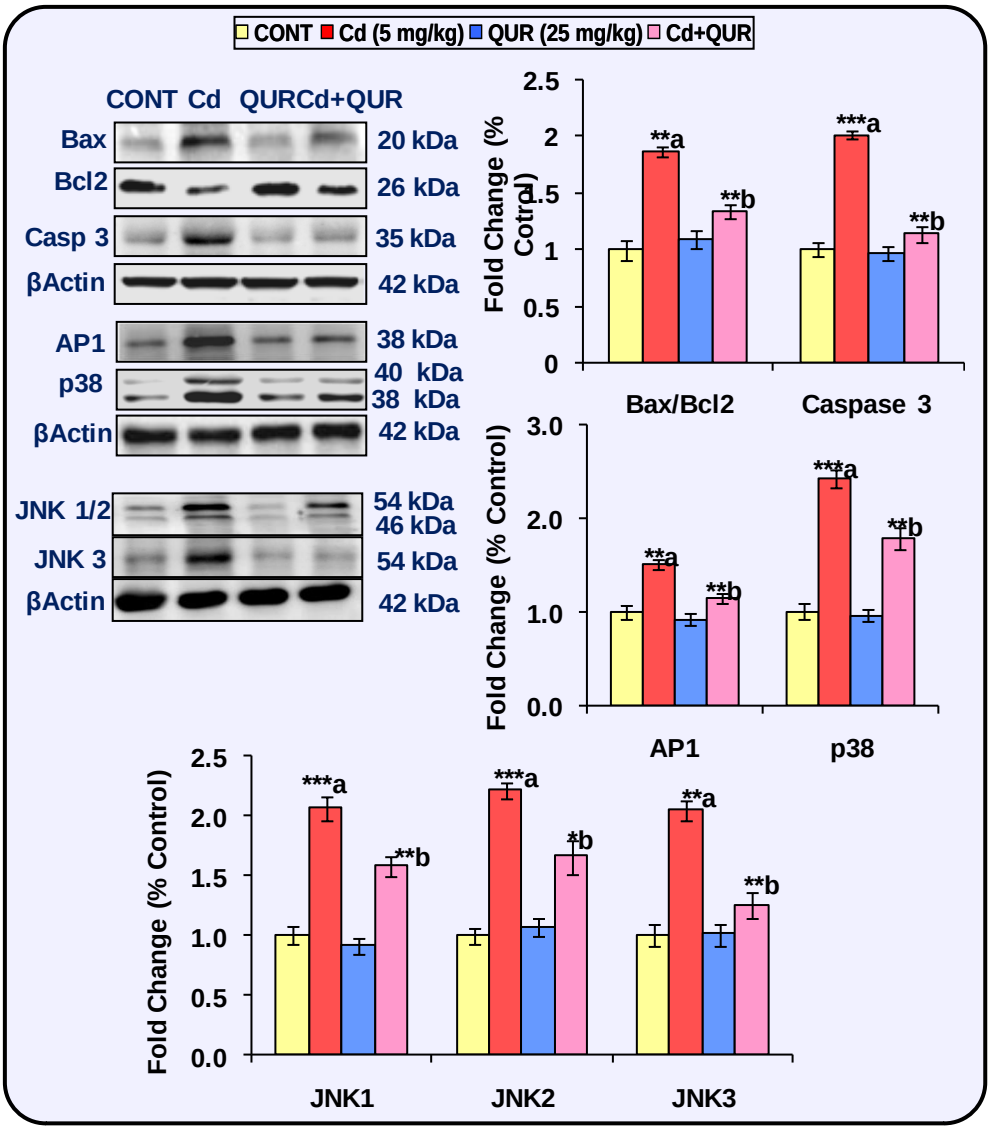


Figure – 4.20. Effect on the expression of apoptotic and MAPKinase proteins in corpus striatum

Values are mean ± SEM of three animals in each group; Significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin; Ratio of Bax/Bcl2 was determined to quantitative apoptosis.

Staining in corpus striatum suggesting that there was no neuronal degeneration (Figure – 4.21).

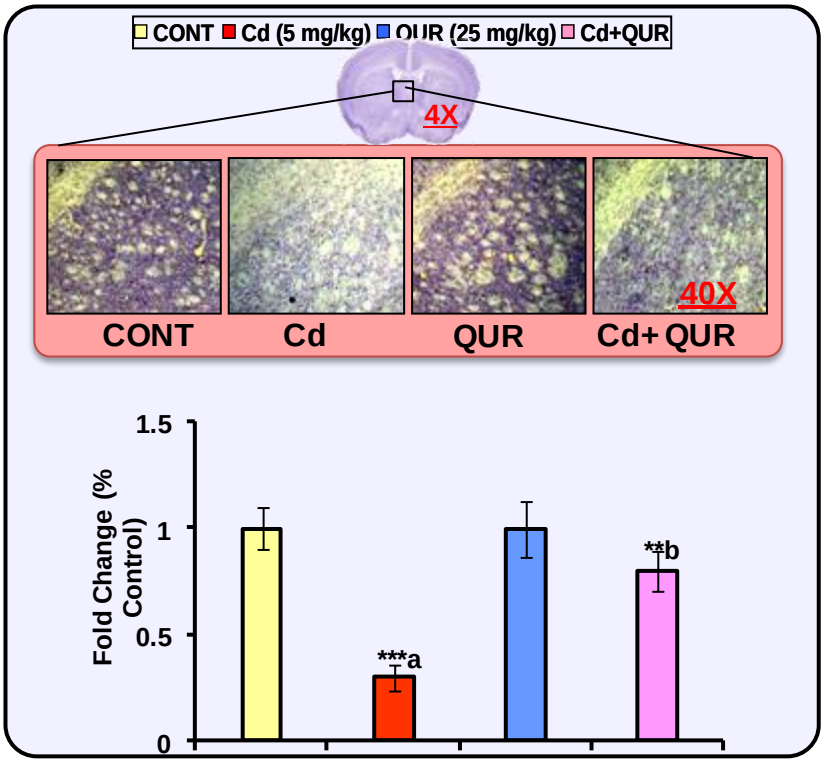


Figure – 4.21. Representative images of Nissl staining of corpus striatum sections of rat illustrating serve degeneration of neurons

Values are mean ± SEM of three rats in each group; Significantly differs (**p < 0.01, ***p < 0.001); a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin Scale bar = 100 µm.

4.2.1.10 Cadmium Induced Alterations in Ultrastructural Changes

Exposure to cadmium in rats for 28 days caused a marked deterioration in corpus striatum as visualized by the loss of neurons, disturbed cell matrix, vacuolization associated with damage to the cell organelles including mitochondria. Disruption in the myelin sheath in corpus striatum was frequently observed on cadmium exposure. Synaptic loss represented by decreased synapse in neuropil region was prominent which could be associated with altered dopaminergic signaling as compared to controls. Simultaneous treatment with quercetin was found to protect cadmium induced changes. There was no change in ultrastuctures in corpus striatum of rats exposed to quercetin alone (Figure –4.22).

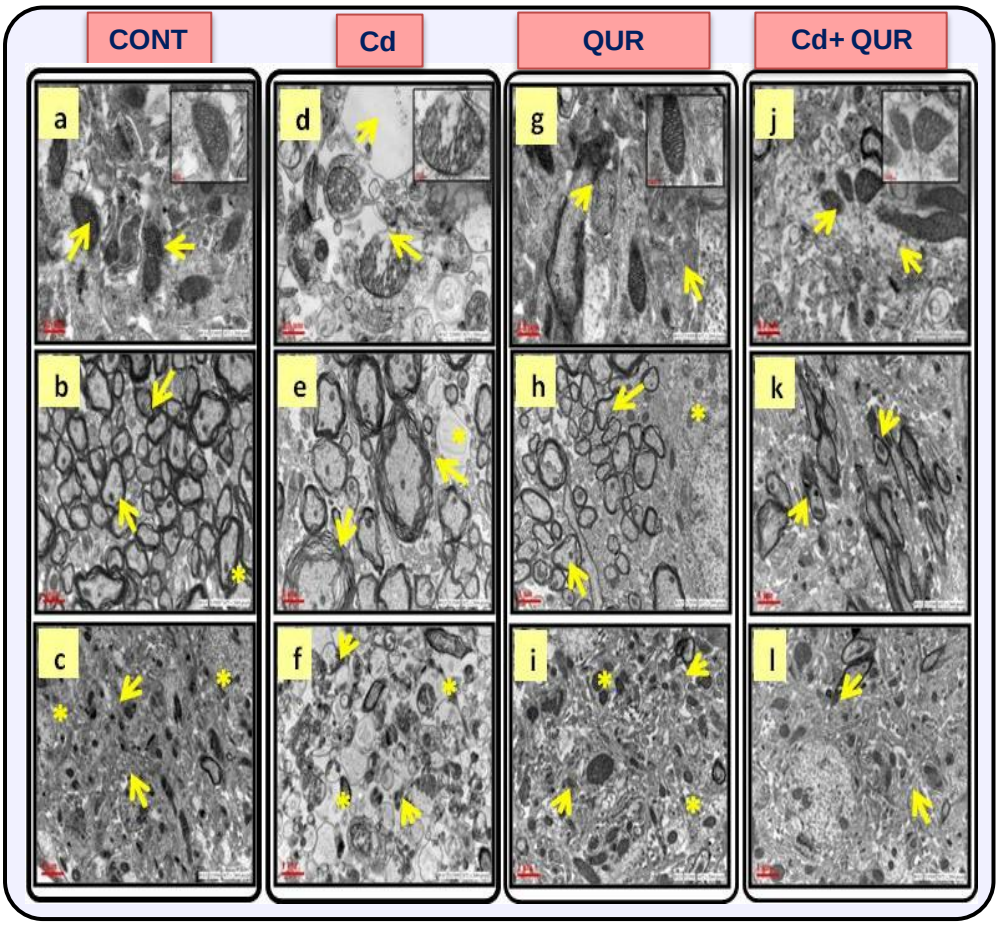


Figure – 4.22. Ultra structural changes in corpus striatum.

Electron micrographs (30000X) and inset view (110000X) have been presented. Figure – a, b, c- control group; a- exhibiting the normal anatomy of unmyelinated neurons showing well developed mitochondria with dense electron create and other organelles; b- well developed myelin sheath; c- completely developed synapse in neuropil regions. Figure –d, e, f- cadmium exposed group; d- the loss in cell organelles, vacuole formation in cytoplasm, mitochondria is damaged, swollen mitochondria with loss in membrane cristae (cracked or missing cristae) along with disruption in mitochondrial membrane in unmyelinated axons; e- demyelination of neurons; f- loss of synapse in neuropil regions. Figure – g, h, i- quercetin exposed group; g- showing normal architecture, with well developed mitochondria and other cell organelles, h- well developed mylineated neurons; i- well developed synapse in neuropil region. Figure – j, k, l- simultaneous exposure to cadmium and quercetin, which is shown to be protected by co exposure to quercetin, with restoration in changes.

4.2.1.11 Behavioral Studies

4.2.1.11.1 Spontaneous Motor Activity

While monitoring the open field activity using Actimot, exposure of cadmium was found to decrease the total distance travelled ($F_{(3,17)}$ = 6.521, 58%, p<0.001), time moving ($F_{(3,17)}$ = 5.553, 43%, p<0.05), rearing ($F_{(3,17)}$ = 2.754, 10%, p>0.05) and stereotypic counts ($F_{(3,17)}$ = 5.651, 38%, p<0.05) associated with increase in time resting ($F_{(3,17)}$ = 5.655,35%, p<0.01) as compared to controls.

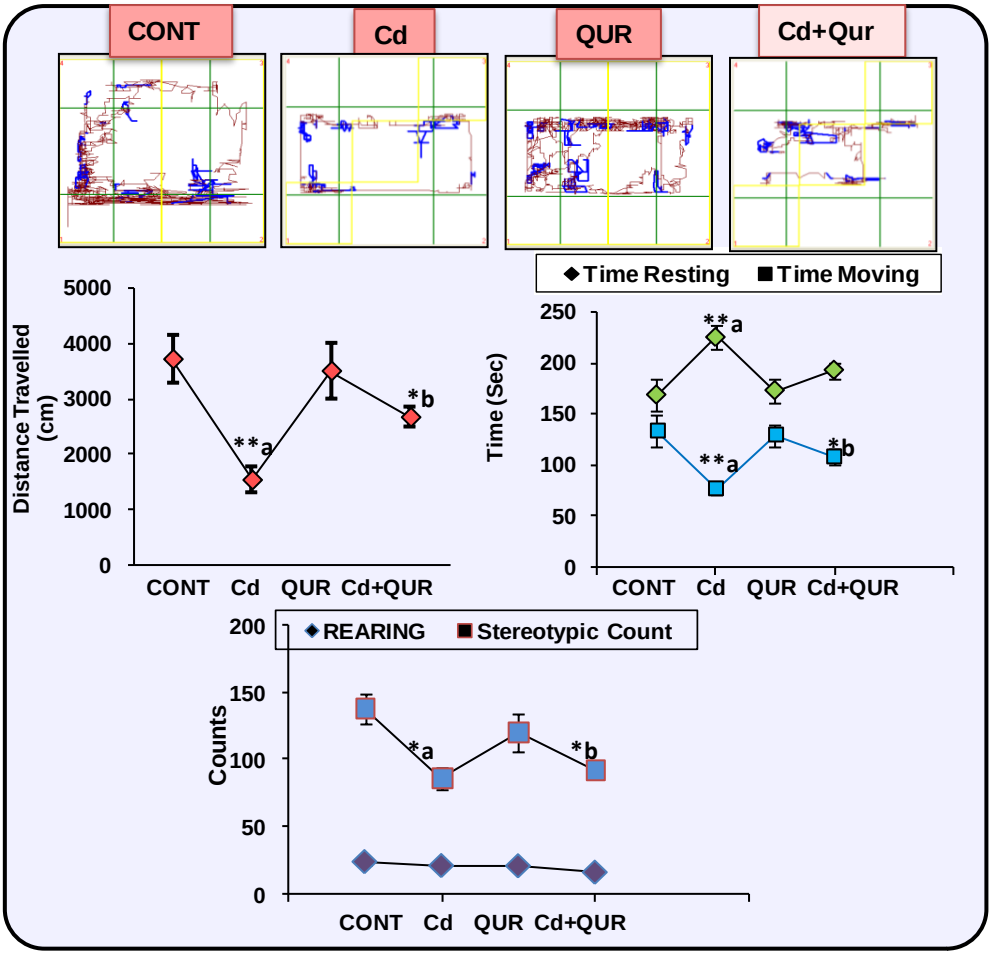


Figure – 4.23. Effect on spontaneous motor activity of rats.

Representative tracking patterns (A-D) of individual treatment groups exhibit the motor deficits in cadmium exposed rats. Values are mean ± SEM of five rats in each group; Significantly differs (*p < 0.05, **p < 0.01); a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin.

Increase in total distance travelled (F(3,17) = 6.521, 73%, p<0.05), time moving (F(3,17) = 5.553, 42%, p<0.05), rearing %(F(3,17) = 2.754, 25, p>0.05) and stereotypic count (F(3,17) = 5.655, 10%, p>0.05) associated with decrease in time resting (F(3,17) = 5.655,16.8% p<0.05) was clearly evident in cadmium treated rats simultaneously exposed with quercetin. No significant change in any of these parameters was observed in rats treated with quercetin alone as compared to controls (Figure –4.23).

4.2.1.11.2 Effect on Rota – Rod Performance

Effect on motor co-ordination was assessed by monitoring the performance using rotamex. Cadmium exposed rats fell earlier from the rotating rod and thus exhibited impaired motor co-ordination ($F_{(3,16)}$ = 12.77, 56%, p<0.001) as compared to controls. Rats simultaneously treated with cadmium and quercetin were found to stay longer on the rotating rod ($F_{(3,16)}$ = 12.77, 55%, p<0.05) as compared to those treated with cadmium alone (Figure –4.24).

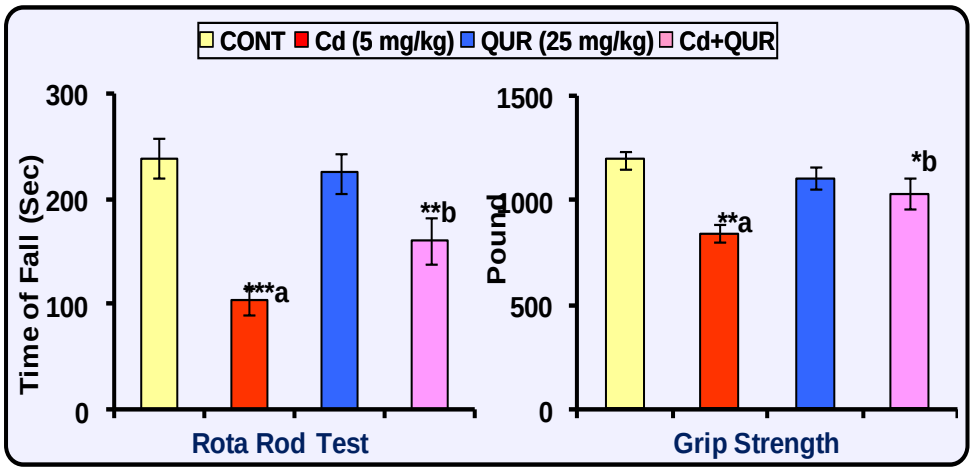


Figure – 4.24. Effect on motor co-ordination and muscle strength.

Values are mean ± SEM of five rats in each group; Significantly differs (**p< 0.01, ***p < 0.001); a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd– Cadmium, QUR – Quercetin.

4.2.1.11.3 Effect on Grip Strength

A significant impairment ($F_{(3,16)}$ = 7.296, 29%, p<0.01) in forelimb strength was

observed in rats exposed to cadmium as compared to control. Although a significant improvement ($F_{(3,16)}$ = 7.296, 22.6%, $p<0.05$) in forelimb strength was observed in co exposed groups as compared to cadmium treated alone. While such changes were not observed in rats exposed to quercetin alone (Figure –4.24).

4.2.2 *In vitro Studies*

4.2.2.1 Neuronal Differentiation of PC12 Cells and Cytotoxicity Studies

PC12 cells were seeded on the poly-L-Lysine coated flasks and differentiated in medium containing NGF (100ng/ml) for 8 days. These cells were fully differentiated into neurons. Representative images of neuronal differentiation of PC12 cells at 0, 2, 4, 6 and 8 days are presented (Figure –4.25). Effect of cadmium and quercetin was assessed at different concentrations (10^{-3} to 10^{-7} M) on four different time points (24 - 96 hr) on the viability of differentiated PC12 cells. Non cytotoxic concentration of cadmium and biological safe concentration of quercetin were determined by MTT assay. Simultaneous exposure with quercetin at 100µm concentration was found to protect cytotoxicity significantly in PC12 cells exposed to cadmium both at 1 and 10 µm as revealed by MTT assay (Figure –4.25)

4.2.2.2 Effect on the Expression of Key Proteins Involved in Presynaptic Signaling

After establishing the non-cytotoxic and cytoprotective concentration of cadmium and quercetin respectively, protective effect of quercetin on the expression of proteins associated with pre-dopaminergic signaling (TH, DAT, and VMAT2) was assessed in cadmium induced toxicity in PC12 cells. Effect on the expression of proteins related with down streaming signaling was also studied. Exposure of differentiated PC12 cells to cadmium (10 µm) for 24 hr resulted to decrease the expression of TH, DAT and VMAT2 as compared to the cells which were not exposed to cadmium (Figure – 4.26). Simultaneous exposure with quercetin (100 µm) resulted to protect cadmium induced decrease in the expression of these proteins associated with pre-dopaminergic signaling.

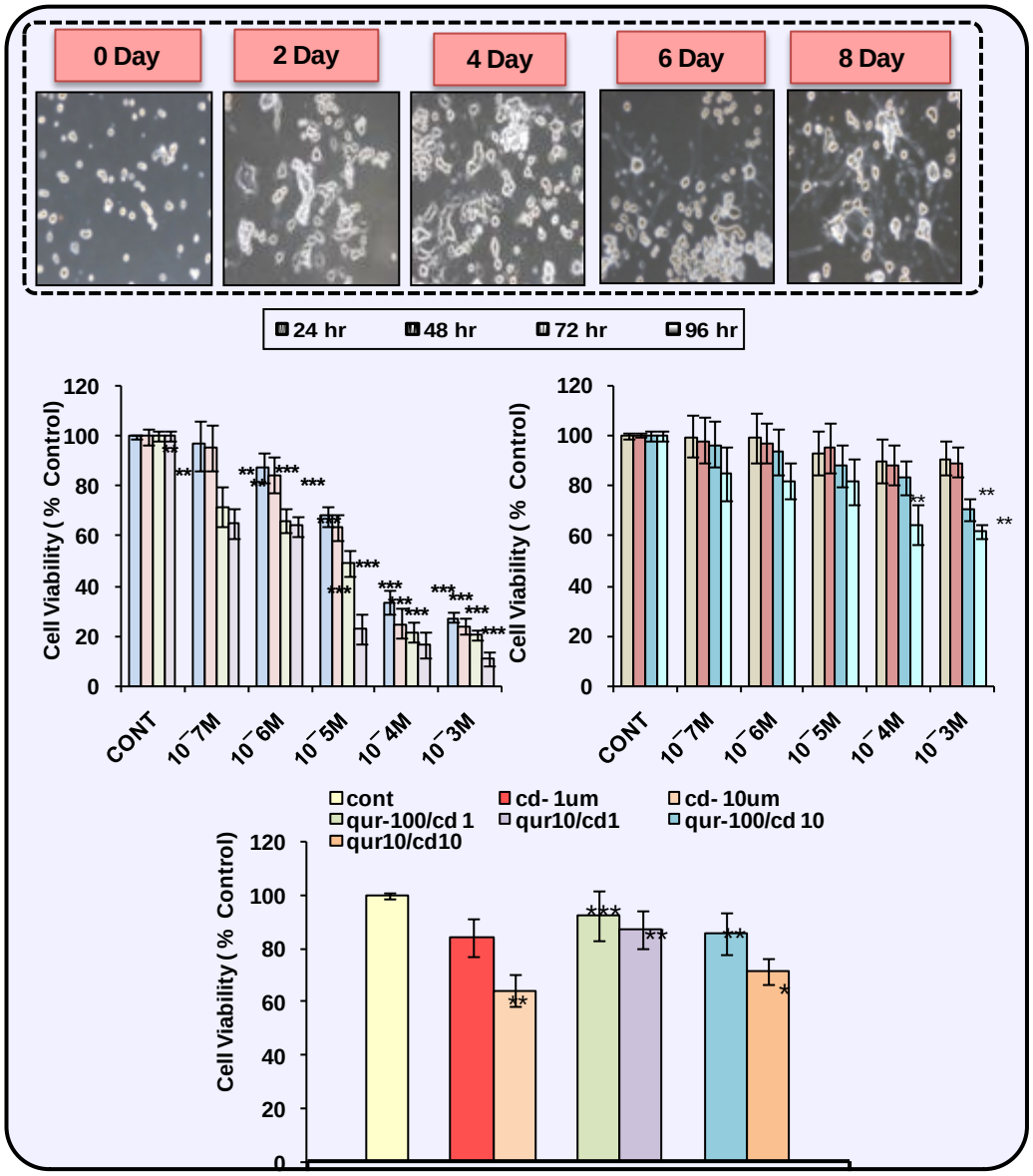


Figure – 4.25. Assessment of non-cytotoxic and cytoprotective doses of cadmium and quercetin.

% cell viability in PC12 cells was assessed by MTT assay using the different concentrations of cadmium and quercetin fat different time intervals (24−96h). Values are mean ± SEM of three experiments each carried out in triplicate; Significantly differs (**p < 0.01, ***p < 0.001); CONT – Control, Cd– Cadmium, QUR – Quercetin

4.2.2.3 Effect on the Expression of Dopamine Receptors

Although there was no change in the expression of DA-D1 receptors on exposure of

differentiated PC12 cells to cadmium (10 μm), decrease in the expression of DA-D2 receptors was clearly evident (Figure – 4.26). Interestingly, changes were found consistent with *in vivo* study and exhibit vulnerability of DA-D2 receptors to cadmium. Simultaneous exposure with quercetin resulted to protect cadmium induced decrease in the expression of DA-D2 receptors and exhibit the potential of quercetin in preventing such changes.

4.2.2.4 Effect on DA-D2 Receptor Mediated Post Synaptic Signaling

Exposure of PC12 cells to cadmium resulted to decrease the expression of pPKA, pDARPP32 and pCREB suggesting that cadmium may affect the DA-D2 receptor mediated down streaming signaling (Figure – 4.26). It was further interesting that expression of pPP1α, a negative regulator of DARPP32 was increased on exposure to cadmium in PC12 cells exhibiting that dopamine dependent signaling is vulnerable on cadmium exposure. Simultaneous treatment with quercetin was found to protect cadmium induced changes in the expression of these proteins (Figure –4.26).

4.2.2.5 Effect on β-Arresetin Mediated Cell Signaling Pathway

Consistent with *in vivo* findings, exposure to cadmium was found to inhibit DA-D2 receptor mediated β-arrestin 2/Akt/GSK 3β survival pathway which plays an important role in DA-D2 receptor mediated behavioral functions. Cadmium exposure in PC12 cells resulted to increase the phosphorylation of Akt associated with decreased expression of GSK-3β. Simultaneous exposure with quercetin was found to protect cadmium induced changes in the expression of Akt and GSK-3β in PC12 cells as compared to those exposed with cadmium alone (Figure – 4.26).

4.2.2.6 Pharmacological Inhibitors Studies

Further, involvement of dopamine receptors and its downstream signaling on exposure to cadmium and quercetin was confirmed using specific pharmacological inhibitors of PKA (H-89) and Akt (A6730). PC12 cells were initially exposed to specific inhibitors of PKA or Akt for 1 hr before being exposed to cadmium (10μM) and quercetin (100μM) for 24

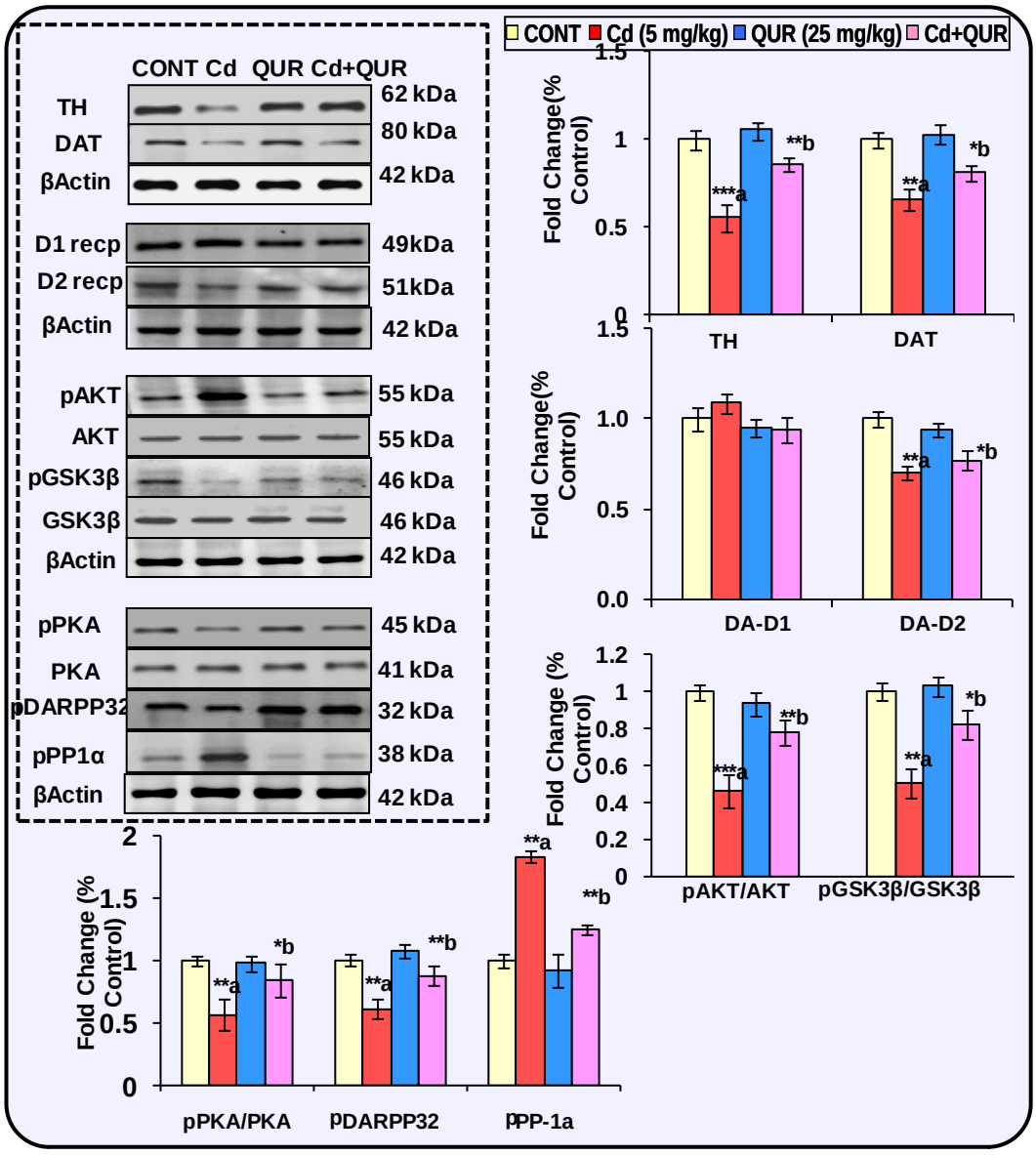


Figure – 4.26. Effect on the expression of dopamine receptors and targets associated with pre and post synaptic signaling in differentiated PC12 cells following the exposures of cadmium (10 µM) and quercetin (100 µM).

β- actin was used as an internal control to normalize the data. The data represent means ± SE of three independent experiments; *p < 0.05; **p < 0.01; a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

h. Cadmium induced changes were more pronounced in cells pre-exposed to specific pharmacological inhibitors of PKA and Akt as compared to PC12 cells exposed to

cadmium alone. It was interesting that in the presence of specific inhibitor of PKA, expression of DARPP32 and PP1α was altered while expression of Akt and GSK 3β remained unchanged suggesting PKA independent mechanism. In the presence of specific inhibitor of Akt, there was significant change in the expression of GSK 3β and CREB phosphorylation as compared to PC12 cells exposed to cadmium alone (Figure – 4.27, 4.28).

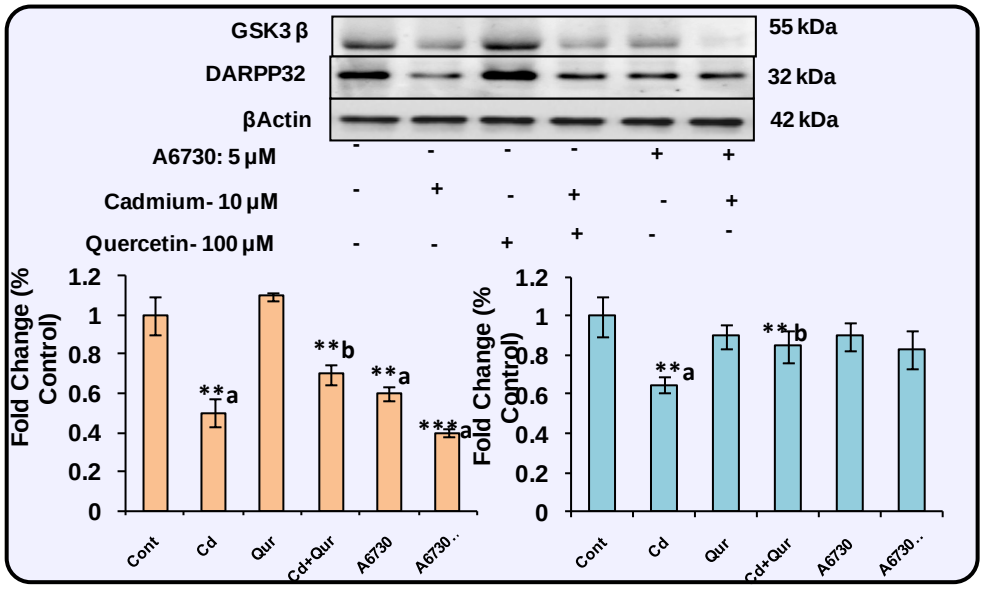


Figure – 4.27. Effect on the proteins related with dopamine receptor mediated down streaming signaling using pharmacological inhibitor against Akt.

β- actin was used as an internal control to normalize the data. The data represent means ± SE of three independent experiments; *p < 0.05; **p < 0.01, **p < 0.001. ; a- compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

4.2.3 *In Silico* studies

4.2.3.1 Computational Modeling and Molecular Docking

4.2.3.1.1 Homology Modeling Studies

3D-structure of DA-D2 receptor for UniProt entry P61169 was modelled using structure of the Human DA-D3 having PDB id – 3PBL. It had 48% identity, 59% similarity and 95% query coverage with D2 receptor for *Rattus norvegicus*. Ramachandran plot analysis

of best model indicated 89.1% residues in favored region, 10.9% region in allowed region and 0.0% residues in disallowed region (Figure – 4.29). For the DA-D1 of *Rattus norvegicus*, having UniProt ID – P18901, homology model was constructed using template 3KJ6 (Methylated Beta2 Adrenergic Receptor) having 36% identity and 57% similarity. Ramachandran plot analysis of best model indicated 89.6% residues in favored region, 8.9% region in addition allowed region and 1.5% residues in disallowed region.

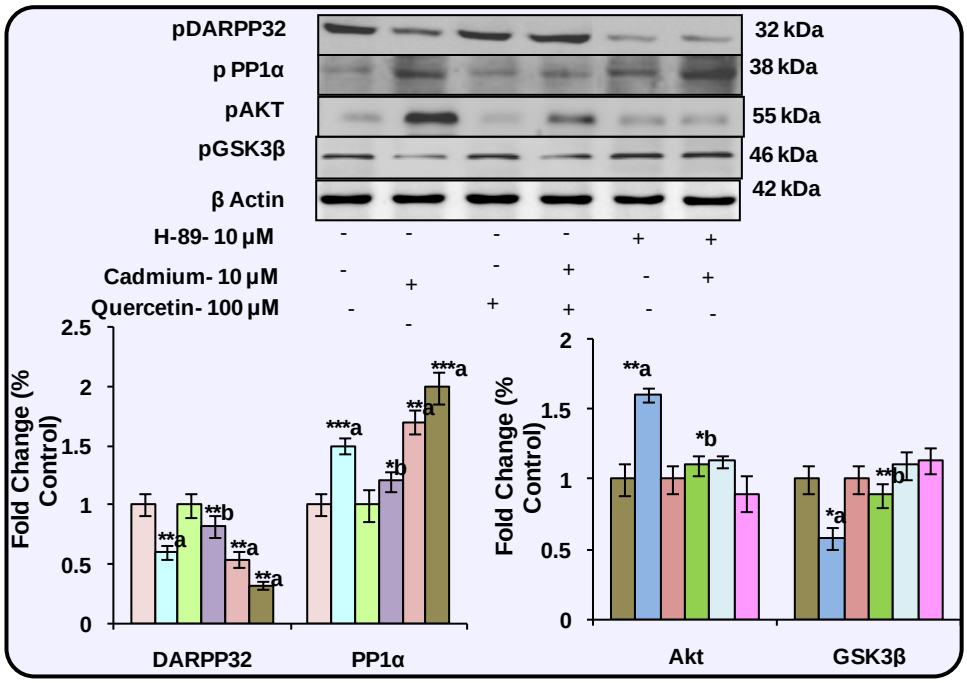


Figure – 4.28. Effect on the proteins related with dopamine receptor mediated down streaming signaling using pharmacological inhibitor against PKA

β- actin was used as an internal control to normalize the data. The data represent means ± SE of three independent experiments; *p < 0.05; **p < 0.01, **p < 0.001; a-compared to control group; b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

4.2.3.1.2 Docking and Interaction Analysis

Top 3 binding sites were used for docking all 3 compounds using CDOCKER module of Discovery Studio version 4.1. Lowest energy pose indicated CDOCKER energy of -61.4419, -46.6889 and -37.419 KJ/mol for compounds CdCl2, quercetin and dopamine respectively. Non-bonded interaction module of Discovery Studio-4.1, identified residues

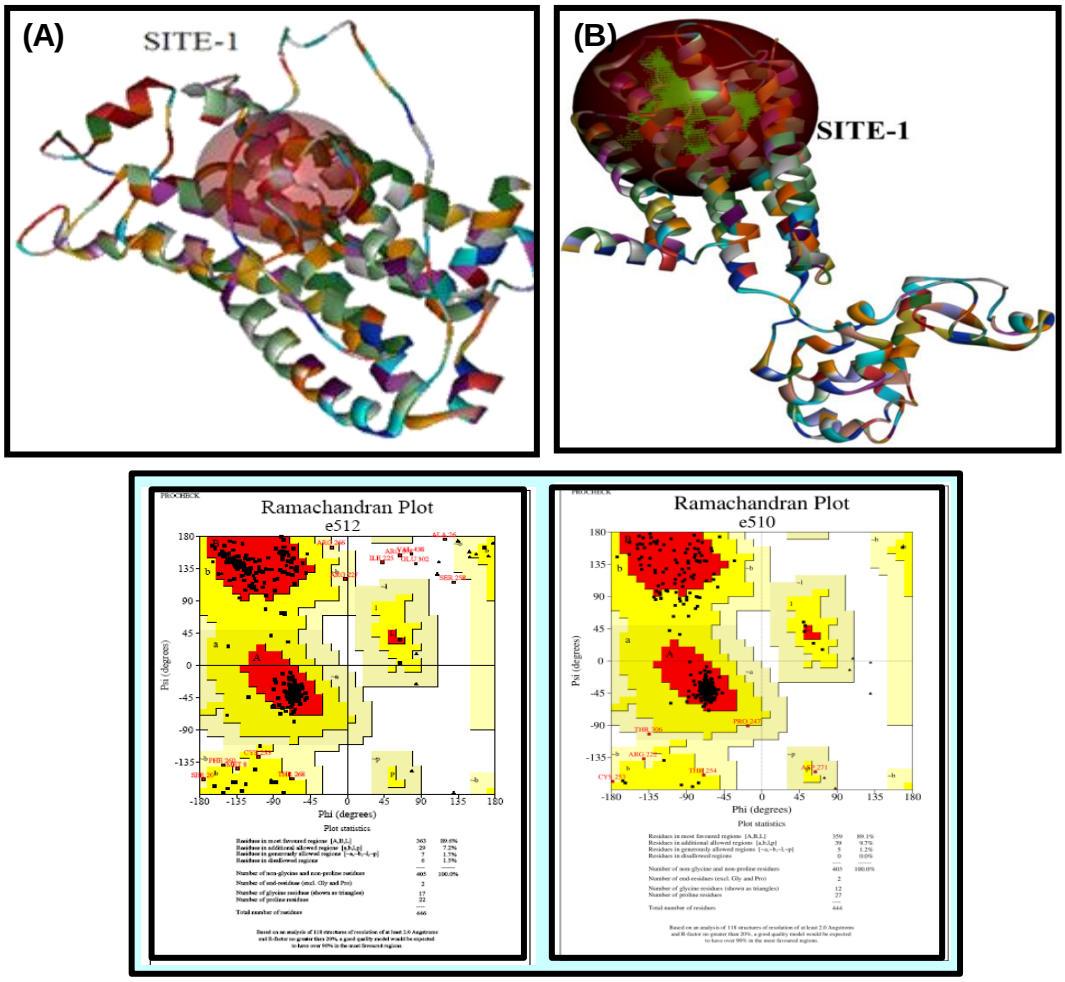


Figure – 4.29. Homology modeling of DA - D1, DA- D2 Receptor

Modelling was done using the modeller 9.15. The representative images and Ramachandran Plot of DA- D1 (A) and DA-D2 (B) receptors has been shown**.**

namely L41, D95, S410, T413, W414 for compound CdCl2. For quercetin, residues identified were V111, D114, V190, S193, Y409 and H394. The dopamine, residues were V91, E95, D114, F409 and T413 (figure – 4.30A, B, C). These non-bonded interactions include hydrogen bonding, hydrophobic interaction, electrostatic and Van der Waal forces. After identification of binding site for DA-D1 model, it was docked with CdCl2, quercetin and Dopamine molecules using CDOCKER module. Both for CdCl2 and quercetin CDOCKER score was 23.5912 and 36.6108 respectively, which was quiet low

compared to that of D2 dopamine receptor, indicating that these molecules preferably interact with the latter. For dopamine molecule CDOCKER energy was 48 .2473 and involves interaction of residues namely Val91, Glu95, Asp114, Tyr409, Thr413.

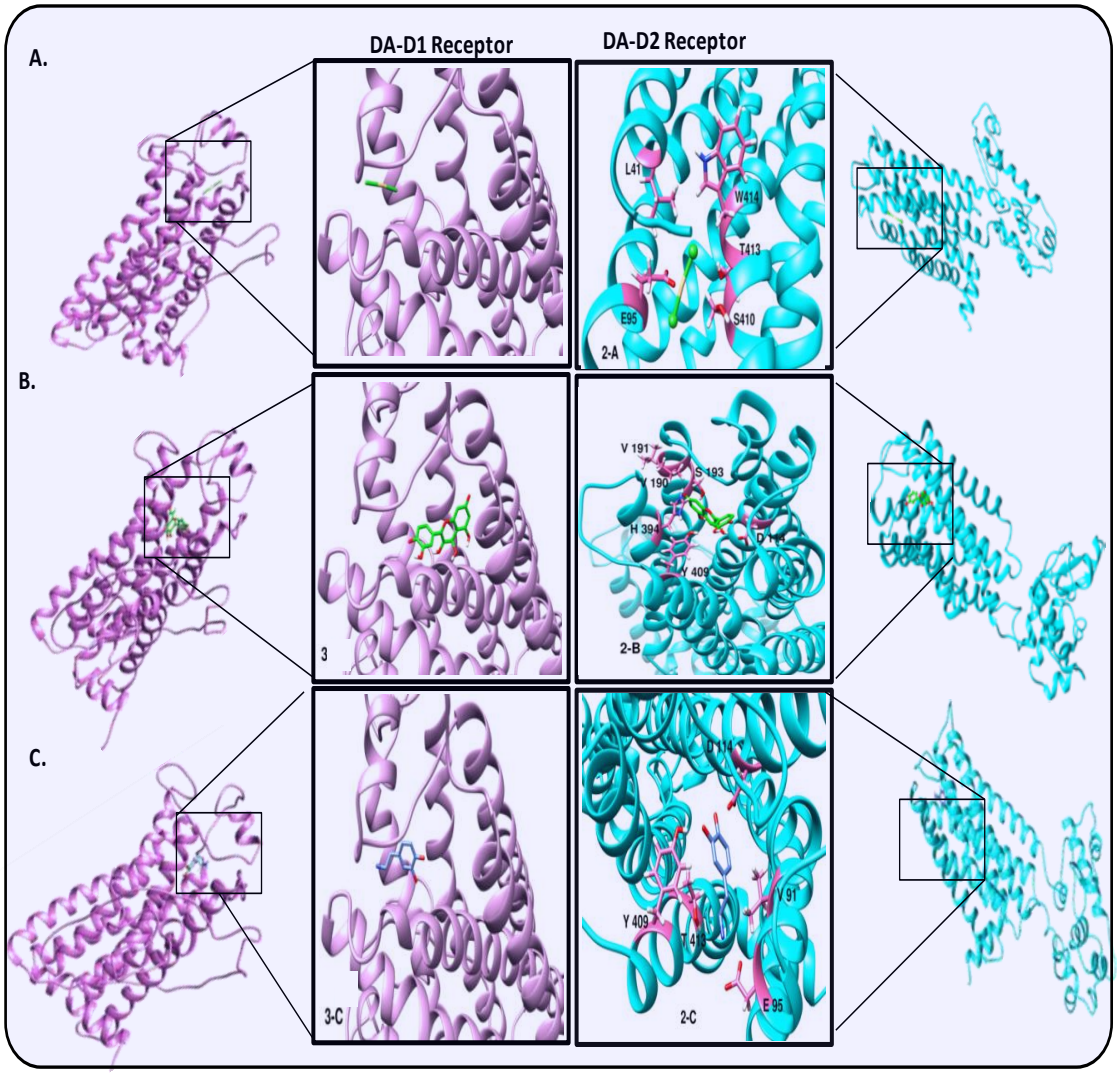


Figure – 4.30. Ligand – protein interaction diagram (Ribbon view model) of DA-D1 and DA-D2 receptor of *Rattus norvegicus*

The residues interacting with their bound partners are indicated in pink color in atom view using Chimera. The docking of DA-D1 and DA-D2 receptors with CdCl2 (A), Quercetin (B) and Dopamine (C) molecules has been shown respectively. The docking was carried out involving CDOCKER.

4.2.3.2 DFT Studies

Firstly, the optimized, stable structure of Quercetin was solved using DFT method **(**Figure – 4.31). On complete optimization of all four parameters (Maximum force, Maximum Displacement, RMS force, RMS Displacement) were converged. As previous studies suggested that the Cadmium ions interact with the Quercetin by interacting with its hydroxyl groups. In light of these studies, DFT calculations were performed against

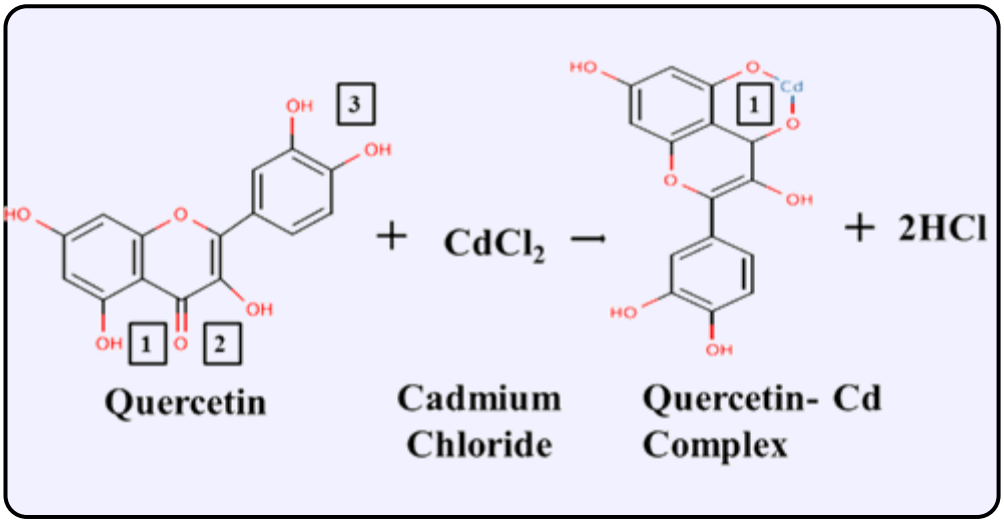


Figure – 4.31. Predicted mechanism of cadmium quercetin interaction using DFT studies.

All the possible sites where Cadmium might possible interact with Quercetin (Figure 1b). In case of site 2 and 3, the energies of the Cadmium-Quercetin complex were unable to converge to minima. While in case of Site 1, all the energies converged to minima, and the structure formed was optimized and stable. The energy calculated was -1151.89985894 a.u. at this Site-1.

4.3 MODULE III- Effect on the Expression of Metallothionein and Levels of Neurotransmitters and Their Metabolites Following Exposure to Cadmium, Quercetin and their Co-Exposure

4.3.1 *In vivo* Studies

4.3.1.1 Effect on the Expression of MT-3 in Brain Regions

Expression of MT-3 was assessed to understand the involvement of metallothionein in cadmium induced toxicity was done using western blot analysis. Increase in the

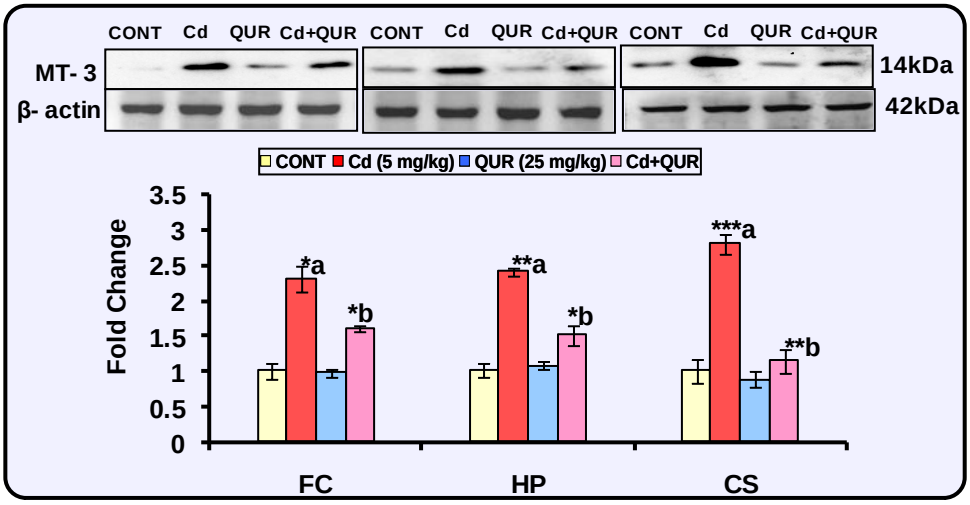


Figure – 4.32. Effect on the expression of MT- 3 in brain regions

Values are mean ± SEM of three rats in each group; Significantly differs (*p < 0.05, **p < 0.01); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin, FC- frontal cortex, HP – hippocampus and CS – corpus striatum.

MT-3 was observed following exposure of rats to cadmium in frontal cortex ($F_{(3,8)}$ = 4.23, 2.1 Fold, p<0.05), hippocampus ($F_{(3,8)}$ = 4.132, 2.4 Fold, p<0.01) and corpus striatum ($F_{(3,8)}$ = 5.123, 2.8 Fold, p<0.0001) as compared to controls. Simultaneous exposure of rats to cadmium and quercetin was found to decrease these changes in frontal cortex ($F_{(3,8)}$ = 3.879, 1.45 Fold, p<0.05), corpus striatum ($F_{(3,8)}$ = 5.265, 1.6 Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 3.567, 1.57 Fold) in comparison to rats exposed to cadmium alone. No significant change was observed in any of the brain regions of rats exposed to quercetin alone as compared to controls (Figure – 4.32).

4.3.1.3 Effect on Biogenic Amines and Their Metabolites in Brain Regions

4.3.1.3.1 Effect on Frontal Cortex

Cadmium exposure for 28 days in rats caused a significant decrease in the levels of NE (35%, p>0.05), DA (33% p<0.01), HVA (35% p<0.001), DOPAC (29%, p<0.05) and increase in the levels of and EPN (73%, p>0.05) in frontal cortex as compared to controls. Levels of 5-HT (32%, p<0.01) in the frontal cortex were found decreased in rats

treated with cadmium. Interestingly, simultaneous exposure of rats to cadmium and quercetin resulted to increase the levels of NE (45%, p>0.05), DA (33%, p<0.05), HVA (34%, p<0.05), DOPAC (22%, p>0.05) and decrease the levels of EPN (26%, P>0.05) in frontal cortex. A significant increase in the levels of 5-HT (38% p<0.05) in frontal cortex was also observed in rats simultaneously treated with cadmium and quercetin as compared to those exposed to cadmium alone. No significant effect on the levels of DA, NE, EPN, 5-HT, DOPAC and HVA was observed in the frontal cortex of rats exposed to quercetin alone as compared to control rats (Figure – 4.33).

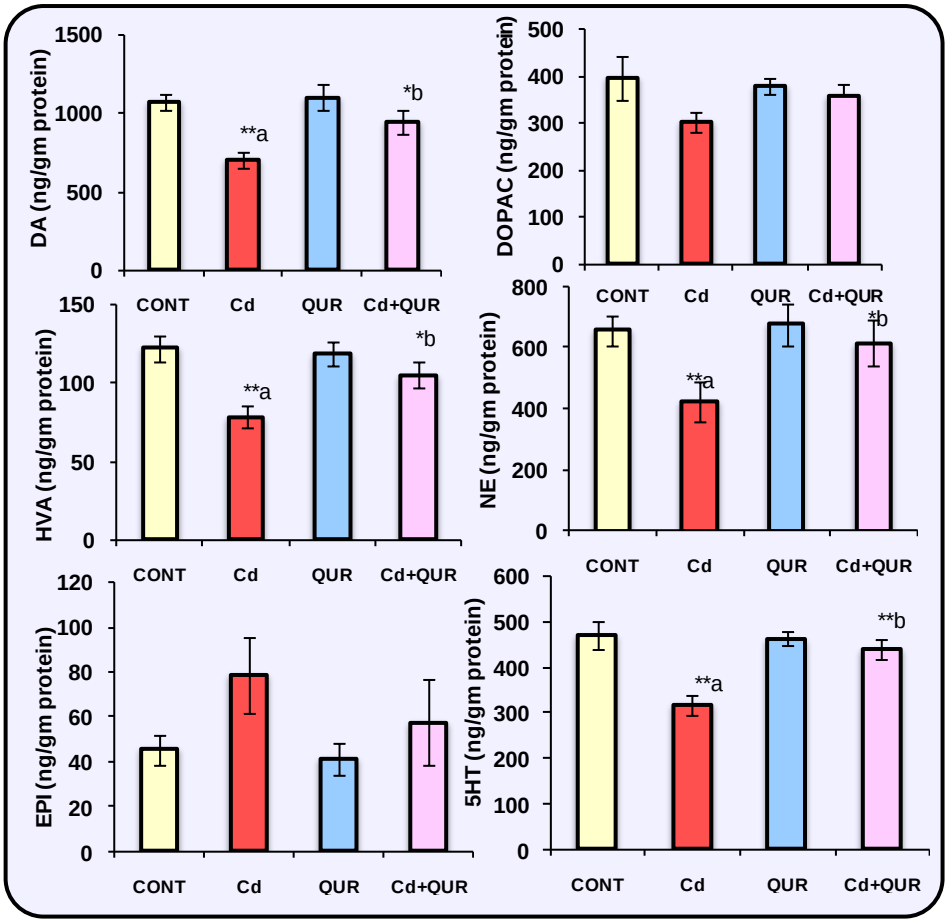


Figure – 4.33. Effect on the level of biogenic amines and their metabolites in frontal cortex

Values are mean ± SEM of five rats in each group; Values are expressed as ng/g tissue weight; significantly differs (*p < 0.05, **p < 0.01, ***p < 0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

4.3.1.3.2 Effect on Hippocampus

Rats exposed to cadmium for 28 days exhibited a decrease in the levels of NE (43%, $p<0.001$), DA (34%, $p<0.01$), DOPAC (41%, $p<0.05$) and HVA (32%, $p<0.05$) in hippocampus as compared to controls. However, increase in the levels of and EPN (27 %) was observed in the hippocampus of rats to cadmium exposure. Levels of 5-HT (34%, $p>0.05$) in the hippocampus were found decreased in rats subjected to

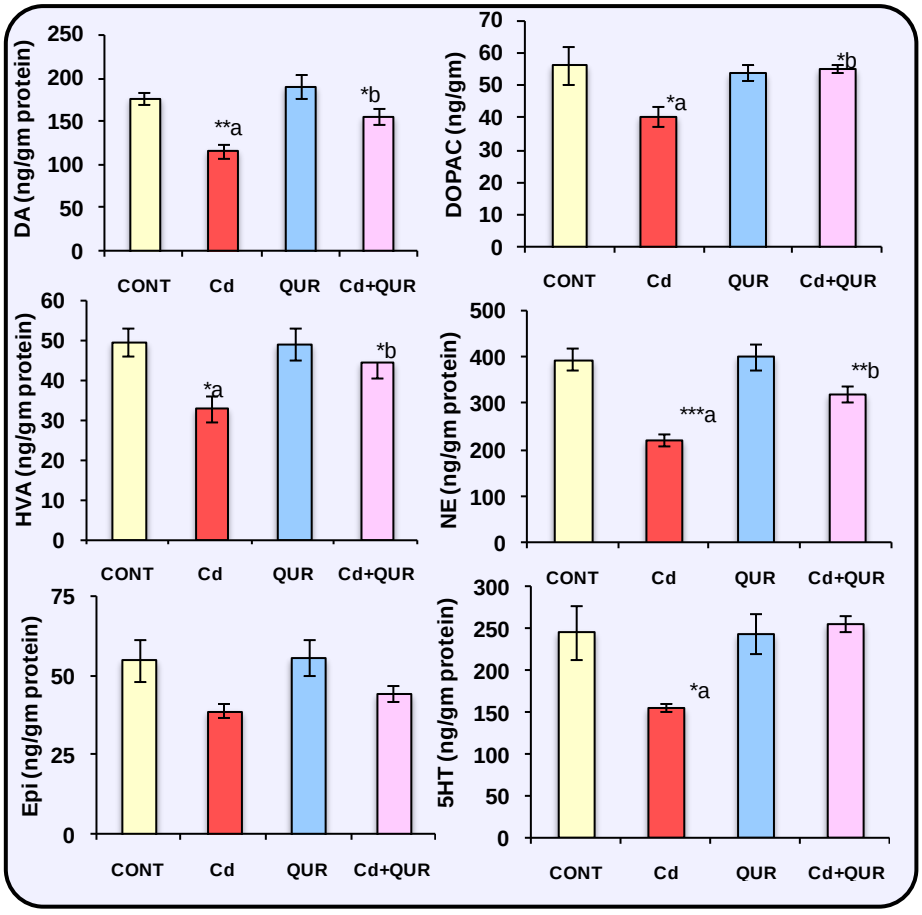


Figure – 4.34. Effect on the level of biogenic amines and their metabolites in Hippocampus

Values are mean ± SEM of five rats in each group; Values are expressed as ng/g tissue weight; significantly differs (*p < 0.05, **p < 0.01, ***p < 0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

cadmium. Interestingly, simultaneous exposure of rats to cadmium and quercetin resulted to increase the levels of NE (44%, $p<0.01$), DA (33%, $p<0.05$), HVA (37%, $p<0.05$), DOPAC (27%, $p<0.05$) and decrease the levels of EPN (15%, $p>0.05$), in comparison to rats treated with cadmium alone. The rats also exhibited significant increase in the levels of 5-HT (28.8%, $p<0.05$) in hippocampus as compared to those treated with cadmium alone. No significant effect on the levels of DA, NE, EPN, 5-HT, DOPAC and HVA in hippocampus was observed in rats treated with quercetin as compared to those in the control group (Figure – 4.34).

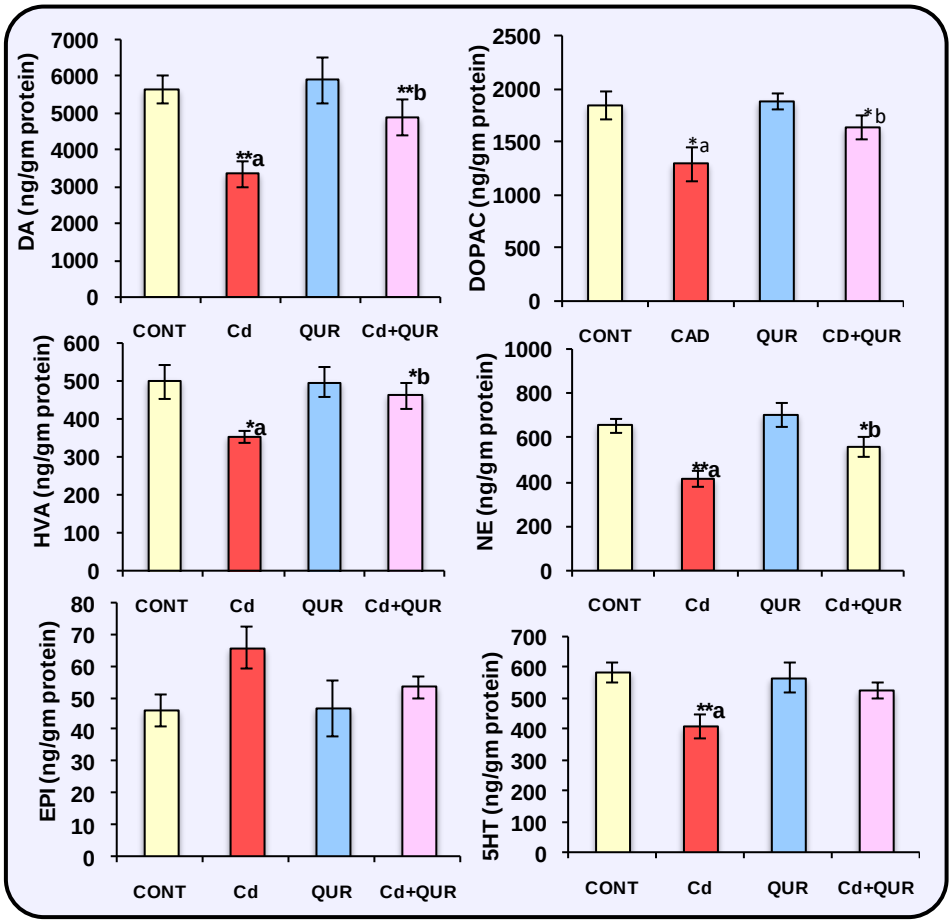


Figure – 4.35. Effect on the level of biogenic amines and their metabolites in corpus striatum

Values are mean ± SEM of five rats in each group; Values are expressed as ng/g tissue weight; significantly differs (*p < 0.05, **p < 0.01, ***p < 0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

4.3.1.3.1 Effect on Corpus Striatum

Exposure of rats to cadmium caused a significant decrease in levels of NE (52% $p<0.01$), DA (40% $p<0.001$), HVA (29.4% $p<0.05$), DOPAC (31.5%, $p<0.05$) 5HT (29%, $p<0.05$) and increase in the levels of EPN (42%, $p>0.05$) in corpus striatum as compared to controls. Simultaneous exposure of rats to cadmium and quercetin resulted to increase the levels of NE (34.6%, $p<0.05$), DA (46%, $p<0.05$), HVA (31.4%, $p<0.05$) and DOPAC (10.5%, $P>0.05$) and decrease the levels of EPN (19%, $p>0.05$) as compared to rats treated with cadmium alone (Figure – 4.35). A significant increase in the levels of 5-HT (28.2%, $p>0.05$) was also evident in rats simultaneously exposed to cadmium and quercetin in corpus striatum as compared to those treated with cadmium alone. No significant change in any of these parameters was observed in rats treated with quercetin as compared to those in the control group

4.3.1.2 Assessment of Cadmium Levels in Brain Regions

Exposure of rats to cadmium resulted to increase the cadmium levels in frontal cortex ($F_{(3, 16)} = 7.637$, 1.9 Fold, $p<0.01$), corpus striatum ($F_{(3, 16)} = 25.99$, 4.8 Fold, $p<0.001$) and

Table – 4.2. Levels of cadmium in different brain regions

Brain region	Treatment groups			
	Control	Cadmium (5mg/kg)	Quercetin 25 mg/kg)	cadmium +quercetin
Frontal cortex	73±9.5	137±11**a	55±14	81±15**b
Hippocampus	36±10	133±21***a	34±11	73±8.2**b
Corpus striatum	63±11	308±10***a	55±8.4	100±33***b

Values are mean ± SEM of five rats in each group; Values are expressed as ng/g wet tissue weight; Significantly differs (**$p < 0.01$, ***$p < 0.001$); a-compared to control group, b-compared to cadmium exposed group.

hippocampus ($F_{(3, 16)}$ = 11.37, 3.7 Fold, $p<0.001$) as compared to controls. Interestingly, simultaneous exposure of rats to cadmium and quercetin caused a significant decrease in cadmium level in frontal cortex ($F_{(3, 16)}$ = 7.637, 1.6 Fold, $p<0.05$), corpus striatum ($F_{(3, 16)}$ = 25.99, 1.32 Fold, $p<0.001$) and hippocampus ($F_{(3, 16)}$ = 11.37, 1.52 Fold, $p<0.01$) as compared to rats treated with cadmium alone. However, No significant effect on cadmium levels in any of brain regions was observed in rats exposed to quercetin alone as compared to controls (Table – 4.2).

4.3.2 *In silico* Studies

Prediction of ADMET parameters of CdCl2 was performed by the admet SAR sever which was mentioned (Table- 4.3). The study showed that CdCl2 posses the Blood Brain Barrier (BBB+ve) absorption properties with the probability of 0.9786 and also predicted the acute oral toxic and warning in carcinogenic properties of CdCl2.According to the derived docking model CdCl2 bind with MT3 protein. The comparative optimization was carried out to find the best docked pose and the energy of CdCl2 with MT3. The best 10 docked poses were identified and analyzed. The figure was generated using program

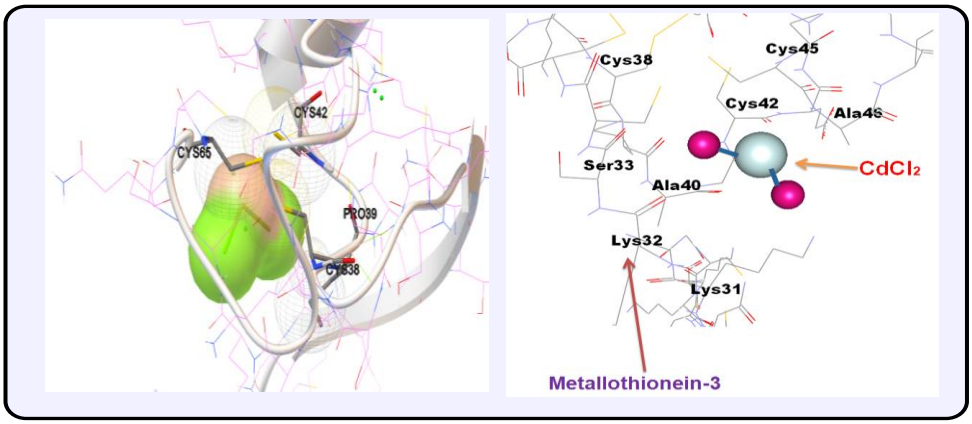


Figure – 4.36. Interaction of cadmium chloride with metallothionein III

Cadmium-Interacts with metallothionein III protein on residues CYS65, CYS42, PRO39, CYS38; With the possible Binding energy (Cdcl2- MT3 complex) -2.55 kcal/mol and Inhibition constant (ki)- 13.49mM

Table -4.3. Toxicokinetic profiling of cadmium

Model	Results	Probability
Absorption		
Blood-Brain Barrier	BBB+	0.9786
Human Intestinal Absorption	HIA+	0.9935
Caco-2 Permeability	Caco2+	0.6190
P-glycoprotein Substrate	Non-substrate	0.9022
P-glycoprotein Inhibitor	Non-inhibitor	0.9785
Renal Organic Cation Transporter	Non-inhibitor	0.9223
Distribution/Metabolism		
CYP450 2C9 Substrate	Non-substrate	0.8005
CYP450 2D6 Substrate	Non-substrate	0.7503
CYP450 3A4 Substrate	Non-substrate	0.7412
CYP450 1A2 Inhibitor	Non-inhibitor	0.5695
CYP450 2C9 Inhibitor	Non-inhibitor	0.7345
CYP450 2D6 Inhibitor	Non-inhibitor	0.8774
CYP450 2C19 Inhibitor	Non-inhibitor	0.7109
CYP450 3A4 Inhibitor	Non-inhibitor	0.9378
CYP Inhibitory Promiscuity	Low CYP Inhibitory Promiscuity	0.9075
Excretion		
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	0.9547
	Non-inhibitor	0.9746
AMES Toxicity	Non AMES toxic	0.9633
Carcinogens	Carcinogens	0.6621
Biodegradation	Ready biodegradable	0.7326
Acute Oral Toxicity	III	0.5846
Carcinogenicity (Three-class)	Warning	0.4769
Toxicity		
Rat Acute Toxicity	2.0135	LD50, mol/kg

Toxicokinetic profiling of cadmium using *in silico* studies was carried out by using ADMET Predicted Profile --- Regression

AutoDock Visualizer (Figure – 4.36) illustrates best docked pose of CdCl2 with MT3 that possessed ΔG of -2.14 Kcal/Mol and Ki value 26.88 mM. This pose identified residues CYS65, CYS42, PRO39, CYS38 were nearest binding residues which exhibit interactions with the CdCl2.

4.4 Module IV – Effect on the Neuroinflammation and Associated Signaling Following Exposure to Cadmium, Quercetin and their Co-Exposure

4.4.1 Effect on the GFAP and IBA Expression

To understand whether cadmium causes astrogliosis and microgliosis, effect on the protein expression of GFAP and IBA was assessed and protective efficacy of quercetin investigated. Statistical analysis revealed that cadmium exposure cause significant increase in the expression of GFAP in frontal cortex ($F_{(3,8)}$ = 4.967, 1.34Fold, $p<0.01$), corpus striatum ($F_{(3,8)}$ = 19.58, 2.08Fold, $p<0.001$) and hippocampus ($F_{(3,8)}$ = 5.671, 1.93Fold, $p<0.05$) of rats as compared to controls. Interestingly, Simultaneous exposure to quercetin in cadmium exposed rats caused a significant down regulation in GFAP in frontal cortex ($F_{(3,8)}$ = 4.967, 1.84Fold, $p<0.05$), corpus striatum ($F_{(3,8)}$ = 19.58, 1.71Fold, $p<0.01$) and hippocampus ($F_{(3,8)}$ = 5.671, 1.82Fold, $p<0.05$) of rats as compared to controls as compared to rats exposed to cadmium alone (Figure –4.37).

Cadmium exposure in rats caused increase microgliosis as observed by increase protein expression of IBA1 in subsequent brain regions frontal cortex ($F_{(3, 8)}$ = 41.13, 1.70Fold, $p<.001$), corpus striatum ($F_{(3, 8)}$ = 54.62, 2.25Fold, $p<0.001$) and hippocampus ($F_{(3,8)}$ = 7.811, 2.40Fold, $p<0.01$) as compared control exposed rats. Further, Simultaneous exposure to quercetin in cadmium exposed rats caused a significant down regulation of IBA1 in frontal cortex ($F_{(3, 8)}$ = 41.13, 1.75Fold, $p<0.01$), corpus striatum ($F_{(3, 8)}$ = 54.62, 1.73Fold, $p<0.001$) and hippocampus ($F_{(3,8)}$ = 7.811, 1.76Fold, $p<0.05$) of rats as compared to controls as compared to rats exposed to cadmium alone.

4.4.2 Assessment of Pro and Anti Inflammatory Cytokines Expression Following Multiplex Bead Assay

Multiplex protein profiling of selected cytokines was done to asses impact of cadmium

brain inflammatory pathways. Using luminex based technology; we found the altered inflammatory cytokines levels following cadmium exposure in various brain regions suggesting cadmium induced Neuroinflammation leading to various neuronal abnormality and death.

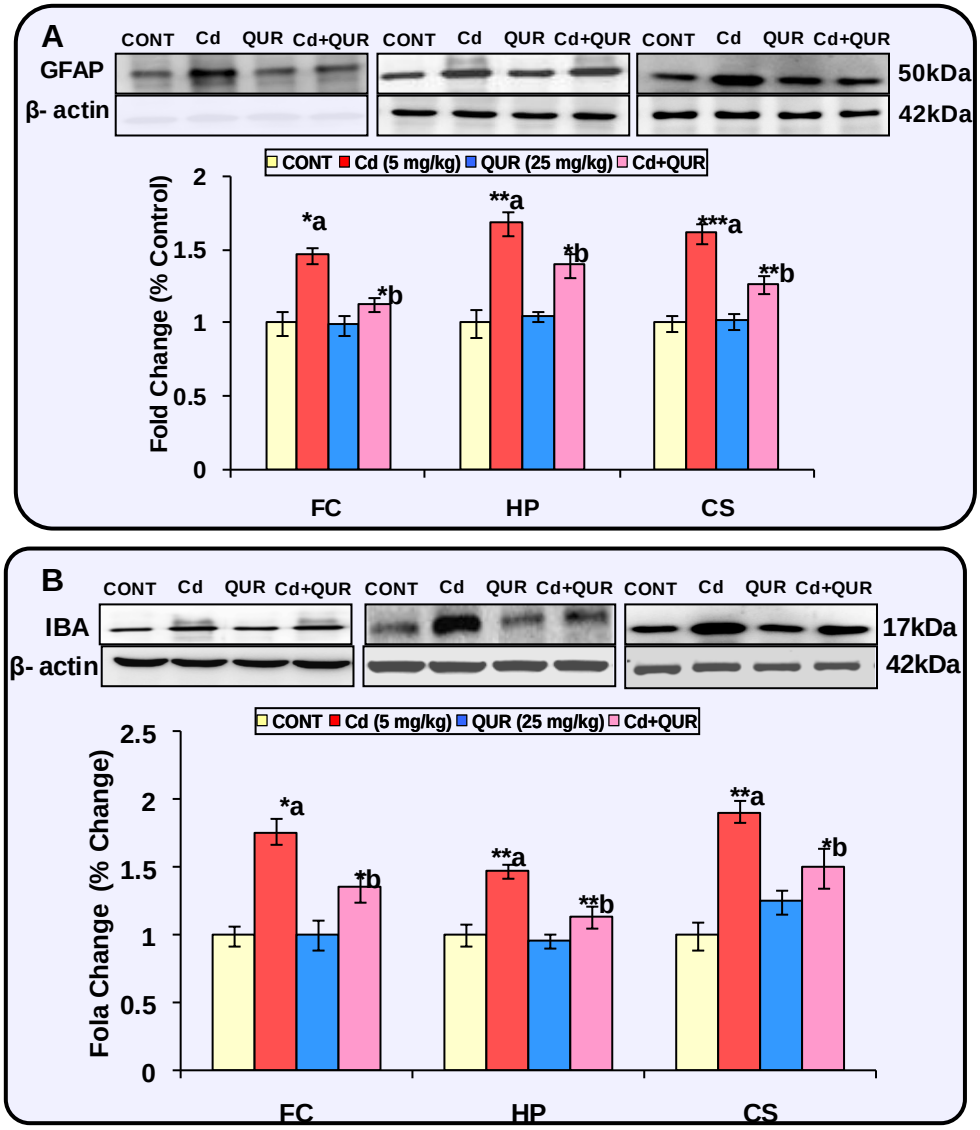


Figure – 4.37. Effect on GFAP (A) and IBA1 (B) protein in frontal cortex, hippocampus and corpus striatum

Values are expressed as mean ± SEM (n=3 rats/group); **p < 0.01; a-compared to control, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin; FC- Frontal Cortex, HP – Hippocampus and CS – Corpus striatum.

4.4.2.1 Level of Inflammatory Cytokines in Frontal Cortex

A significant increase in the expression of TNF-α ($F_{(3,15)}$ = 4.419, 57%, p<0.05), IL-1β ($F_{(3,15)}$ = 5.061, 50%, p<0.05), IL-6 ($F_{(3,15)}$ = 6.261, 40%, p<0.01) and decrease expression of anti-inflammatory cytokine IL-10 ($F_{(3,15)}$ = 9.668, 42%, p<0.001) was

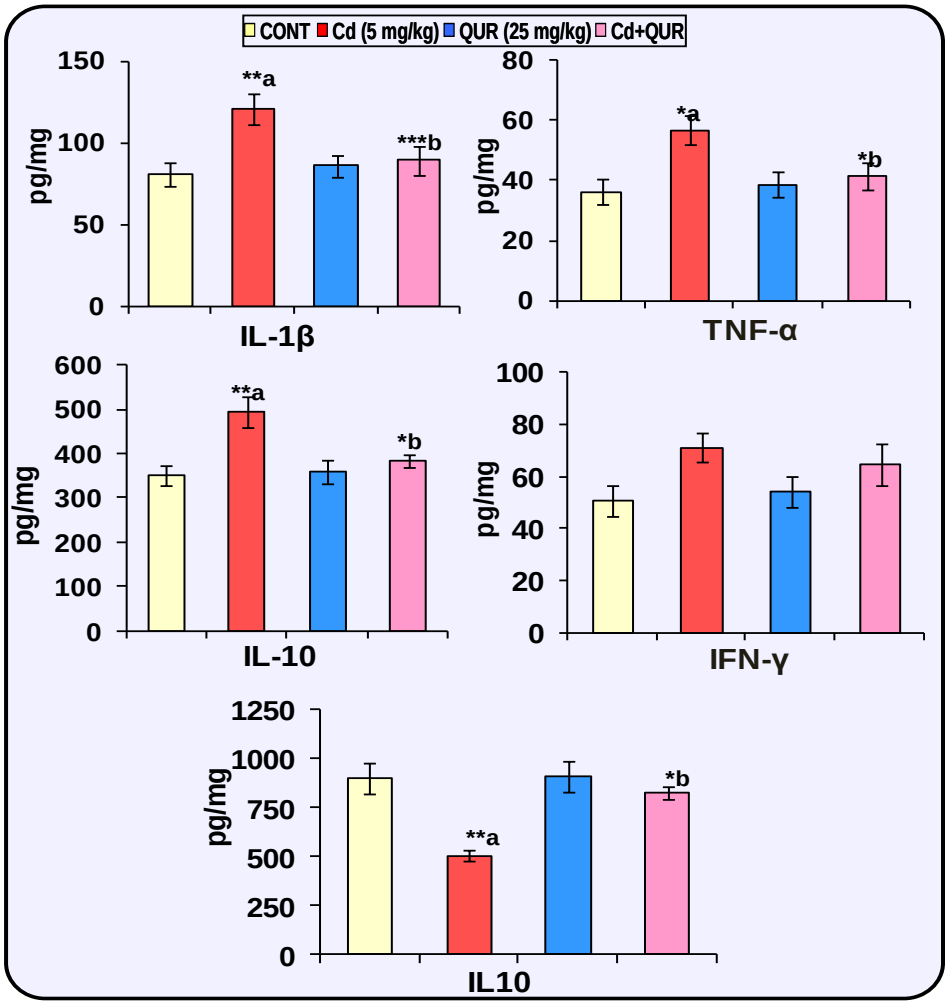


Figure – 4.38. Assessment of pro and anti-inflammatory cytokines by multiplex bead assay in frontal cortex

Values are expressed as mean ± SEM (n=5 rats/group); **p < 0.01; a-compared to control, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

observed following cadmium exposure. An increasing trend of IFNγ ($F_{(3,15)}$ = 2.097, 39%, p>0.05) was found in cadmium exposed rats. Simultaneous exposure with quercetin in

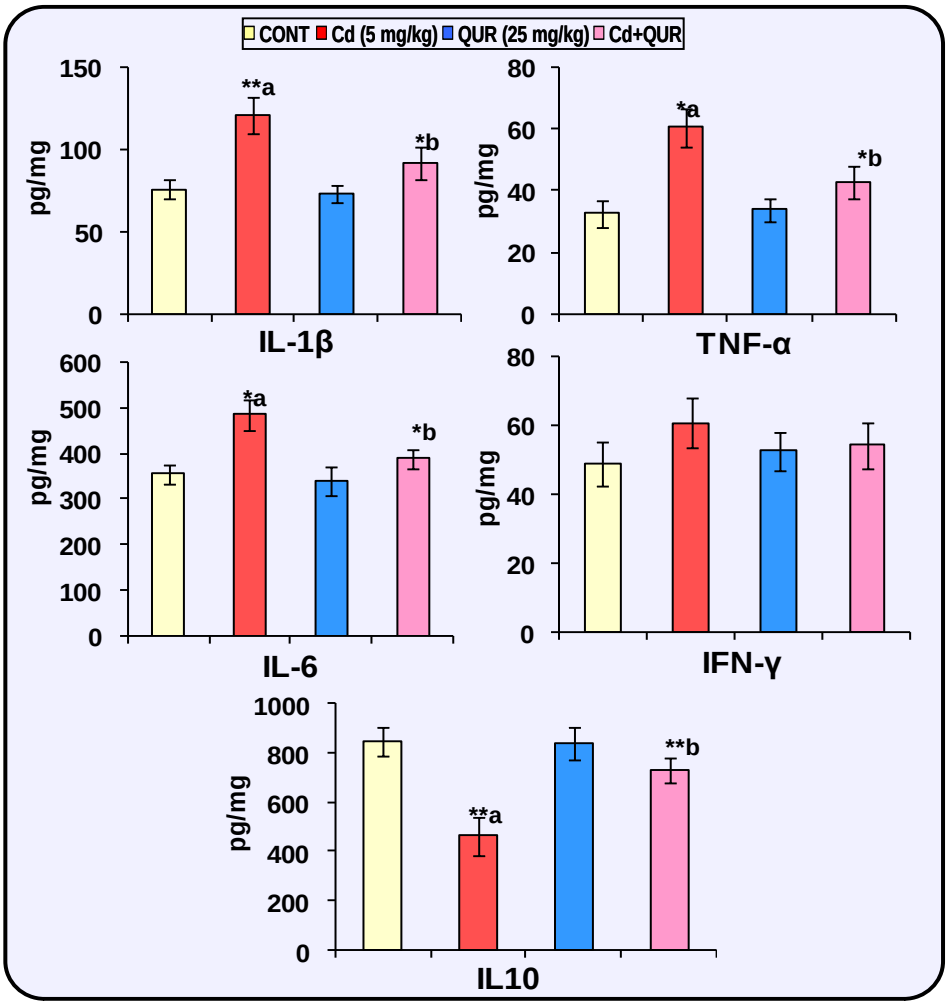


Figure – 4.39. Assessment of pro and anti-inflammatory cytokines by multiplex bead assay in hippocampus

Values are expressed as mean ± SEM (n=5 rats/group); **p < 0.01; a-compared to control, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

cadmium exposed rats was found to decrease the expression of TNF-α ($F_{(3,15)}$ = 4.419, 27%, p<0.05), IL-1β ($F_{(3,15)}$ = 5.061, 26%, p<0.05), IL-6 ($F_{(3,15)}$ = 6.261, 22%, p<0.01) and decrease expression of anti-inflammatory cytokine IL-10 ($F_{(3,15)}$ = 9.668, 64%, p<0.01) as compared to rats exposed to cadmium alone. While no significant change in the expression of IFNγ ($F_{(3,15)}$ = 2.097, 9%, p>0.05) in combine exposure group (Figure – 4.38).

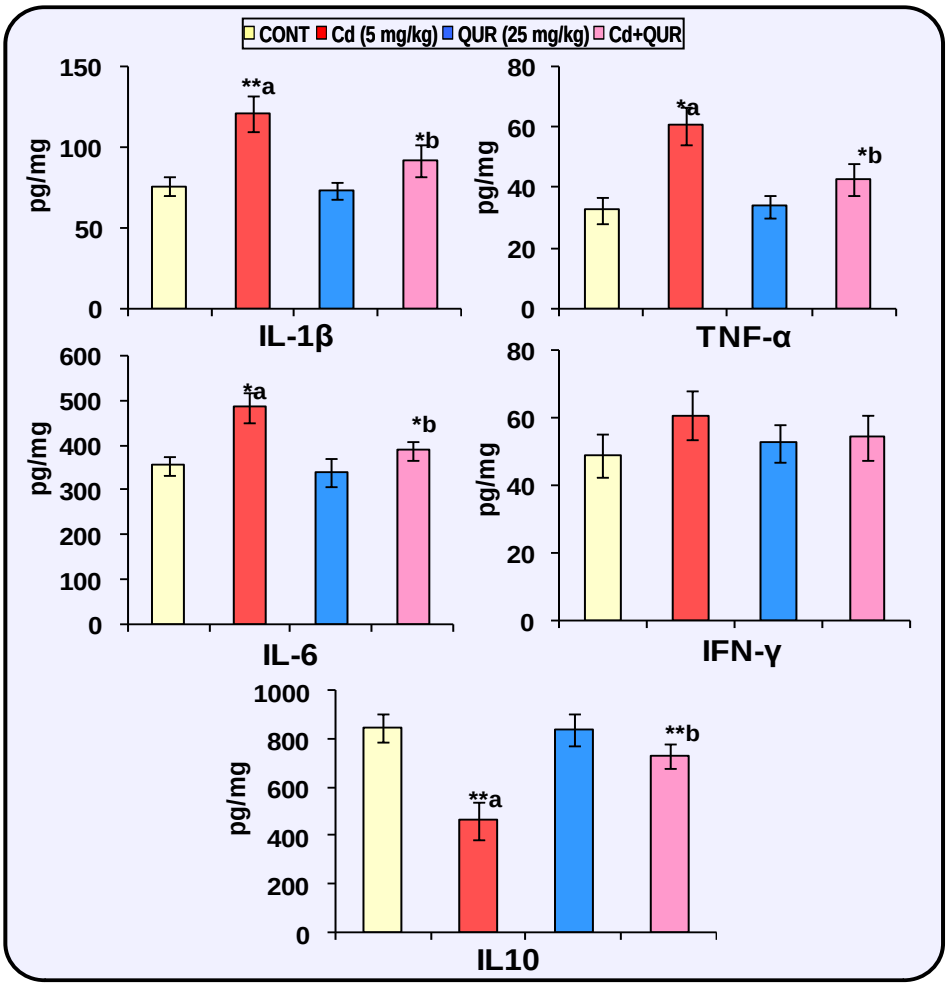


Figure – 4.40. Assessment of pro and anti-inflammatory cytokines by multiplex bead assay in corpus striatum

Values are expressed as mean ± SEM (n=5 rats/group); **p < 0.01; a-compared to control, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

4.4.2.2 Level of inflammatory cytokines in hippocampus

A significant increase in the expression of TNF-α ($F_{(3,15)}$ = 7.111, 85%, p<0.01), IL-1β ($F_{(3,15)}$ = 6.930, 59%, p<0.01), IL-6 ($F_{(3,15)}$ = 5.965, 37%, p<0.01) and decrease expression of anti-inflammatory cytokine IL-10 ($F_{(3,15)}$ = 7.905, 45%, p<0.01) was observed following cadmium exposure. An increase trend of IFNγ ($F_{(3,15)}$ = 0.5722, 24%, p<0.05) was found in cadmium exposed rats. Simultaneous exposure with quercetin in cadmium exposed rats was found to decrease the expression of TNF-α ($F_{(3,15)}$ = 7.111,

23%, p<0.01), IL-1β ($F_{(3,15)}$ = 6.930, 24%, p<0.05), IL-6 ($F_{(3,15)}$ = 5.965, 23%, p<0.05) and decrease expression of anti-inflammatory cytokine IL-10 ($F_{(3,15)}$ = 12.47, 54%, p<0.01) as compared to rats exposed to cadmium alone. While no significant change in the expression of IFNγ ($F_{(3,15)}$ = 0.5722, 11%, p>0.05) in combine exposure group (Figure – 4.39).

4.4.2.3 Level of Inflammatory Cytokines in Corpus Striatum

Exposure to cadmium caused a significant increase in the expression of TNF-α ($F_{(3,15)}$ = 4.328, 59%, p<0.01), IL-1β ($F_{(3,15)}$ = 10.99, 52%, p<0.001), IL-6 ($F_{(3,15)}$ = 14.31, 47%, p<0.001) and decrease expression of anti-inflammatory cytokine IL-10 ($F_{(3,15)}$ = 12.47, 47%, p<0.001) as compared to controls. Also, increase in the levels of IFNγ ($F_{(3,15)}$ = 3.457, 55%, p<0.05) was found in cadmium exposed rats. while, Simultaneous exposure with quercetin in cadmium exposed rats was found to decrease the expression of TNF-α ($F_{(3,15)}$ = 4.328, 23%, p<0.05), IL-1β ($F_{(3,15)}$ = 10.99, 23%, p<0.01), IL-6 ($F_{(3,15)}$ = 14.31, 23%, p<0.01) and increase expression of anti -inflammatory cytokine IL-10 ($F_{(3,15)}$ = 12.47, 51%, p<0.01) as compared to rats exposed to cadmium alone. While no significant change in the expression of IFNγ ($F_{(3,15)}$ = 3.457, 20%, p>0.05) in combine exposure group (Figure – 4.40).

4.4.3 Levels of Nitric Oxide in Brain Regions

A significant increase in the levels of nitric oxide in the frontal cortex ($F_{(3, 15)}$ = 18.10, 2.64Fold, p<0.01), corpus striatum ($F_{(3, 15)}$ = 24.29, 3.1Fold, p<0.0001) and hippocampus ($F_{(3,15)}$ = 12.47, 2.99Fold, p<0.0001) was observed in rats on exposure to cadmium as compared to controls. Simultaneous treatment with cadmium and quercetin caused a significant decrease in the nitric oxide levels in frontal cortex ($F_{(3,15)}$ = 18.10, 1.53Fold, p<0.05), corpus striatum ($F_{(3,15)}$ = 24.29, 2.01Fold, p<0.01) and hippocampus ($F_{(3,15)}$ = 12.47, 1.97Fold, p<0.01) in comparison to rats exposed to cadmium alone. Exposure to quercetin caused no significant changes in the levels of nitric oxide in any of brain region as compared to control rats (Figure - 4.41).

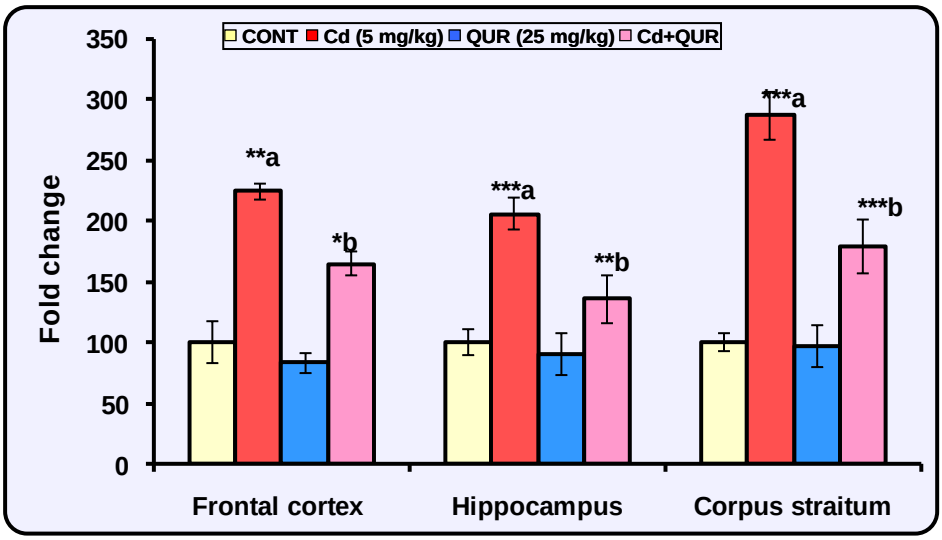


Figure – 4.41. Effect on the nitric oxide levels in brain regions

Values are mean ± SEM of five rats in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

4.4.4 Effect on the Expression of iNOS and nNOS

Involvement of iNOS and nNOS in cadmium induced neurotoxicity and protective efficacy of quercetin was assessed in frontal cortex, hippocampus and corpus striatum. Exposure to cadmium resulted to increase the expression of iNOS and nNOS in frontal cortex ($F_{(3,8)}$ = 6.465, 1.65 Fold, p<0.01; $F_{(3,8)}$ = 5.6, 1.64 Fold, p<0.01), corpus striatum ($F_{(3,8)}$ = 7.125, 2.29 Fold, p<0.01; $F_{(3,8)}$ = 4.987, 1.94 Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 3.234, 1.39 Fold, p<0.05; $F_{(3,8)}$ = 3.906, 1.44 Fold, p<0.05) as compared to controls. Simultaneous exposure to cadmium and quercetin was found to decrease the expression of iNOS and nNOS in frontal cortex ($F_{(3,8)}$ = 6.234, 1.59 Fold, p<0.05; $F_{(3,8)}$ = 5.345, 1.56 Fold, p<0.05), corpus striatum ($F_{(3,8)}$ = 4.894, 1.39 Fold, p<0.05; $F_{(3,8)}$ = 5.345, 1.64 Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 4.590, 1.54 Fold, p<0.05; $F_{(3,8)}$ = 3.097, 1.34 Fold, p<0.01) in comparison to rats exposed to cadmium alone. No significant change in any of these proteins was observed in any of the brain region of rats exposed to quercetin alone as compared to rats in the control group (Figure – 4.42).

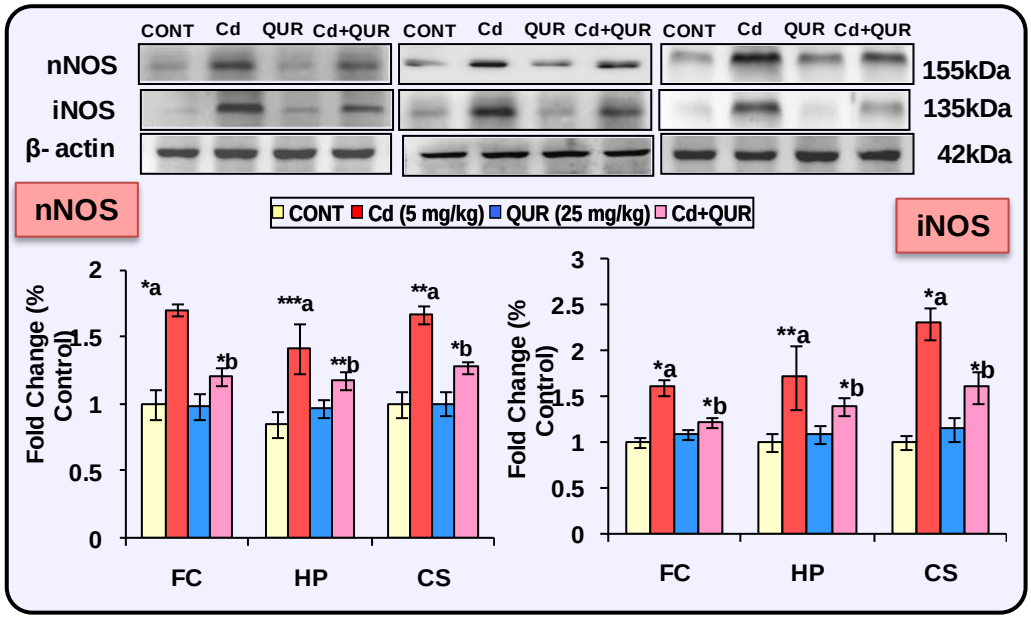


Figure – 4.42. Effect on the expression of nNOS (A) and iNOS (B) in brain regions

Values are mean ± SEM of five rats in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin.

4.4.5 Expression of Selected Proteins Associated with Neuroinflammation

Exposure to cadmium resulted to increase the phosphorylation of CamkIIα and ERK1/2 frontal cortex ($F_{(3,8)}$ = 6.954, 1.65 Fold, p<0.05; $F_{(3,8)}$ = 7.456, 1.85 Fold, p<0.01),corpus striatum ($F_{(3,8)}$ = 44.85, 1.65 Fold, p<0.001; $F_{(3,8)}$ = 51.23, 2.01 Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 10.85, 1.65 Fold, p<0.01; $F_{(3,8)}$ = 23.67, 1.92 Fold, p<0.001) respectively as compared to control. Interestingly, simultaneous exposure with quercetin significantly decreases the expression of CamkIIα and ERK1/2 frontal ($F_{(3,8)}$ = 6.954, 1.34 Fold, p<0.05; $F_{(3,8)}$ = 7.456, 1.85 Fold, p<0.05), corpus striatum ($F_{(3,8)}$ = 44.85, 1.52 Fold, p<0.001; $F_{(3,8)}$ = 51.23, 2.01 Fold, p<0.01) and hippocampus ($F_{(3,8)}$ = 10.85, 1.65 Fold, p<0.05; $F_{(3,8)}$ = 23.67, 1.32 Fold, p<0.05) as compared to cadmium treated rats (Figure – 4.43).

4.4.6 Glial Morphology- Ultrastructural Changes

Cadmium Exposure in rats affects the integrity of glial and neuron cell as visualized by

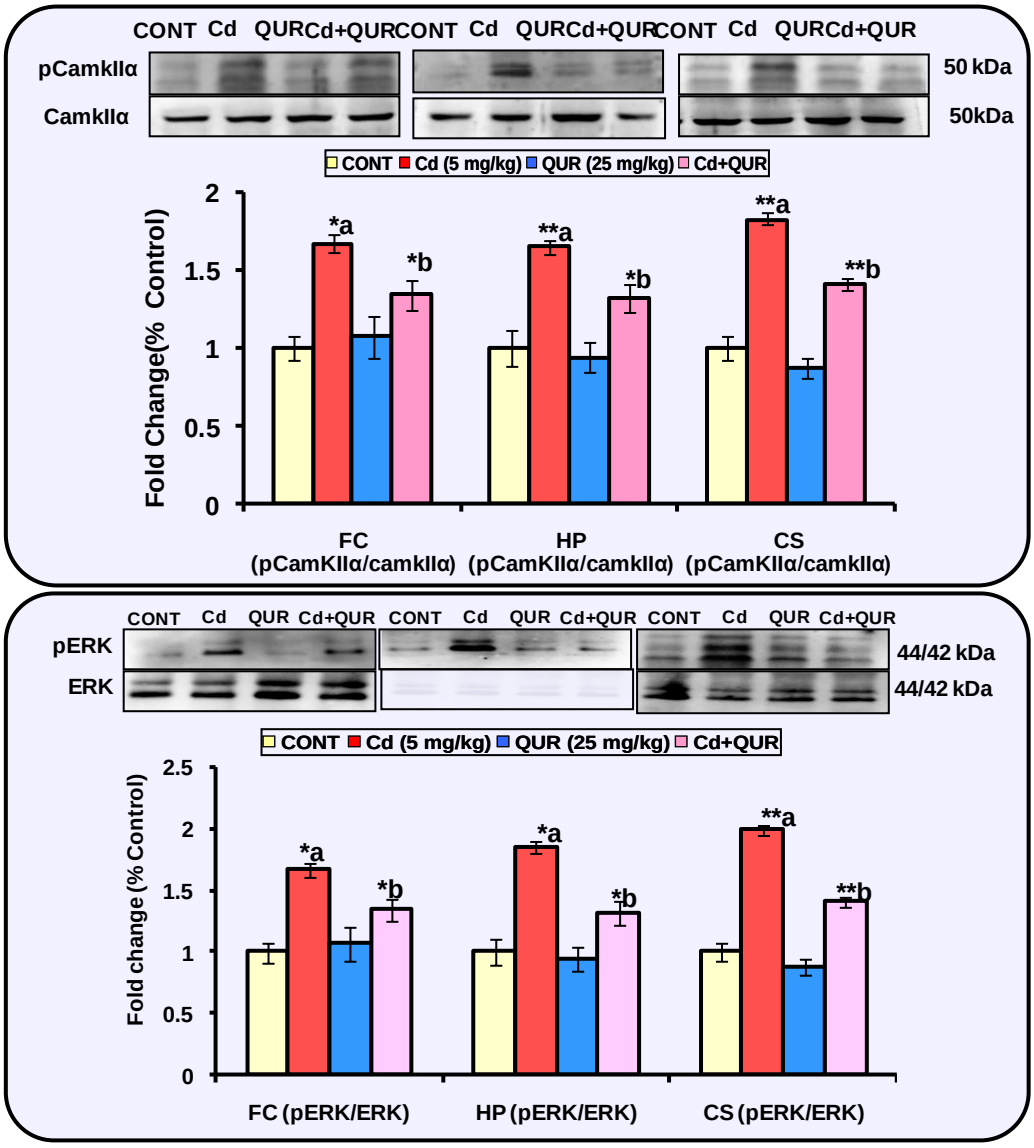
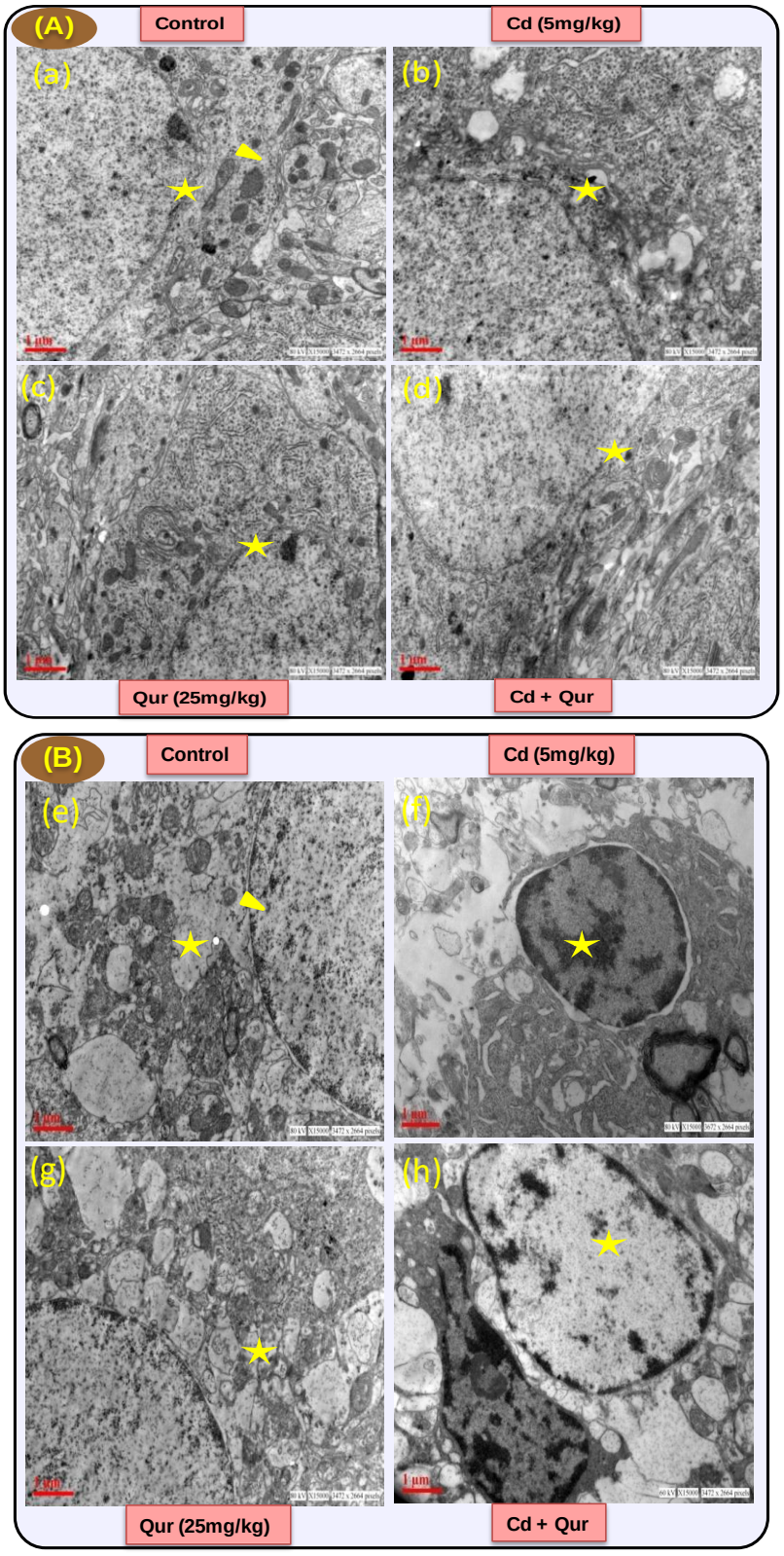


Figure – 4.43. Effect on the expression of CamkIIα and ERK in selected brain regions

Values are mean ± SEM of five rats in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

electron microscopy. The glial cell was found to be highly active, dense and packed cell with increase in ribosomes was observed in cadmium treated rats. The endoplasmic reticulum was dilated and fragmentation in cisternae along with disruption in the astrocytes membrane was observed. The mitochondria were damaged along with the loss


(A)
Control
Cd (5mg/kg)
(a)
(b)
(c)
(d)
Qur (25mg/kg)
Cd + Qur
(B)
Control
Cd (5mg/kg)
(e)
(f)
(g)
(h)
Qur (25mg/kg)
Cd + Qur

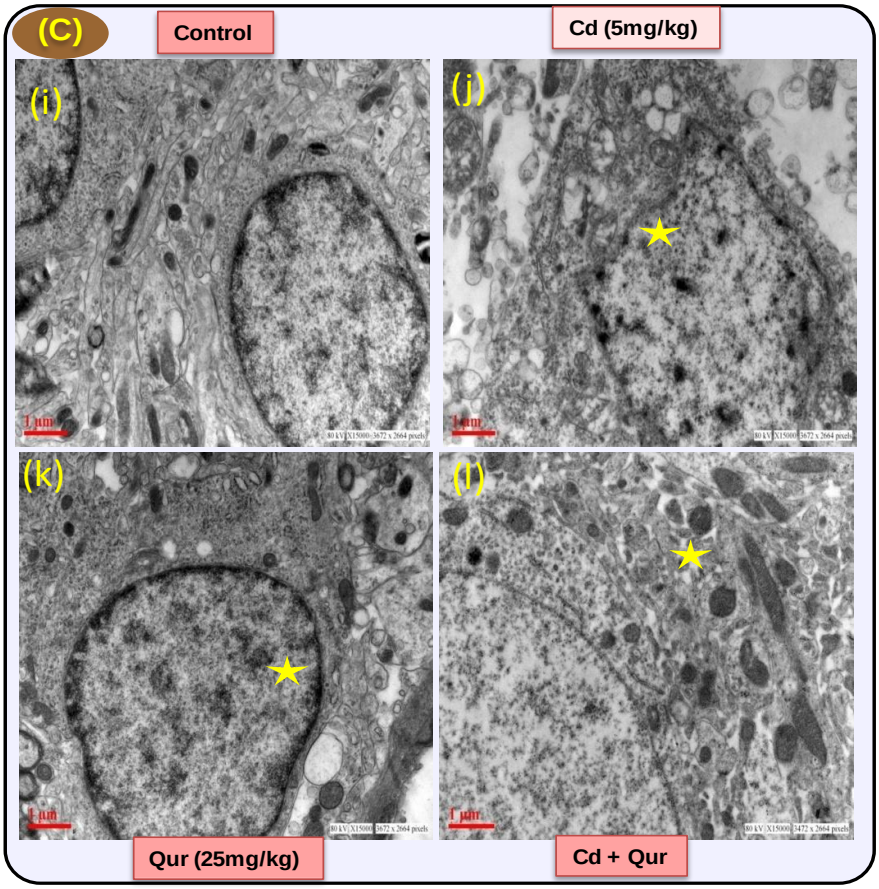


Figure – 4.44. Ultrastructural changes in the frontal cortex (A), hippocampus (B) and corpus striatum (C) regions

cadmium exposure in rats causes the loss of cell organelles, increased astrocytes and microglia activity. Reactive fibrous astrocytes containing developed abundant rough endoplasmic reticulum (Astrick), swollen mitochondria with low electron density. Abundant bundles of gliofilaments (arrow) and some lipid droplets. The Fragmentation or dilation of the cisternae of Golgi apparatus in the soma has also been observed in the rats. While, Quercetin exposure in cadmium treated rats protects such changes.

cristae and electron density in the selected brain regions that is hippocampus, frontal cortex and corpus striatum as compared to control rats. However, rats simultaneously treated with cadmium and quercetin showed the better Ultrastructure as compared to rats treated with cadmium alone (Figure – 4.44).

4.5 Module V – Effect on the Autophagy Process Following Exposure to Cadmium, Quercetin and their Co-Exposure

4.5.1 *In vivo* Studies

We observed that cadmium increased the neurodegeneration in corpus striatum; in this regard, we further hypothesized that whether it could be due to alteration in cell survival of autophagy which leads to neuronal death**.** The autophagy is considered as protective mechanism in the cell by removing the unwanted and misfolded proteins with lysosomes.

4.5.1.1 Expression of LC3-1/LC-3II and p62

To study, the effect of cadmium on autophagy makers, we carried out the western blotting analysis. We found that cadmium treatment in rats significantly increases the expression of autophagy protein LC3-II and decreases the expression of p62 proteins as compared with controls. The increased expression of LC3-II suggests the increase in the autophagosmoes formation. Interestingly, co exposure of cadmium and quercetin reduces the cadmium increased expression of LC3-II and increase levels of p62 (Figure – 4.45).

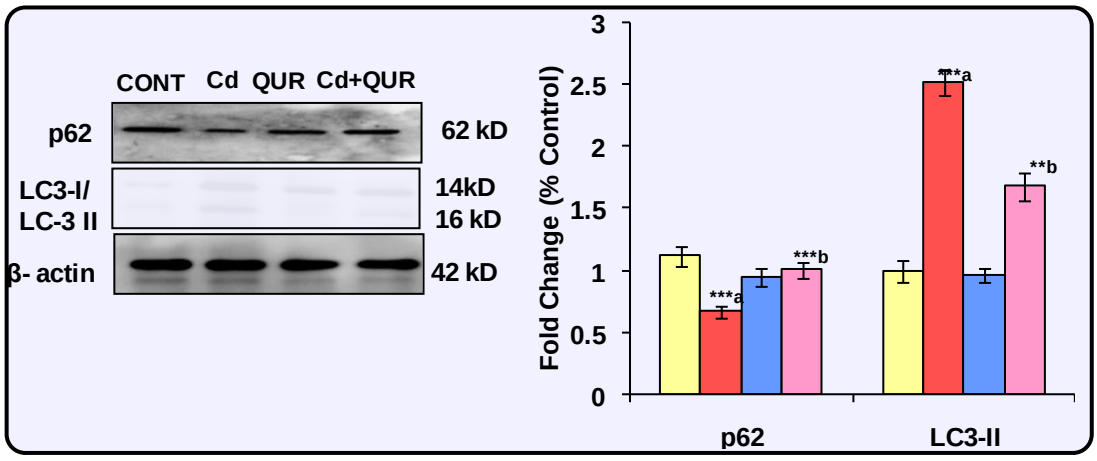


Figure – 4.45. Effect on the expression of p62 and LC3II in corpus striatum

Values are mean ± SEM of three rats in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

4.5.1.2 Expression of Autophagy Markers

Cadmium treatment in rats for 28 days found to increase the expression of autophagy proteins Beclin-1, Atg5, Atg12, Atg7 and Atg3 as compared with controls. However, no significant change in the expression of Atg16 was observed on cadmium treatment at dose of 5mg/kg in rats. Further, interesting to it, simultaneous treatment with the quercetin in cadmium exposed rats down regulates the expression of cadmium induced increase in the Beclin-1 and Atg like proteins shows the homeostatic levels of autophagy (Figure – 4.46).

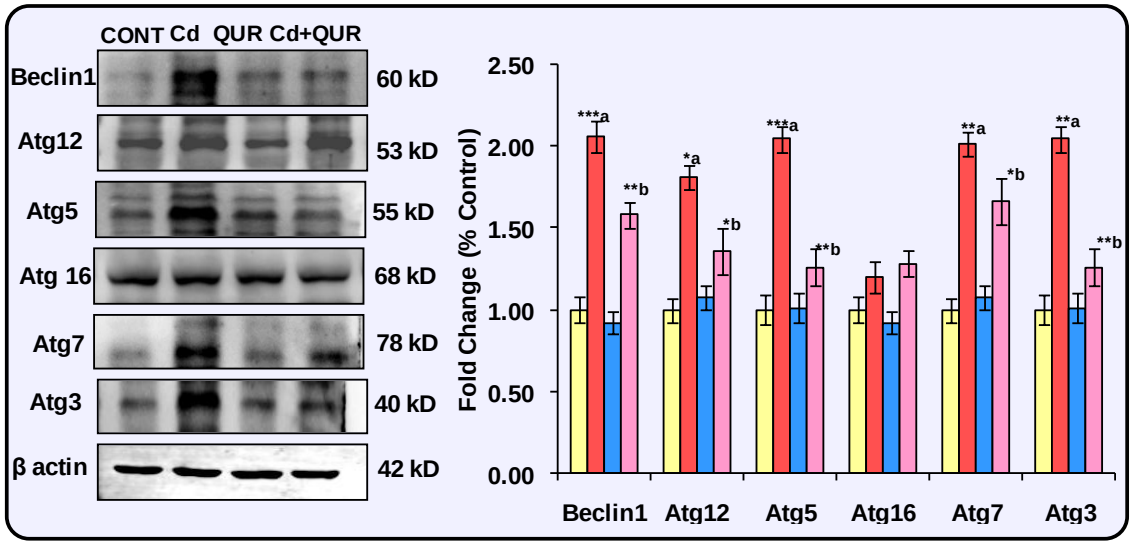


Figure – 4.46. Effect on the expression of Beclin1 and Atg like proteins in corpus striatum.

Values are mean ± SEM of three rats in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

4.5.1.3 Expression of Lamp2 Expression

Importantly, Lamp2a which is considered as important determinants in autophagy process as it indicates as the marker of lysosomes present in the cell. In case an altered autophagy process more numbers of lysosomes are present to digest the increased number of autophagosomes and thus maintain the basal levels of autophagy. Interestingly,

increased expression of Lamp2a was found in the present following the cadmium exposure as compared to controls. However, quercetin treatment in cadmium induced rats reduces the expression of Lamp2a in corpus striatum in rats (Figure – 4.47).

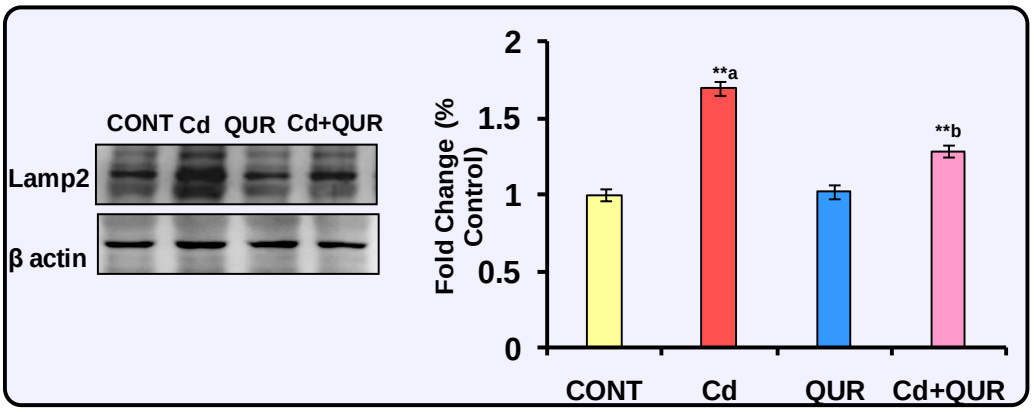


Figure – 4.47. Effect on the expression of Lamp2a protein in corpus striatum brain regions

Values are mean ± SEM of three rats in each group; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control,

4.5.2 *In vitro* Studies

In vitro studies were further carried out to assess the mechanism of autophagy following cadmium treatment using SHYSY-5Y cells**.**

4.5.2.1 Cell Viability Studies

Effect of cadmium and quercetin was assessed at various concentrations (10^{-3} to 10^{-7} M) on four different time points (24 - 96 hr) on the viability the cells. The mitochondrial MTT assay using the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was carried out to established the non-cytotoxic dose of cadmium and biological safe concentration of quercetin. We found that the cadmium significantly decrease the cell viability above 10µM in 24 h. The 10µM dose of cadmium was non-cytotoxic dose and selected for the study. However, quercetin was found to be safe at dose of 100 µM and was further used for the studies. Interestingly, cell viability

assay carried out further suggests that simultaneous exposure with quercetin at 100µm concentration was found to protect cadmium induced decrease in the cell viability. The images were shown in (Figure – 4.48, 4.49).

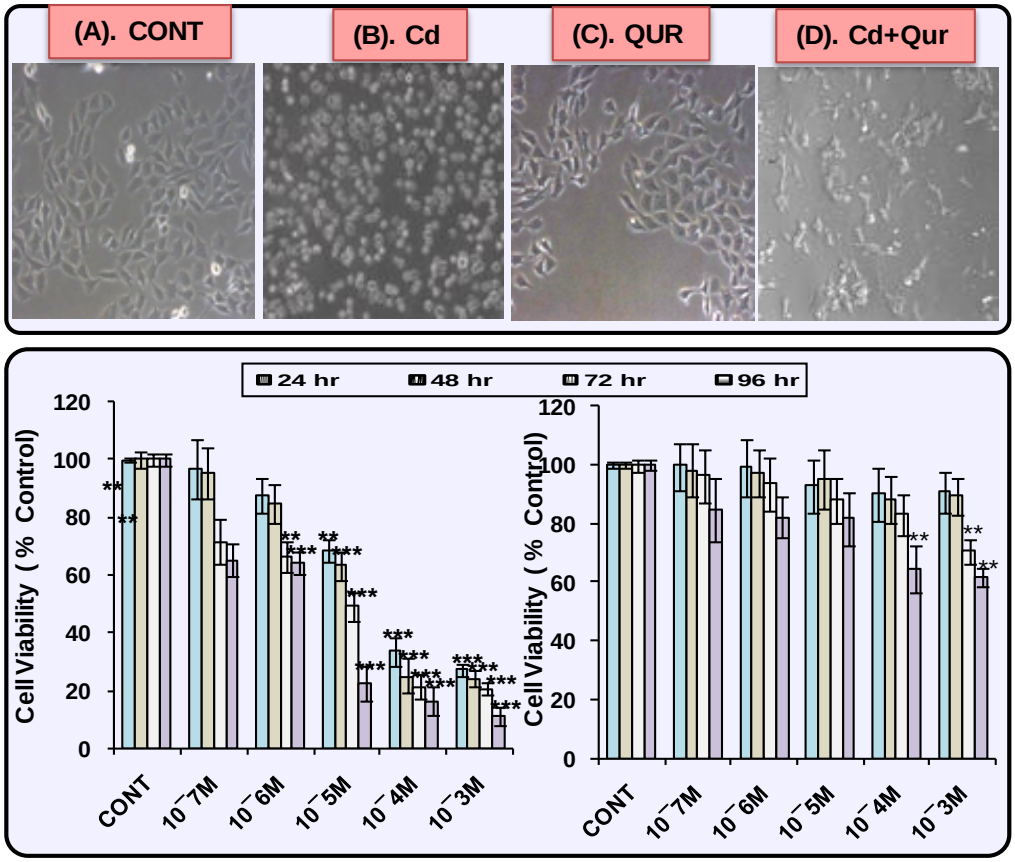


Figure – 4.48. Assessment of cell viability of SHYSY5Y following exposure to cadmium and quercetin

Representative images of cadmium and quercetin exposure (A-D); Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); * - Compared to controls.

4.5.2.2 Cadmium Induces Autophagy in the Neuronal SHYSY5Y Cells *In vitro*

After identifying the non-cytotoxic and cytoprotective doses of cadmium and quercetin respectively, further studies were carried out to assess whether cadmium induces autophagy, cells were treated with the various concentrations of cadmium (5 µM, 10 µM,

20 µM and 25 µM). A significant upregulation in the levels of LC3-II and Beclin1 and down regulation of p62 levels was found in the dose dependent manner (Figure – 4.50) as compared to unexposed cells. We further treated the cells to cadmium at various time points (3hr, 6hr, 12hr and 24hr) at dose of 10 µM. Cadmium treatment in cells increases the levels of LC3II and Beclin1 and decrease the level of p62 as compared to controls.

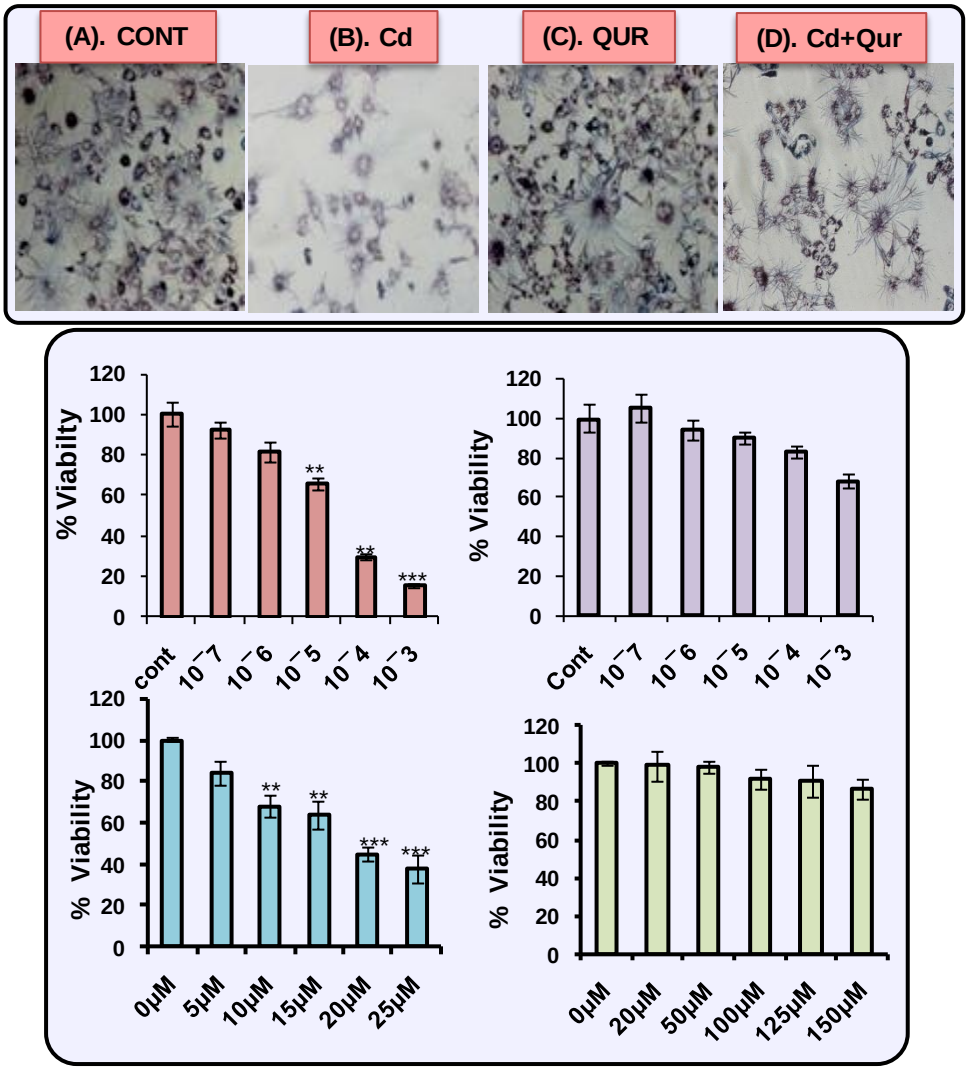


Figure – 4.49. Assessment of cytotoxic and cytoprotective doses of cadmium and quercetin

Representative images of formazon crystal following cadmium and quercetin exposure (A-D) Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); Compared to controls

The significant increase in the expression of autophagy proteins was also found at 3h which was further augmented till 24hr (Figure – 4.50).

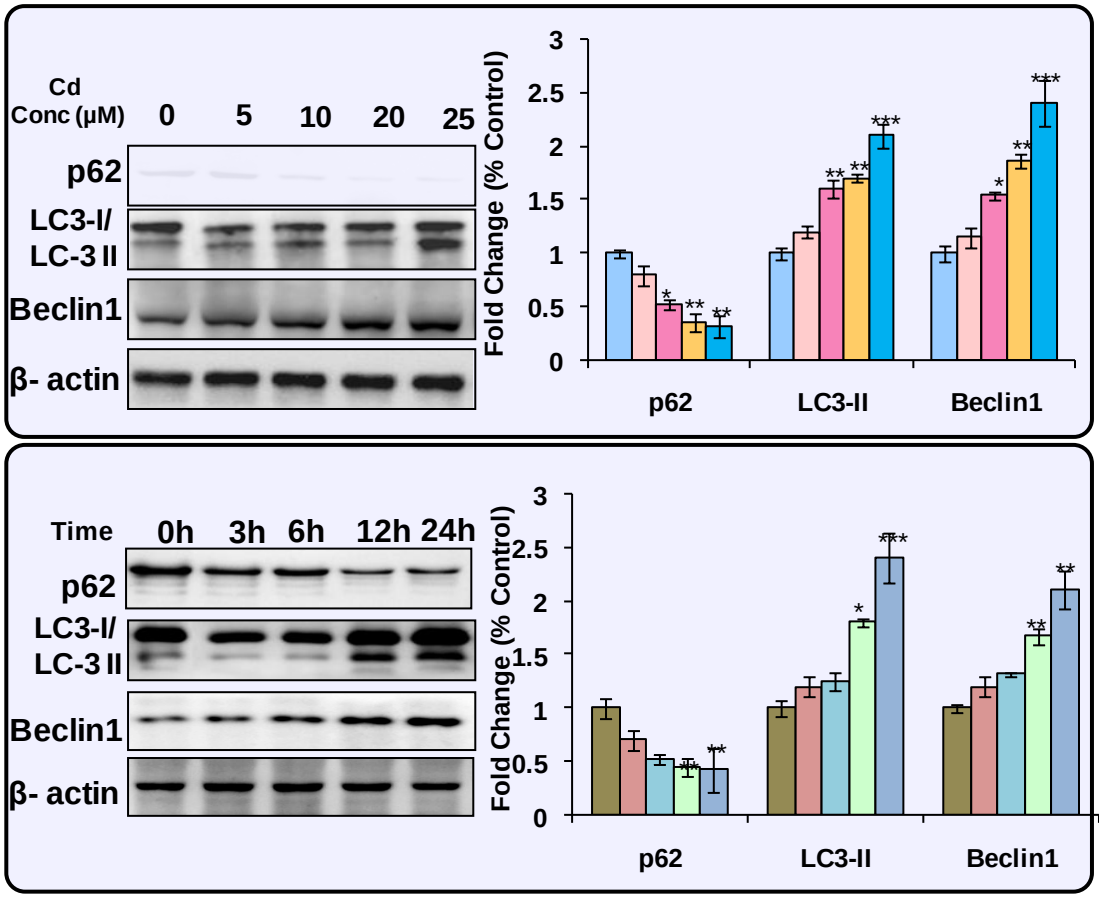


Figure – 4.50. Effect on the expression of autophagy maker proteins (p62, LC3II and Beclin) at various concentrations and time points in cells

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); Compared to controls

4.5.2.3 Cadmium Induces Increase in the GFP-LC3 Puncta

Further studied the effect of cadmium the formation of GFP-LC3 puncta in the transfected cells. GFP-LC3 puncta is considered as an indicator of formation of autophagosomes in the cells. We found that cadmium treatment in cells increased number of GFP-LC3 puncta in the transfected cells in as compared to controls. While treatment with quercetin ameliorates such changes (Figure – 4.51).

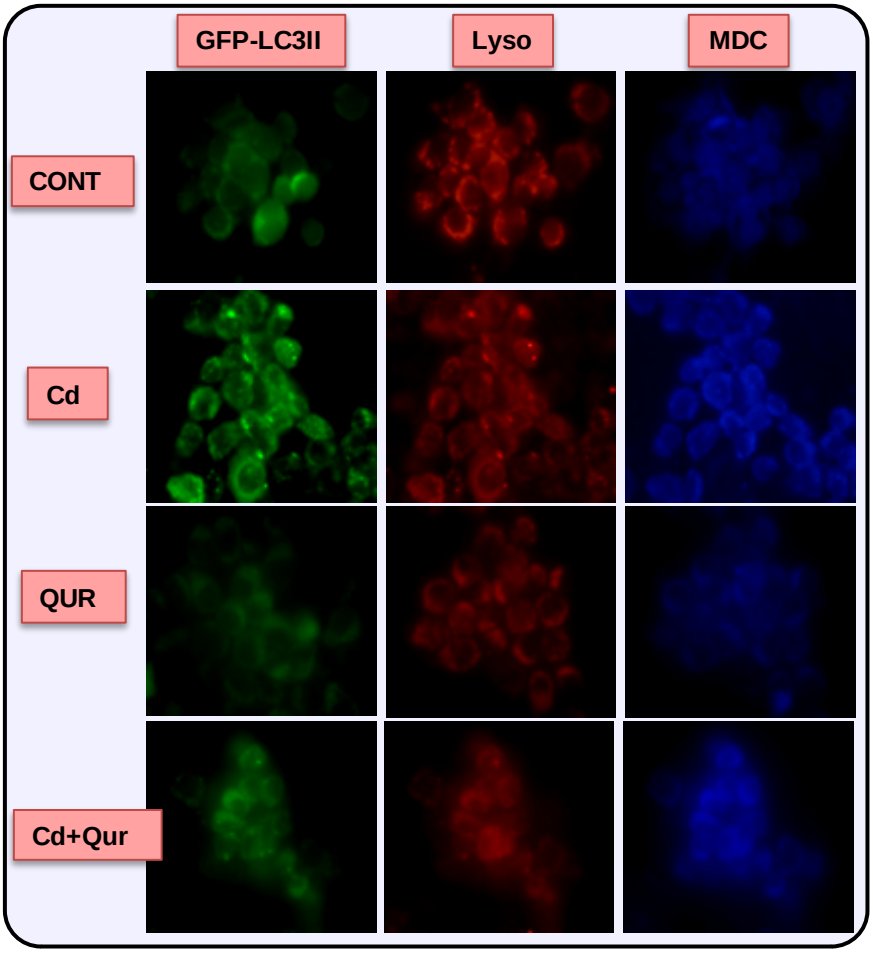


Figure – 4.51. Effect on the GFP LC3II puncta following exposure to cadmium and quercetin

Representative images have been shown following exposure to cadmium, quercetin individually and simultaneously. Co localization of mitochondria and lysosome with LC3-GFP positive autophagosomes; Green fluorescence represents for GFP LC3II puncta, Red fluorescence for Lysotracker, blue fluorescence for mitotracker (MDC)

4.5.2.4 Effect of Cadmium in Autophagy Protein Markers

The effect of cadmium on various autophagy proteins was further studies to establish that the effect of cadmium on autophagy process and effect of quercetin in cadmium induced toxicity *in vitro* in neuronal cell. We found that cadmium treatment in the cells at a dose 10µM for 24 hr increased the expression of various autophagy proteins Beclin-1, Atg5,

Atg12, Atg7 and Atg3 as compared with controls. Further, treatment of quercetin at dose of 100 µM down regulates the cadmium induced activation of autophagy proteins. The results suggest that quercetin have potential to combat the cadmium induced toxicity (Figure – 4.52, 4.53 and 4.54).

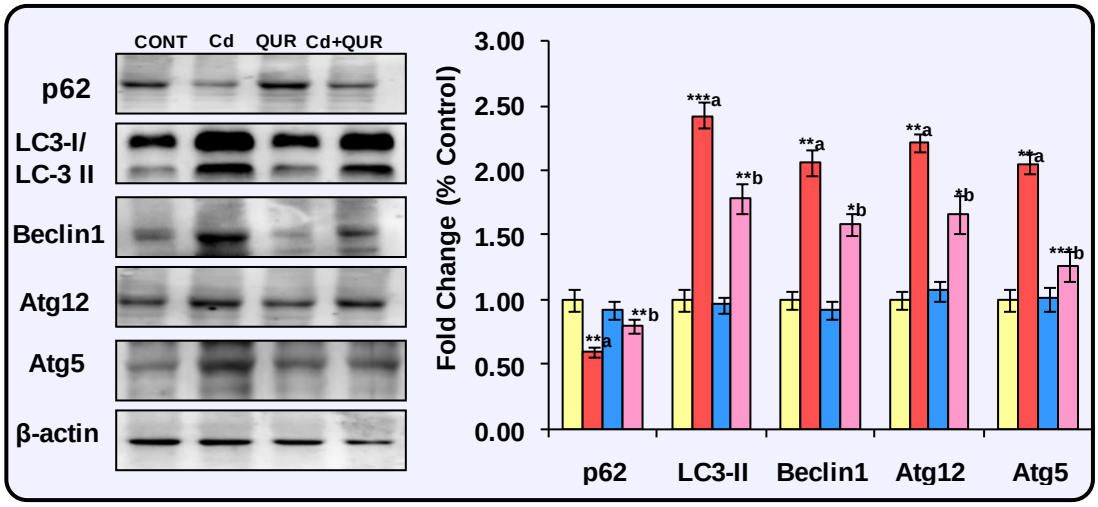


Figure – 4.52. Effect on the expression of p62, LC3II, Beclin and Atg like proteins

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin

4.5.2.5 Effect of Cadmium on LC3-II Lipidation in Presence/Absence of Autophagy Activator

Further, effect of cadmium on autophagy markers LC3-II lipidation and p62 in presence/absence of autophagy activator rapamycin and autophagy inhibitor 3MA was validated. We found an interesting result that cadmium exposure in presence of rapmaycin enhances the levels of LC3-II and P62 in the cells as compared to control and cadmium exposed cells. However, the presence of quercetin the changes was rescued such changes. These results provide an important confirmation that cadmium in presence/absence on autophagy activator further modulates the autophagy markers LC3-II and p62 levels (Figure – 4.55).

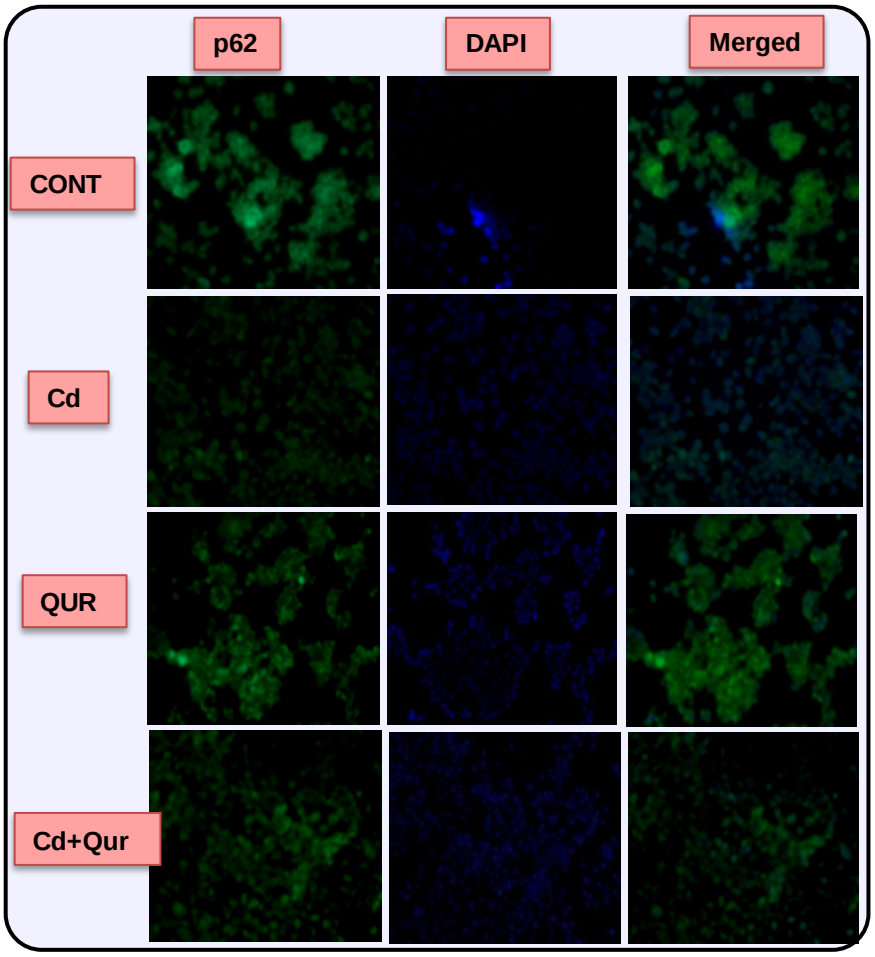


Figure – 4.53. Effect on the expression of autophagy marker p62

Representative images have been shown following exposure to cadmium, quercetin individually and simultaneously; Green fluorescence represents for p62, blue fluorescence for DAPI and Merged are presentation for p62 and DAPI both

4.5.2.6 Effect of Autophagy on Cadmium Induced ROS Generation in Presence/Absence of Autophagy Activator/Inhibitor

Exposure of cadmium to the cells causes the ROS generation, resulting the activation various signaling cascade and cell death. We therefore, study the role of autophagy in cadmium mediated increase in ROS generation. Exposure to cadmium increased the ROS generation in time dependent manner. Further cadmium induced increase in the

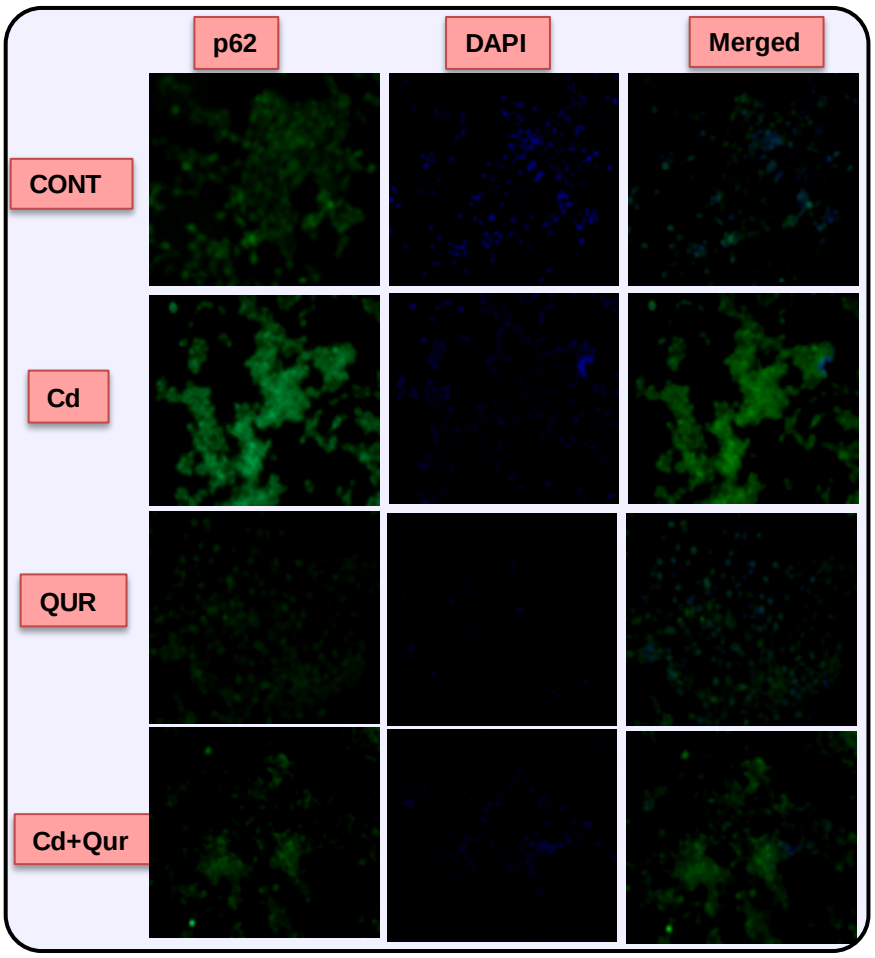


Figure – 4.54 Effect on the expression of Beclin1

Representative images have been shown following exposure to cadmium, quercetin individually and simultaneously; Green fluorescence represents for p62, blue fluorescence for DAPI and Merged are presentation for p62 and DAPI both.

ROS generation was decreased in presence of autophagy activator rapmaycin and increases in the presence of autophagy inhibitor 3MA. Further, in presence of quercetin, an antioxidant the levels of ROS were decreased in time dependent manner. These results together complies that inhibition of autophagy may further aggravate the cadmium induced ROS generation and other parameters associated (Figure – 4.56).

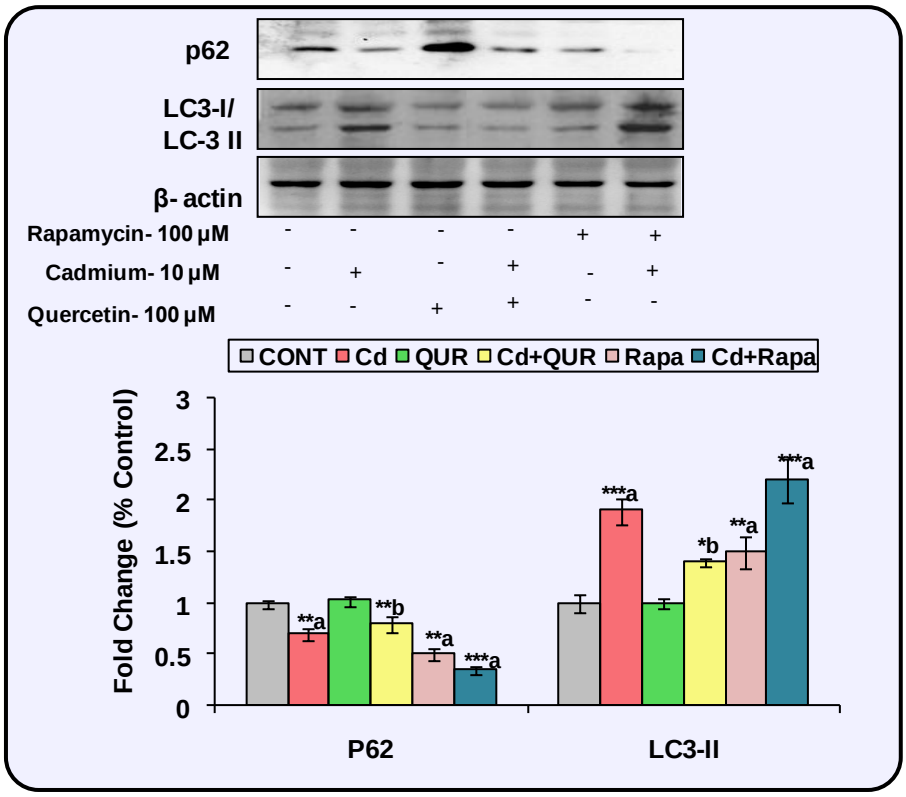


Figure – 4.55. Effect on the p62 and LC3II using the autophagy activator.

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group.

4.6 Module VI - Assessment of Protective, Prophylactic and Therapeutic Approaches of Quercetin Vs Nanoquercetin

4.6.1 Synthesis and Characterization of DA NPs and *In Vitro* Release Profile

Formulation was considered as the initial step. For which, the suitable vehicle in the most crucial step in the preparation of nanoparticles so we have selected the PLGA for the preparation of quercetin nanoparticles. The schematic representation of quercetin nanoparticles was given in (Figure – 4.57). PLGA emulsified with quercetin and PVA

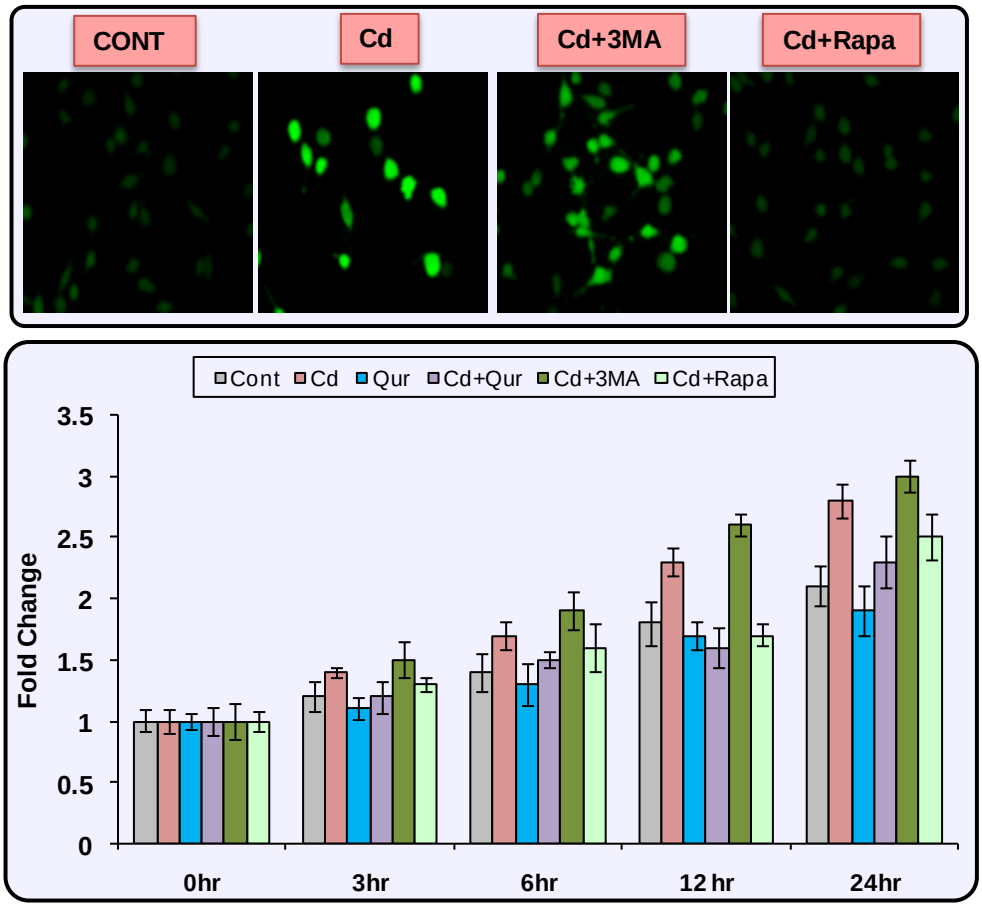


Figure – 4.56. Effect on ROS generation using autophagy activator and inhibitor at various time points

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin, Rapa- Rapamycin, 3MA- 3 methyl amide

and assembles to give nanoparticles of quercetin by the emulsion solvent evaporation quercetin release from nanoparticles. The release graph predicated that 50% of quercetin was method. The size of the quercetin nanoparticle was measured by DLS was 121.2 ± 2.3 nm and polydispersity index (PDI) was 0.284. The DLS measures the particles size of nanoparticles in its hydrodynamic stage that is the core particle along with the solvent layer attached to it. The *in vitro* drug release kinetics of quercetin nanoparticles was studied for 3 days in phosphate saline buffer at 37^{o} C and observed the continuous

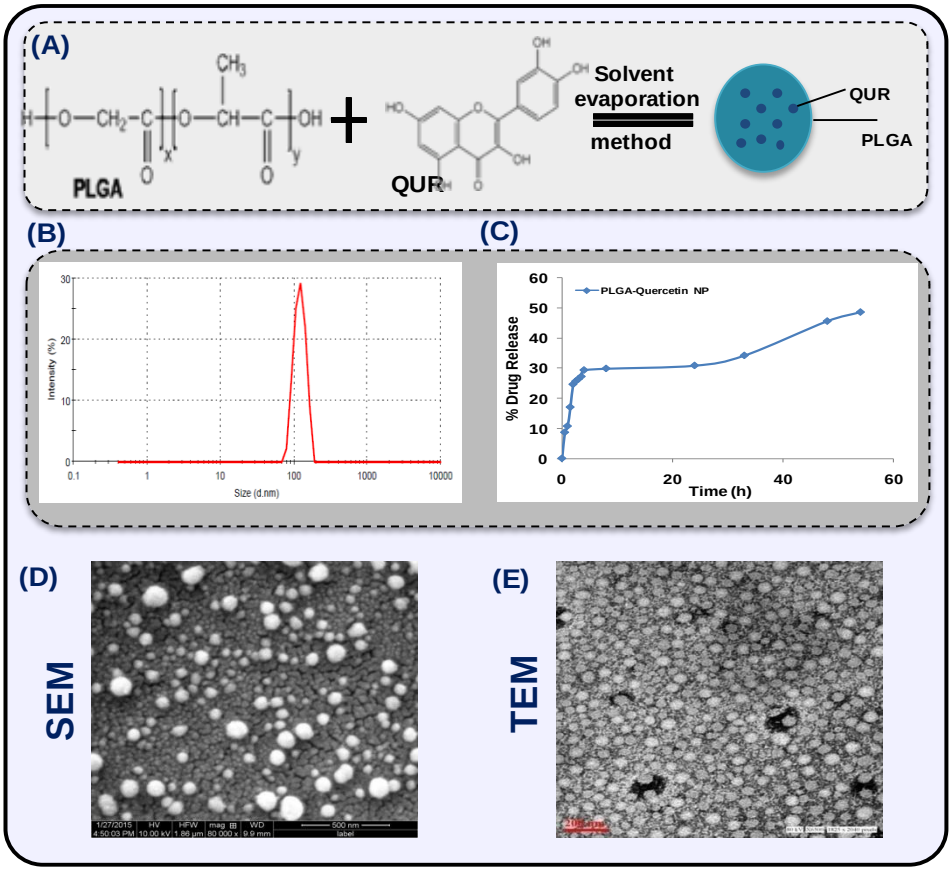


Figure – 4.57. Synthesis and characterization of QUR nanoparticles

Schematic representation of the quercetin nanoparticles (A) by the emulsion solvent evaporation method; the determination of size, PDI of nanoparticles by DLS (B); Invitro release kinetics of quercetin nanoparticles (C); Photomicrographic representation of quercetin NPs through Scanning Electron Microscope and Transmission Electron Microscopy (D & E); PDI- polydispersity index, DLS- dynamic laser light scattering, QUR- Quercetin Scale bar -10 and 20 μm for SEM and 50 nm for TEM.

released in the time. The release pattern showed the biphasic response. The surface morphology of quercetin nanoparticles was confirmed using the TEM and SEM studies, the size obtained through TEM analysis was 58-72 nm further confirm that nanoparticles was in nano range as it was found in DLS studies. The SEM and TEM analysis showed the smooth and spherical shape of the nanoparticles.

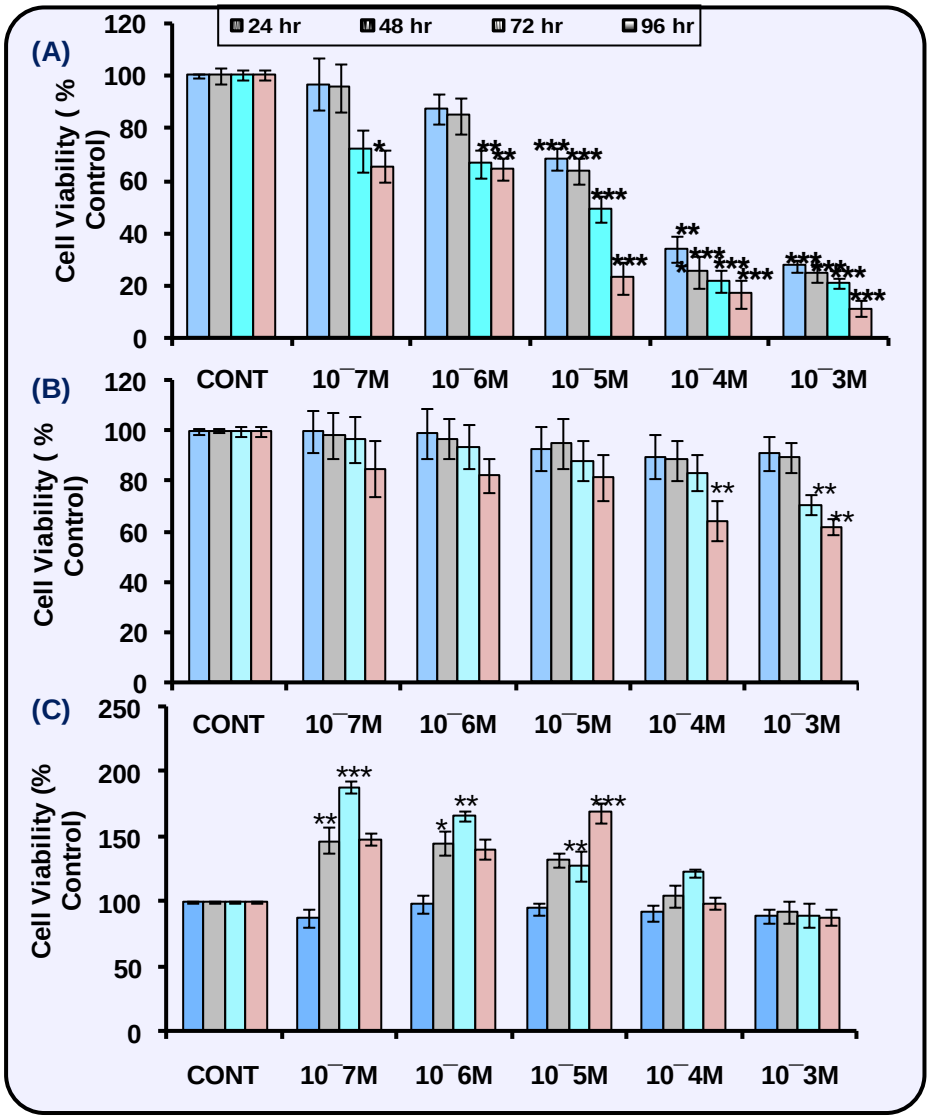


Figure – 4.58. Assessment of cell viability of cadmium, quercetin and nanoquercetin using differentiated pc12 cells

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); compared to control group.

4.6.2 Neuronal Differentiation of PC12 Cells and Cytotoxicity Studies

PC12 cells were seeded on the poly-L-Lysine coated flasks and cells were differentiated in medium containing NGF (100ng/ml) for 8 days. These cells were fully differentiated into neurons. (Figure – 4.58). Effect of cadmium, quercetin and nanoquercetin was examined at different concentrations (10^{-3} to 10^{-7}M) at various time points (24 - 96

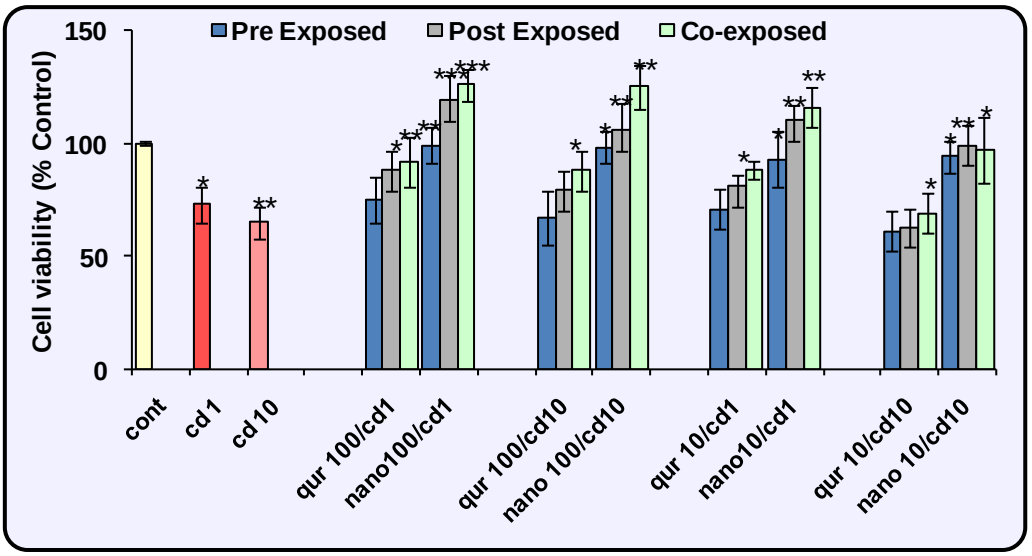


Figure – 4.59. Assessment of prophylactic, protective and therapeutic efficacy of quercetin and nanoquercetin

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); a-compared to control group, b-compared to cadmium exposed group; CONT – Control, Cd – Cadmium, QUR – Quercetin, NanoQ- Nanoquercetin

hr) on the viability of differentiated PC12 cells to assess the non cytotoxic dose of cadmium and biological safe dose of quercetin and nano-quercetin using MTT assay. The changes in the mitochondrial activity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The percent cell viability was found to be more increased in nano-quercetin exposed cells at various doses as of the bulk quercetin. Further, it is interesting to note that, the viability was more pronounced in nano-quercetin at 48 hr ($p < 0.01$). To assess the prophylactic, protective and therapeutic efficacy of bulk vs nano-quercetin.

The cell viability assay was again performed using mitochondrial MTT assay. The results suggests that nano-quercetin exposed cell was found to be more viable as to of bulk quercetin. Simultaneous exposure with quercetin and nano-quercetin at doses of 100 and 10 µM for 48 hr in cadmium exposed cells at dose of 1 and 10µM was found to more viable as pre and post exposure (Figure – 4.59).

4.6.3 Assessment of TH Activity

The exposure of cells to cadmium at dose of 10µM causes a significant decrease

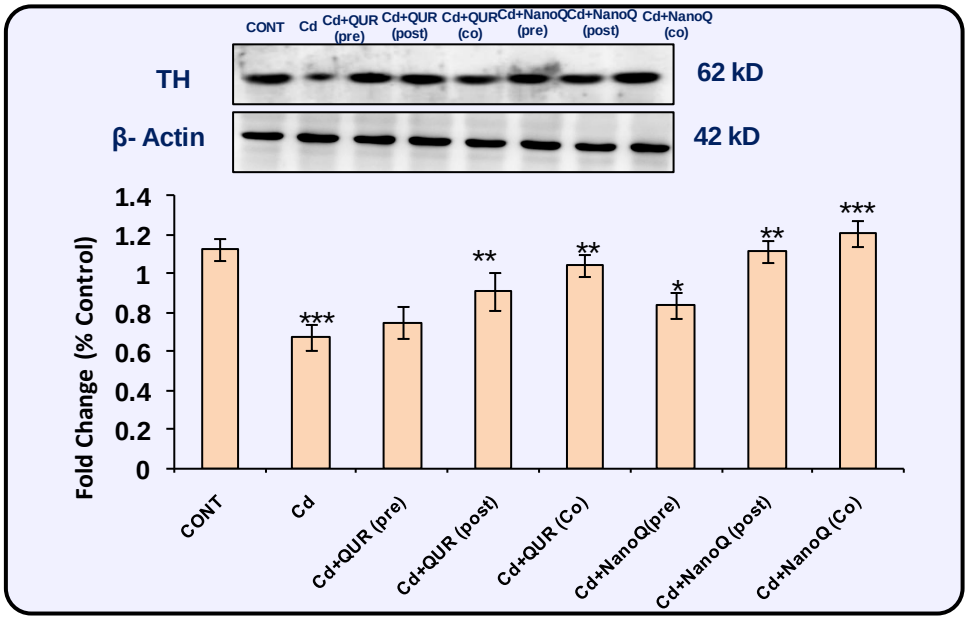


Figure – 4.60. Effect on the expression of TH protein

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); compared to controls

($p<0.01$) the protein expression of tyrosine hydroxylase (TH), a rate limiting enzyme in dopamine synthesis as to unexposed one. The pre, post and co-exposure of these cells to bulk and nano-quercetin were showed a significant protection in the TH activity as compared to cadmium exposed cells. However the intensity of changes was more in nano-quercetin as of bulk quercetin showing the slow and continuous release of quercetin from nanoparticles at longer duration and the changes were more marked in co-exposed group as of pre and post exposed cells (Figure – 4.60).

4.6.4 Assessment of DA-D2 Receptors

Cadmium exposure to differentiated PC12 cells showed the marked decrease in DA-D2 receptor as compared to controls. Further, the exposure of bulk and nano-quercetin for 48 hr exhibited a trend of recovery in such groups. Interestingly, the changes were more marked in co-exposed group both in bulk and nano-quercetin exposed as compared to pre and post treatment group (Figure – 4.61).

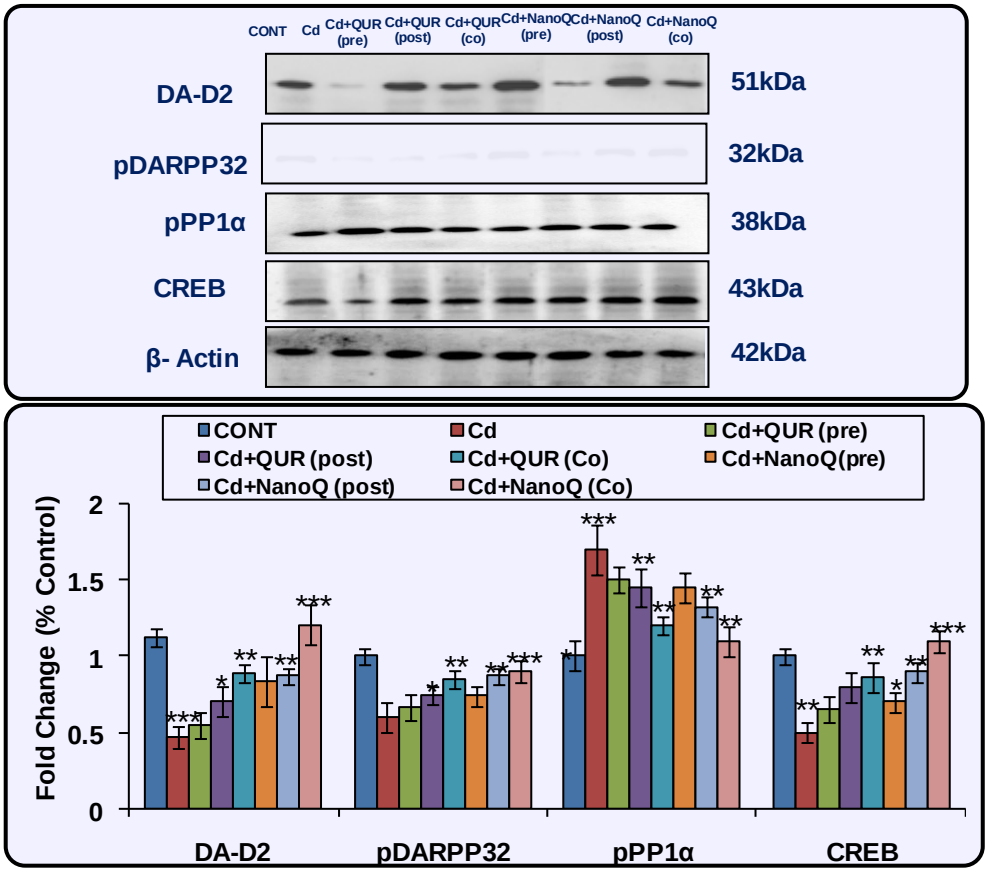


Figure – 4.61. Effect on the expression of Dopamine D2 receptors and related post synaptic signaling molecules

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); compared to controls

4.6.5 Assessment of DA-D2 Mediated Post Synaptic Signaling Proteins

The DA-D2 receptor mediated postsynaptic DARPP32/PP1α/ CREB signaling is considered to be the important mechanism in dopaminergic neurotransmission. The

exposure to cadmium to cells for 48 hr resulted to decrease the phosphorylation of DARPP32 at thr34 and CREB at ser31 and increase in the expression of PP1α as compared to controls.

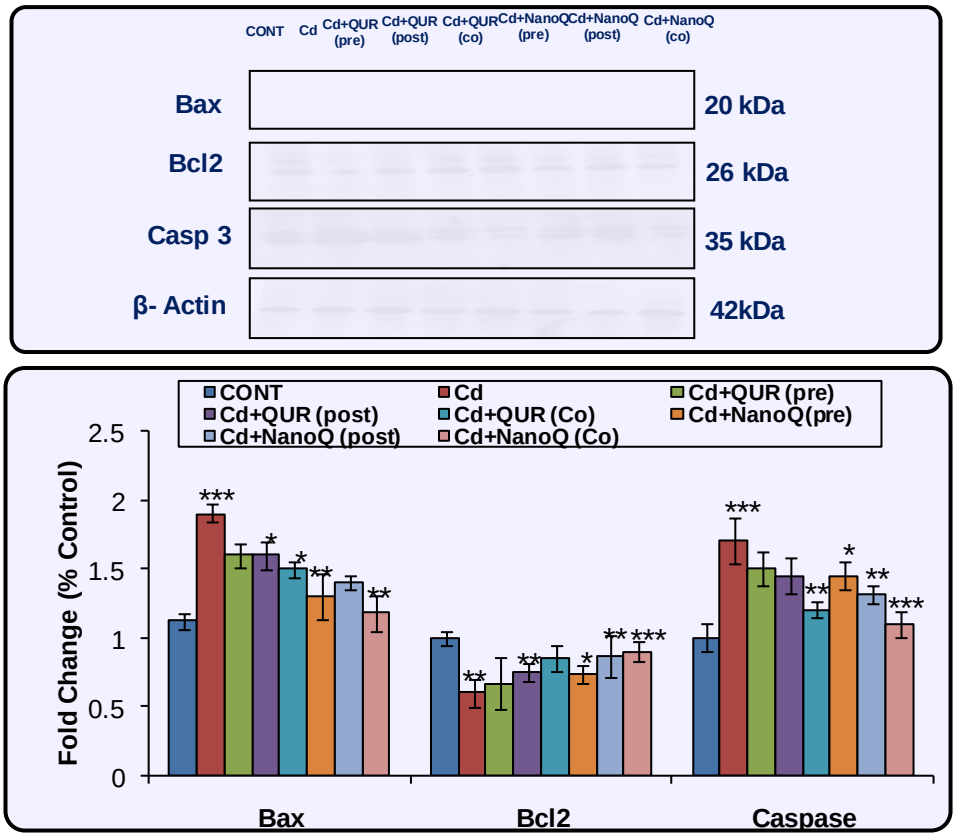


Figure – 4.62. Effect on the expression of apoptotic marker proteins

Values are mean ± SEM of three Individual experiments; Significantly differs (*p < 0.05, **p < 0.01,***p<0.001); *- compared to controls

While bulk and nano-quercetin at dose of 100µM significantly reversed such alteration as compared to cadmium exposed cells. Interesting to it, the protection was more marked in nano-quercetin exposed cells. However, pre exposure with bulk quercetin not showed the significant (p>0.05) protection in such proteins while co-exposed group with nano-quercetin exhibit the higher trend of recovery suggesting the effectiveness of nanoparticles as of bulk quercetin (Figure – 4.61).

4.6.6 Apoptotic Pathway Study

The apoptotic pathway is an important phenomenon and any dysregulation in such process may lead to cell death. The study was further carried out to assess the effectiveness of nano-quercetin over bulk quercetin on apoptotic markers. A significant increase in the expression of BAX and caspase-3 and subsequent decrease in the expression of Bcl2 was observed in cells exposed to cadmium as unexposed ones. Treatment with bulk and nano-quercetin in cadmium exposed cells showed the trend of recovery in such parameters. While the protection was more significant in co-exposed group (Figure – 4.62).

Chapter V

Discussion

In view of vulnerability of cholinergic neurons to cadmium, studies have been carried out to understand its impact on cholinergic receptors, AChE activity and other parameters associated with the integrity of cholinergic functions. Decrease in AChE activity in cerebral cortex and hippocampus, increase in hypothalamus and no change in striatum and cerebellum was observed on cadmium exposure (2.5 mg/kg body weight for 45 days) in rats (Abdalla et al., 2014). In another study, Abdalla et al. (Belyaeva et al., 2012) found increase in synaptic AChE activity in rats exposed to cadmium (2.5 mg/kg body weight) for 45 days. Further, increase in brain AChE activity was also found in rats exposed to cadmium (1 mg/kg body weight) either intraperitoneally or intramuscularly for 14 days or 4 months respectively (Carageorgiou et al., 2004; Carageorgiou et al., 2005). Cadmium induced decrease in AChE activity has been reported *in vitro* (Gkanti et al., 2014). While there are evidences exhibiting that cadmium exposure may cause alterations in brain AChE activity, consistent changes have not been observed. Variations in AChE activity on cadmium exposure have been largely attributed due to differences in

the dose, duration and route of exposure. Use of different animal species, cellular preparations, approaches either *in vivo* or *in vitro* and assay protocols could also be associated with variability in the results (Zarros et al., 2013). Of various factors suggested to be associated with the alteration of AChE activity, deactivation of the enzymatic site directly or occupation of active site of the enzyme by cadmium are quite convincing (Casalino et al., 1997). It has been found that cadmium may affect the cholinergic transmission by inhibiting the activity of ChAT, a marker for the integrity of cholinergic neurons (Dwivedi, 1983). (Del Pino et al., 2014) found a dose dependent decrease in ChAT activity in SN56 cells on cadmium exposure *in vitro*.

There are five distinct subtypes of muscarinic cholinergic receptors which are distributed differentially in brain regions and play important role in modulating pharmacological functions. Presence of M1, M2 and M4 cholinergic receptors has been found to be more in hippocampus and cortex and their role in modulating cognitive functions is well demonstrated in experimental and clinical studies (Abrams et al., 2006). Exposure to cadmium resulted to decrease muscarinic cholinergic receptors in rat brain both *in vivo* and *in vitro* (Hedlund et al., 1979). It was found that cadmium may inhibit the sensitivity of M1 receptors as pharmacological effect in response to acetylcholine and pirenzapine, a selective antagonist for M1 receptors was decreased. In an interesting study, del Pino et al. 2014 found that cadmium induced cell death in septal SN56 basal forebrain cholinergic neurons is mediated through inhibition of M1 receptors. Further, cadmium induced inhibition on M1 receptors was associated with over expression of GSK-3b and AChE-S and decreased expression of AChE-R (Del Pino et al., 2014). Effect on other muscarinic cholinergic receptor subtypes which could have given the complete picture of effect of cadmium was not studied by them. In the present study, significant decrease in mRNA expression of M1, M2 and M4 and no change in M3 and M5 cholinergic receptors was observed both in hippocampus and frontal cortex. Decrease in the binding of ^{3}H-QNB, known to label muscarinic cholinergic receptors in hippocampus and frontal cortex as observed in the present study is consistent with transcriptional changes associated with decreased mRNA expression of M1, M2 and M4 cholinergic receptors. It

is further interesting that transcriptional changes in ChAT and AChE in the present study are consistent with translational changes on cadmium exposure and thus exhibit vulnerability of cholinergic neurons. Decreased expression of AChE in frontal cortex and hippocampus on cadmium exposure in the present study indicate reduced activity and may have contributed to enhance acetylcholine levels resulting in the down regulation of cholinergic-muscarinic receptors. Further, reduction in the expression of ChAT both in frontal cortex and hippocampus on cadmium exposure may be due to autofeed back regulation as a result of increased acetylcholine levels.

Role of brain cholinergic receptors in modulating the learning and memory is well established (Levey, 1996). Loss of cholinergic neurons associated with selective loss of AChE and ChAT activities and alterations in muscarinic cholinergic receptors in hippocampus and frontal cortex has been found to impair learning and memory (O'Reilly and Rudy, 2001; Nyakas et al., 2011; Schliebs and Arendt, 2011). Both frontal cortex and hippocampus are cholinergic rich areas and their role in modulating learning and memory is well documented (Whishaw, 1989; Jarrard, 1993). Further, muscarinic-cholinergic receptors which are important in regulating memory circuits are highly distributed in both these brain areas (Levey, 1996; Van der Zee and Luiten, 1999). In view of this, the present study is focused to assess the protective potential of quercetin in cadmium induced cholinergic alterations in hippocampus and frontal cortex.

Several isoforms of PKCs are distributed in the brain and their role in modulating learning and memory is well accepted (Bank et al., 1988; Alkon et al., 2007). Based on the molecular cloning, 12 isoforms of PKC identified are broadly classified into three major classes – conventional (cPKCα,β, βII, and γ), novel (nPKC δ, μ, ε, θ, and η) and atypical (aPKCκ and λ). It has been found that activation of PKC modulates learning involving conventional PKC isoforms. PKCα, PKC β/βII are largely postsynaptic while PKCγ is also presynaptic (Abeliovich et al., 1993). Role of PKCβ1 is well established in modulating learning and memory (Paratcha et al., 2000; Weeber et al., 2000; Wu et al., 2007). Involvement of PKCβ1 in early synaptic events and in modulating the avoidance

learning has also been demonstrated (Paratcha et al., 2000). in an interesting study, decrease in PKCβ resulted to impair learning in mice (Weeber et al., 2000). Moreover, decrease in brain muscarinic cholinergic receptors and expression of PKCβ1 on exposure of rats to arsenic or those subjected to stress and exposed to lambda-cyhalothrin has been correlated with decreased learning and memory (Wu et al., 2007; Chandravanshi et al., 2014; Shukla et al., 2015). Impairment in the spatial memory and learning as assessed by Y-maze and passive avoidance task in cadmium exposed rats in the present study may be linked with decrease in brain cholinergic muscarinic receptors and expression of PKC β-1 and increased oxidative stress.

Like other heavy metals, cadmium may affect the integrity of mitochondria as observed by decrease in the mitochondrial membrane potential and ATP levels. While studying the effect on mitochondrial functions, Belyaeva et al. 2012 found that cadmium inhibited the FCCP-uncoupled respiration. Further, cadmium resulted to cause mitochondrial membrane permeabilization associated with alterations in the activity of complex I and III which are involved in modulating the ETC learning and (Belyaeva et al., 2012). Alterations in mitochondrial bioenergetics, important for energy production through oxidative phosphorylation may enhance ROS generation, suggested to be one of the potential mechanisms in cadmium neurotoxicity. It has been found that cadmium induced ROS generation may enhance lipid peroxidation in cortical neurons (Lopez et al., 2006). Alterations in the activity of anti-oxidant enzymes which could counteract the generation of free radical species on cadmium exposure has also been reported and associated with enhanced oxidative stress (Kumar et al., 1996). Thus, increased ROS generation associated with impairment in the activity of complex I, II-III and IV and decreased mitochondrial membrane potential on cadmium exposure in frontal cortex and hippocampus in the present study are consistent with these reports and suggest that cadmium may affect the mitochondrial integrity and associated functions.

Cadmium being prooxidant in nature has been found to enhance expression of Bax, a proapoptotic protein and Bcl2, an antiapoptotic protein. Activation of caspase-3, an

executor protein and a crucial mediator in apoptosis has also been observed either independently by activating the death receptor pathway or releasing cytochrome C. It has been found that ERKs, JNKs and p38 are important constituents of MAPkinases and play important role in modulating cellular processes including differentiation, survival and apoptosis (Chen et al., 2008). Of three isoforms of JNKs identified, JNK3 is specific to neuronal cells while JNK1/2 are distributed in other tissues (Jing and Anning, 2005). JNKs are stress activated proteins and are known to trigger the apoptotic signals by modulating the expression of mitochondrial pro and antiapoptotic proteins either by phosphorylation or indirectly modulating the proapoptotic proteins by transactivation of transcription factor c-jun on N-terminal Ser-63 and Ser-73. Further, JNK dependent mitochondrial apoptosis requires activation of JNK at phosphorylation sites at Thr-183 and Tyr-185 and involved in neuronal death. While investigating the mechanism of cadmium induced oxidative stress in pancreatic beta cells, cadmium was found to target mitochondrial dependent apoptotic pathway (Chang et al., 2013). Enhanced release of cyto C associated with decreased expression of Bcl-2 resulted to activate caspases and affect the mitochondrial functions. Further, enhanced oxidative stress was found to affect the downstream JNK signaling and contributed in apoptosis. Cadmium induced neuronal death has also been observed via targeting JNK and PTEN- Akt/mTOR network (Chen et al., 2014). In the present study, activation of pJNK and pp38 in hippocampus and frontal cortex on cadmium exposure appears to contribute in the activation of transcription factor AP1. Interestingly, activation of JNK may cause Bax translocation associated with the release of cyto C and contribute in neuronal apoptosis on cadmium exposure as evident in the present study.

Ultrastructural and morphological changes in brain have been frequently reported on exposure to environmental chemicals (Liu et al., 2014). Blebbing of mitochondria and damaged mitochondrial cristae as observed on cadmium exposure may be responsible for mitochondrial dysfunctions. Loss of synapses both in hippocampus and frontal cortex further indicates that ultrastructural integrity could be affected on cadmium exposure. Further, abnormalities in mitochondria in frontal cortex and hippocampus, the cholinergic

rich areas in the brain exhibit their vulnerability on cadmium exposure. Consistent with this, decreased Nissl staining in hippocampus and frontal cortex provide a histological evidence of damage of neurons in these cholinergic rich areas of brain on cadmium exposure.

Quercetin is preferred over other flavonoid in view of its broad pharmacological spectrum due to strong radical scavenging potential, high anti-oxidant activity associated with anti-inflammatory and anti-chelating effects (Morikawa et al., 2003). Protective role of quercetin in cardiovascular and metabolic disorders has been reported by a number of investigators and associated with decreased oxidative stress (Rivera et al., 2008). In view of the fact that quercetin crosses the BBB, a number of studies have been carried out to assess its protective efficacy in experimental models of neurological disorders and chemical induced neurotoxicity. Interestingly, quercetin was found to attenuate the neurotoxic effects of rotenone and 6-OHDA in hemi-parkinsonian rats by inhibiting oxidative stress (Haleagrahara et al., 2011). Quercetin was found to reduce tauopathy and beta-amyloidosis and improve learning and memory in triple transgenic mouse model of Alzheimer's disease due to decrease in oxidative stress (Ansari et al., 2009). Brain dopaminergic dysfunctions due to PCB induced oxidative stress were also found to be attenuated by quercetin in rats (Bavithra et al., 2012). Protective role of quercetin in aluminum-induced cholinergic deficits was found to be associated with decreased oxidative damage (Sharma et al., 2013). Unsal et al. (Unsal et al., 2013) also found that quercetin may protect cadmium induced neuronal damage by inhibiting the oxidative stress and apoptosis. The protective effect of quercetin was associated with scavenging of hydroxyl radicals associated with increase in the capacity of anti-oxidant enzymes. Alteration in the activity of brain AChE on cadmium exposure was found to be protected by quercetin earlier (Abdalla et al., 2013). Although effect on other important targets of cholinergic system was not studied, the pharmacological restoration in AChE activity by quercetin was attributed to its antioxidant potential.

As role of mitochondrial dysfunctions in the etiology of neurodegenerative diseases is well demonstrated, a number of studies have been carried out to assess effect of quercetin on mitochondrial bioenergetics. Quercetin has been found to improve mitochondrial dysfunctions in rat model of Huntington's disease induced by nitropropionic acid as evident by reversal in the activity of mitochondrial complexes associated with decreased oxidative stress (Sandhir and Mehrotra, 2013). Chakraborty et al. (Chakraborty et al., 2014) further found that quercetin could protect from inflammatory changes in rat model of Huntington's disease as evident by decreased microglia proliferation and increased astrocytic numbers although striatal lesion induced by nitropropionic acid was persistent. Quercetin at low dose was found to enhance mitochondrial biogenesis both in brain and muscles associated with increased maximal endurance and physical running activity in mice. Based on this, it was suggested that use of quercetin may be explored in neurodegenerative and metabolic disorders involving mitochondrial dysfunctions (Davis et al., 2009). Interestingly, quercetin was found to up regulate complex I activity in mitochondria in rotenone induced hemi-parkinsonian rats (Karuppagounder et al., 2013). In the present study, cadmium induced alterations in brain cholinergic receptors and expression of ChAT and AChE were protected on simultaneous treatment with quercetin. The protective effect on brain cholinergic targets appear to be associated with antioxidant potential of quercetin which could reduce the ROS generation and protect the integrity of mitochondria as evident by increase in the activity of mitochondrial complex I, II-III and IV and mitochondrial membrane potential. Further, preserving the integrity of mitochondria may have reduced oxidative stress. The protective changes of quercetin may further be attributed to its chelating effect resulting to decrease cadmium levels both in hippocampus and frontal cortex as observed by us in an ongoing study.

Quercetin has been found to modulate mitochondrial dependent apoptotic pathway. While investigating the protective effect of quercetin in focal cerebral ischemia in rats, Yao et al. (Yao et al., 2012) found increased expression of Bcl-2 and decreased expression of Bax and cleaved caspase-3 proteins in cortex suggesting decreased apoptosis. It was found that quercetin enhanced levels of BDNF and Trk-β which could

stimulate the PI3K/AKT neuronal survival pathway. Quercetin was also found to protect OPCs from oxygen glucose deprivation induced apoptosis *in vitro*. Enhanced expression of Bax and caspase-3 and decreased expression of Bcl-2 due to oxygen glucose deprivation was found to activate the PI3K/AKT signaling pathway (Wang et al., 2011). Programmed cell death in hemi-parkinsonian rats was also found to be protected by quercetin. Simultaneous treatment with quercetin resulted to decrease the expression of Bax and enhance the expression of Bcl-2 and caspase-3 which were found to be altered on cadmium exposure both in hippocampus and frontal cortex. Further, increase in the expression of proteins associated in MAPkinase signaling specially JNKs, pp38 and AP1 in hippocampus and frontal cortex of rats on cadmium exposure was found to be reduced in rats simultaneously treated with quercetin suggesting that quercetin may decrease apoptosis. Interestingly, quercetin has been found to exhibit antiapoptotic properties and modulate MAPK Signaling and PI3/Akt pathway in a number of studies on chemical induced neurotoxicity and experimental models of neurodegenerative diseases (Wang et al., 2011; Yao et al., 2012; Sandhir and Mehrotra, 2013). It is possible that in normal situations, quercetin maintains the cellular homeostasis because of its antioxidant properties and thus may not interfere with the cell signaling pathways. However, in case of neurotoxicity or in neurodegenerative state, it modulates pathways and targets including those associated with MAPKinases, cell survival and apoptosis. This could possibly be the reason that treatment with quercetin alone in rats had no effect on the levels of cytochrome c and expression of MAPKinases both in hippocampus and frontal cortex in the present study.

It is further interesting to note that blebbing in mitochondria and disrupted cristae in hippocampus and frontal cortex of rats on cadmium exposure was found to be protected by quercetin. Also loss of synapses as evident on cadmium exposure both in hippocampus and frontal cortex was found to be reduced suggesting that quercetin could protect the ultrastructural integrity of mitochondria.

While role of brain cholinergic system in regulating the learning and memory is well accepted, quercetin has been found to protect spatial memory by increasing the antioxidant capacity (Sun et al., 2007). Memory impairment by scopolamine was also found to be prevented by quercetin (Richetti et al., 2011). Decreased expression of PKC β-1 on cadmium exposure was also found to be protected by quercetin.

To our knowledge, this is the first report that demonstrates evince role of DA-D2 receptor mediated postsynaptic signaling in regulating cadmium induced motor dysfunctions. Further, the mechanism associated with protective potential of quercetin, a polyphenolic flavonoid in cadmium induced dopaminergic deficits and motor impairment has also been unraveled. Involvement of DA-D1 and DA-D2 type receptors in dopaminergic neurotransmission and in the integration of motor behavior and other pharmacological functions is largely accepted(Beaulieu and Gainetdinov, 2011). In the present study, exposure to cadmium decreased mRNA and protein expression of DA-D2 receptors in corpus striatum while there was no change both in mRNA and protein expression of DA-D1 receptors. Further, decrease in the expression of DA-D2 receptors and no effect on DA-D1 receptors was also evident on cadmium exposure in PC12 cells in vitro. Interestingly, *in silico* studies carried out by us have complemented the findings exhibiting selective effect of cadmium on DA-D2 receptors. Consistent with transcriptional and translational changes, decrease in the binding of ^{3}H-spiperone in the present study further confirms that cadmium affects the sensitivity of DA-D2 receptors in corpus striatum. While brain dopamine receptors are easy target of environmental chemicals. Selected changes either in DA-D1 and DA-D2 receptors have also been reported. Chronic exposure to arsenic at high dose (5 mg/L) in mice was found to decrease mRNA expression of DA-D2 receptors while there was no change in DA-D1 receptors in striatum (Moreno Ávila et al., 2016).

Of several factors associated with decreased binding of dopamine receptors, availability of dopamine at the synapse is important regulator. Decrease in dopamine levels in corpus striatum of developing rats prenatally exposed to cadmium has been reported (Antonio et

al., 1999). Romero et al 2011 also found decreased dopamine levels in hypothalamus of cadmium exposed rats while studying protective effect of melatonin. In ongoing studies by us to assess the protective effect of quercetin in cadmium induced alterations in brain biogenic amines, we also found that exposure to cadmium results in decreased dopamine levels in corpus striatum. Further, *in silico* study provides interesting evidence that decrease in DA-D2 receptors could be due to direct binding of cadmium at the competitive or non-competitive sites of dopamine on DA-D2 receptors.

Integrity of pre and postsynaptic signaling is important for normal dopaminergic neurotransmission. Role of tyrosine hydroxylase, a rate limiting enzyme in dopamine synthesis and marker of dopaminergic neurons is well accepted (Shahnawaz Khan et al., 2012). Decrease in TH expression in corpus striatum has been associated with reduced dopamine levels on exposure to a number of chemicals and drugs. Effect on DAT has been associated with decreased dopamine levels in clinical cases of Parkinson's disease (Nutt et al., 2004). Decrease in the expression of TH and DAT in corpus striatum on cadmium exposure in the present study may be correlated with reduced dopamine levels. Another important paradigm in dopaminergic signaling is VMAT2, a specific protein present in presynaptic terminals that transports cytoplasmic monoamine neurotransmitters including dopamine into synaptic vesicles. Although cadmium affected the expression of VMAT2, changes were not significant which suggests that transport of dopamine into vesicles may not be affected. It is further interesting that similar changes were observed in vitro on exposure of PC12 cells to cadmium.

Exposure to cadmium in the present study resulted to decrease expression of PKA and pDARPP-32 at (Thr 34) with increased phosphorylation of PP1α which led to decrease the expression of CREB and affect motor activity and motor co-ordination in rats. As there was no change in the expression of DA-D1 receptors in corpus striatum on cadmium exposure, the PKA/DARPP32/PP1α pathway appears to be modulated by DA-D2 receptors both in vivo and in vitro. During the course of signaling, it may affect the nuclear translocation of CREB, a transcription factor also known to be regulated by PKA.

Inhibition in the expression of DARPP-32 was associated with decrease in DA-D1 receptors in corpus striatum of developing rats exposed to manganese 32. Recently, decrease in DA-D2 receptors on arsenic exposure in adult rats resulted to inhibit expression of PKA and DARPP-32 and increase the expression of PP1α although there was no change in the expression of DA-D1 receptors. Role of DARPP-32 and CREB in modulating motor activity and motor co-ordination is well reported (Barone et al., 1994). Decreased motor activity in mice exposed to cannabinoids has been associated with impairment in PKA dependent phosphorylation of DARPP-32 (Thr34).

Besides canonical signaling, multiple evidences exhibit involvement of β-arrestin-2/Akt/GSK-3β pathway in the regulation of dopamine dependent behavior (Beaulieu et al., 2004; Beaulieu et al., 2005). It has been found that both cAMP dependent PKA/DARPP32/PP-1α and cAMP independent β-arresetin/AKt/GSK-3β signaling could take place simultaneously in dopaminergic neurons and contribute equally in regulating motor functions. Pharmacological activation of Akt or inhibition of GSK-3β results in reduction of dopamine associated locomotor function in DAT-KO mice and wt mice treated with amphetamine (Gould and Manji, 2005). Further inhibition of GSK-3β inhibitors can reduce locomotor hyperactivity. Conversely, transgenic GSK-3β mutant mice showed locomotor hyperactivity phenotype that is reminiscent of DAT-knockout mice. Akt1- knockout mice support the involvement of Akt inhibition in DA-D2 receptor mediated behavioral response(Beaulieu et al., 2007). In the present study, increase phosphorylation of Akt (ser 473) and decrease GSK3β (ser 9) in corpus striatum exhibit involvement of AKt/GSK-3β pathway in cadmium induced motor dysfunctions. It further suggests that decrease in DA-D2 receptors not only affects the phosphorylation of PKA but may also affect phosphorylation of Akt and GSK-3β in cadmium exposed rats. Further, pharmacological studies involving specific inhibitors of PKA and Akt in vitro revealed that pretreatment of PC12 cells with PKA inhibitor affected the expression of DARPP32 and PP1α on cadmium exposure while there was no change in the expression of Akt and GSk-3β. Inhibition of Akt pathway in PC12 cells affected the phosphorylation of GSk-3β but not of DARPP32 and PP1α. It clearly indicates that both pathways are

independent as inhibition of one pathway did not affect the integrity of other. These findings are interesting and suggest that besides canonical signaling mediated by cAMP dependent PKA/DARPP32/PP1α pathway, cadmium may also affect the Akt/GSk3β signaling pathway and affect motor functions as evident by decrease in distance traveled due to alterations in DA-D2 receptors in corpus striatum. Ultrastructural changes in the present study exhibit synaptic loss in the neuropil region of corpus striatum and thus indicate that cadmium affects the neuronal integrity. Apart from the loss of myelin sheath and vacuolization, mitochondrial swelling associated with damaged cristae indicate loss of permeability and cellular architecture. Significant decrease in Nissl's positive neurons in corpus striatum further suggests that cadmium may cause neuronal degeneration.

In view of ameliorative effect of quercetin in experimental models of Parkinson's and Huntington's disease (Sandhir and Mehrotra, 2013) its protective potential was investigated in cadmium induced dopaminergic dysfunctions. Combined treatment with quercetin and fish oil was found to protect depletion of dopamine levels in striatum of rats chronically exposed to rotenone(Sandhir and Mehrotra, 2013; Joseph, 2015). El-Horany et al. (2016) found that attenuation of rotenone induced neurotoxicity in rat model of Parkinson's disease on quercetin treatment was due to augmentation of autophagy associated with decreased oxidative stress and apoptosis. Neuronal death associated with enhanced astrogliosis and motor deficits in rats treated with 3-nitropropionic acid were also restored on supplementation with quercetin(Sandhir and Mehrotra, 2013). Being lipophilic due to multiple methylation of hydroxyl groups, quercetin may penetrate into the brain and modulate pharmacological functions. The protective changes of quercetin were attributed to its antioxidant potential and capability to preserve mitochondrial integrity in rotenone treated rats (El-Horany et al., 2016). In the present study, simultaneous treatment with quercetin resulted to protect cadmium induced decrease in DA-D2 receptors both in rat corpus striatum and PC12 cells. Further, cadmium induced alteration in the expression of TH, DAT and DA-D2 receptor mediated PKA signaling in corpus striatum was also protected by simultaneous exposure with quercetin both in vivo and in vitro. The reason behind such modulation by quercetin may

be due to its potent antioxidant and metal chelating properties. More interestingly, DFT studies carried out by us suggest that quercetin may have the tendency to form complex with cadmium. It could therefore be attributed to the metal chelating property of quercetin and may have reduced free cadmium on simultaneous treatment.

Role of neurotransmitters in the etiology of neurological and psychiatric disorders is well documented (Coyle et al., 1983; Choi, 1988; Sulzer et al., 2005). Involvement of dopamine in Parkinson's disease and schizophrenia and serotonin in depression and anxiety has been quite convincing and characterized (Joca et al., 2007).With the demonstration that acetylcholine is associated in cognitive deficits, a number of studies have converged attention to understand the etiology of synaptic transmission. While these neurotransmitters are distributed in different brain regions, selective loss or increase in any of the brain region may affect the associated functions. Further, multiple neurotransmitters in selected or different brain regions could also be affected with the progression and severity of diseases (Hritcu et al., 2007; Frederick and Stanwood, 2009). Changes in the levels of biogenic amines however are not consistent and have been found to vary with age, duration and timing of exposure. Further, the dose of exposure to chemical may also be associated with variability in the levels. As biogenic amines are considered to be primary messengers, any fluctuation in their levels may disrupt the process of synaptic transmission (Frederick and Stanwood, 2009). Moreover, alteration in the levels of neurotransmitters may also affect further signaling through ionotropic or metabotropic receptors (Davis and Kahn, 1991)and thus affect the integrity of synaptic transmission.

Disturbance in the levels of biogenic amines and their metabolites has also been reported in chemical induced neurotoxicity. A number of studies have found that exposure to lead, arsenic and manganese may affect the levels of biogenic amines and their metabolites in brain and their regions (Cory-Slechta, 1995; Tripathi et al., 1997; Minami et al., 2001). As these neurotransmitters also modulate behavioral functions, there has been a lot of interest to understand the effect of chemical exposure on the integrity of

neurotransmitters in brain regions. Alteration in the levels of neurotransmitters has been observed in developing rats on prenatal exposure to cadmium exposure (Antonio et al., 1998; Antonio et al., 1999). In the present study, exposure to cadmium resulted to alter the levels of dopamine and its metabolites – HVA and DOPAC in corpus striatum, frontal cortex and hippocampus. Further, dopamine turn over was also found to be affected on exposure to cadmium. Alteration in the levels of norepinephrine and serotonin in corpus striatum, frontal cortex and hippocampus was also evident while no significant effect on the levels of epinephrine was observed.

While antioxidant and metal chelating properties of quercetin are well accepted, a number of studies have found that quercetin may modulate brain neurotransmitters in neurodegenerative diseases (Chakraborty et al., 2014). Interestingly, simultaneous treatment with quercetin has also been found to protect cadmium induced alterations in neurotransmitter levels in frontal cortex, hippocampus and corpus striatum.

The toxicity of cadmium in body system is very high as it accumulates in body organ like liver, kidney and lung and leading to damage the organs and tissues. Studies suggest that cadmium easily crosses the blood brain barrier and thus accumulates in brain. Because of high bioavailability and long half life of cadmium in human body, results are cumulative. As brain is considered to be the soft target, so the accumulation of cadmium in brain is highly responsible for its neurotoxic effect. In the present study we found high level of cadmium in various brain regions including the frontal cortex, hippocampus and corpus striatum. The accumulation of cadmium in brain regions varies with the dose, time and route of exposure. High accumulation of cadmium in various brain regions may be responsible for the altered neurotransmitter levels in the present study. The results are consistent with the previous studies carried out showing accumulation of cadmium in brain Further, metallothionein a cysteine containing protein, shows the protection against cadmium induced toxicity. Presence of cadmium in the body induces the synthesis of metallothionein in liver, forming cdmt complex which reduces the toxicity of cadmium. Studies carried out previously shows the MTnull mice show the high toxicity

towards cadmium while control mice show less to cadmium. With the various isofroms of MT, MTIII is present specifically in brain both in neurons and glias and thought to play a role in Zinc homeostasis in neurons. Montoliu et al., 2000 suggests the protection of cerebellar neurons form glutamate toxicity and nitric oxide toxicity by MTIII. In the present study the increase expression of MTIII in brain frontal cortex, hippocampus and corpus striatum is correlated with the cadmium neurotoxicity. Increase expression of MTIII served as a marker of enhances cadmium toxicity. Studies carried out demonstrate the MTIII as a potential target to reduce the toxicity of heavy metals especially cadmium.

Multiple properties of quercetin as antioxidant, anti inflammatory and metal chelating is well characterized. A number of studies suggests the neuroprotective potential of quercetin against various traumatic brain injury stroke models as well as in against various neurodegenerative disorders like Alzheimer's and Parkinson's. Further, quercetin is found to be effective against various chemical induced neurotoxicity as well as against various animal disease models like rotenone, 3NP etc. The antioxidant and free radical scavenging properties may be responsible for its neuroprotective potential. The simultaneous treatment with quercetin found to protect altered neurotransmitter levels in cadmium exposed rats. The increase in the level of biogenic amines and their metabolites as dopamine, DOPAC and HVA was found with quercetin exposure in rats exposed to cadmium. Further, ameliorative effect of quercetin was also found as demonstrated by increase in serotonin and norepinephrine in the present study. Joseph et al., 2015 found the increase in straital dopamine content following exposure to fish oil and quercetin in chronic rotenone rat model. Increase in the straital dopamine and antioxidant enzyme levels was also found in 6-hydroxydopamine rat model following quercetin exposure preclinical models of Parkinson's disease by modulating the dopamine content in striatum region but the status of their metabolites and other neurotransmitter levels was not assessed properly. The present study gives a clear glimpse of protective potential quercetin in alerted neurotransmitter levels and related neurotransmission.

Another important data reported in the study that quercetin in found to be effective in decreasing the cadmium level in various brain regions in the present study. We found that

simultaneous exposure of cadmium and quercetin reduces the level of cadmium in brain frontal cortex, hippocampus and corpus striatum as compared to cadmium exposed rats alone. Indeed data indicate that the possible mechanism behind may be metal chelating properties of quercetin forming the complex with cadmium, which reduces the cadmium toxicity. Further, studies are needed to completely understand the mechanism.

The involvement of inflammatory regulators in cadmium mediated dopaminergic neuronal death. The finding of the present study supports and established that (i) cadmium exposure in rats activates the brain glial cells leading to increase in the level proinflammatory cytokines (TNFα, IL-1β and IL-6) and decrease in anti-inflammatory cytokines levels (ii) the excessive release of proinflammatory cytokines leads to the activation of other signaling molecules like STAT3, COX2, ERKs and iNOS/NO system leading to activation of iNOS/NO/CamKIIα pathway which may be associated with neurodegeneration in rats. (iii) because of increase in ROS and RNS, the mitochondrial biogenesis was altered which lead to activation of intrinsic mitochondrial apoptotic pathway &MAPKs leading to neuronal death (v) as well as these changes was protected with the simultaneous treatment with quercetin, a polyphenolic flavonoid. The finding of the present study concurrently explains the intricate involvement of neuroinflammation in cadmium mediated dopaminergic neuronal death. In our knowledge, this is the first study illustrating the area of Neuroinflammation in cadmium mediated neuronal death in rat models.

The extensive review literature enlightens the importance of neuroinflammation in the development of neurodegenerative diseases (Frank-Cannon et al., 2009; Chen et al., 2016). Neuroinflammation plays a role in the cascade of events leading to nerve cell death, thus propagating the neurodegenerative process. Although it has been shown that various chemicals, environmental pollutants and brain toxic insults activates the inflammatory process, however it is not clear how they modulates the pathology and neurodegeneration. Although number of the reports shows that cadmium exerts the neurotoxicity by increasing the oxidative stress, by modulating the mitochondrial

integrity and MAPkinase signaling still the molecular mechanism of cadmium induced inflammation has not been established. In the present study we demonstrated the mechanism and pathway involved in cadmium mediated neurotoxicity targeting via inflammatory molecule in rat models. Further, the protective potential of quercetin in modulating such changes has also been established.

Since over last years, number of studies published the indicated that inflammatory and cytokine mediated neurotoxicity are the major contributing factor in motor dysfunctions and Parkinson like symptoms (Mosley et al., 2006; Hirsch and Hunot, 2009). Further, it has also been suggested that dopaminergic neuronal death in Parkinson's through apoptotic cell death is also governs by neuroinflammation. Additionally, inflammation and activated microglia have been generally implicated in Parkinson's pathology (Streit et al., 2004). In continuation to earlier studies carried out by us, the present study has been further carried out to assess the impact of neuroinflammatory pathway in cadmium mediated dopaminergic neuronal death through the involvement of mitochondria and associated signaling.

Neuroinflammation is generally considered a compensatory mechanism in toxic insults in brain by upregulating of proinflammatory mediators and by downregulating the anti-inflammatory cytokines. Glial cells are the principal cells that are initial mediator against inflammatory response. The activation of native glials cells into active microglia is the essential feature of neuroinflammation which lead to release of array of cytokines, as a immune responser. In controlled and regulated manner inflammatory process has clear beneficial effects only (Finnie et al., 2013). However, under toxic insults conditions it can contribute to neurodegeneration and neurological disorders (Lucin and Wyss-Coray, 2009). Increase in the expression of GFAP and IBA1 in the present indicates activation glial cells (astrocytes and microglia respectively) in rats exposed to cadmium which indicate the native feature of neuroinflammation in the present study. Further, the increased levels of pro-inflammatory cytokines such as TNF, IL-1β and IL-6 and decrease level of anti inflammatory cytokines IL-10 has been found following cadmium

exposure in the present study suggesting that potential involvement of inflammatory cytokines in cadmium mediated toxicity. Compiling evidences also provides an evidence of altered levels of cytokines in worsen the dopaminergic neurons both *in vitro* and *in vivo* . *I*ncreased levels of proinflammatory cytokines has also been observed in the cerebral spinal fluid and striatum of Parkinson's patients when compared with healthy age-matched controls (Nagatsu et al., 2000). The Dysregulation in cytokines is a central feature in the development of neuroinflammation which lead to the neurodegeneration. The excess amount of proinflammatory cytokines due to cadmium exposure lead to activation of various signaling cascade either via activation of death related signaling and/or by activating the cytokines receptors present in the neurons leading to neuronal death. The increase release of cytokines gives and important lead in cadmium mediated mechanism of neurotoxicity via involving the inflammatory process. It has widely been accepted that cytokines mediates the signaling via cytokines receptors leading to activation of the secondary messengers and other signaling cascade (Ihle, 1995). Cytokines are majorly act through the modulation of JAK- STAT pathways. This is the prorogated signaling cascade which is takes place by series of phosphorylation and dephosphroylations sequences. Interesting to this, the increased phosphorylation of STAT3 in cadmium exposed rats was observed in the present study may further lead to activation of various signal cascade.

Numerous studies have shown proinflammatory cytokines primarily activates the iNOS/No systems both in neurons and in glial cells (Kim et al., 2008). These inflammatory cytokines in turns activates various signaling cascades in neurons and in glial cells itself, which are directly or indirectly responsible for neuronal death. We found the increase levels of NO and increased expression of iNOS and nNOS in various brain regions suggesting that inflammatory cytokines may impairs the iNOS/ NO system following cadmium exposure. The excessive amount of may account to the activation of various signaling in neurons leading to the neuronal death. The excessive amount of NO causes the nitosylation of proteins like tyrosine hydroxylase, and alters their function in dopaminergic neurons. Of relevance to the potential role of iNOS in the pathogenesis of

Parkinson's is the demonstration that the density of glial cells expressing iNOS is markedly increased in the Parkinson's patients compared with control subject. Direct genetic evidence has now demonstrated that iNOS is essential to dopaminergic cell demise in MPTP-intoxicated mice. The deleterious activity of NO on dopaminergic neurons was further demonstrated in the present study where it targets the various cascades. Reactive oxygen species and reactive nitrogen species play pivotal roles in neuronal damage. ROS and/or RNS are, at least in part, produced by cerebral parenchymal cells, including neurons and glial cells, as well as peripheral inflammatory cells such as leukocytes.

As mitochondria is considered as most susceptible target of environmental chemicals, studies carried out in recent years suggest that mitochondria is the prime target of cadmium. Multiphased studies carried out in recent years suggest the involvement of mitochondria in various neurodegenerative diseases. In the present study, mitochondrial integrity was found to be affected due to cadmium exposure. A extremely disturb mitochondrial biogenesis as assessed through various complexes involved shows that release of cytokines and secondary messenger like NO, COX2 may be a causative factor in affecting the mitochondrial bioenergetics in the present study. Further generation of ROS are also a considering factor in mitochondrial integrity. A disturbance in mitochondrial complexes led the disturb the mitochondrial architecture leading to disturb the mitochondrial membrane potential leading to release of Cyto C and activation of caspase cascade. Cadmium induced changes in the membrane potential may explain the fact. These changes can further be correlated with increased cytokines levels in the brain regions. Number of investigator also showed that mitochondria is the major target in cadmium induced neuronal death. Cadmium being prooxidant in nature may lead to the translocation of cytosolic Bax into mitochondria affect the Bax/Bcl2 complex leading to activation of caspase cascade. Overwhelming evidence has indicates that the caspases families are the crucial mediator of apoptosis among them caspase-3 an important key player in cell death either by releasing Cyto C or independently by activating death receptor pathway. The increase in the Bax and decrease in the Bcl2 was observed in rats

exposed to cadmium. Further, activation of caspase3 has also been reported in the present study. These may regulated by inflammatory targets especially by cytokines. However, a wealth of studies over the past decade established that TNF has causes the rapid decreases in mitochondrial membrane potential and coincident increases in reactive oxygen species. The ultrastructral carried out also exhibit the serve degeneration of dopaminergic neurons in the corpus striatum area where disturb archicture in corpus striatum, neuronal loss and disturb mitochondrial integrity was also evident in electron micrographs observed in TEM. Together these findings confirm that cadmium exposure in rats affect dopaminergic modalities and related functional changes and quercetin have ability to protect such changes.

Various experimental and clinical studies suggest that autophagy plays an important role in neurodegenerative disorders (Hara et al., 2006; Nixon, 2006, 2013). It has been suggested that autophagy works in a coordinated manner with apoptosis to maintain cellular homeostasis and cell survival (Shen et al., 2011). However, in stress and nutritional deprivation conditions the cell activates the protective autophagy to remove the debris and aggregates from the cell with the help of lysosomes. Thus, autophagy represents a compensatory, neuroprotective mechanism to clear toxic accumulation of misfolded proteins, aggregates, other waste and toxic material from the cells and thus plays a protective role in the cells. Role of autophagy in many neurological and neurodegenerative diseases is well accepted. Because the brain consumes the large amount of oxygen and glucose thus generating the huge waste products and toxic materials. During the exposure to toxic chemicals, the process of autophagy gets activated as to protect the cell from the damage.

A number of studies have been carried out recently showed the activation of autophagy in chemical induced neurotoxicity (Kanzawa et al., 2003; Ngwa et al., 2011; Agarwal et al., 2015). Studies have been shown that cadmium induced generation of ROS and activation of apoptosis in associated with cadmium induced neurotoxicity in various animal models. however, its role on other form of cell death mechanism that is autophagy is not known.

Here in, we first time explored the potential involvement of autophagy in cadmium mediated neurotoxicity. With this, we examined the molecular mechanism underlying autophagy in cadmium mediated neurotoxicity. We reported that cadmium activates the autophagic flux in the cells through the mTOR pathway both *in vivo* studies using rat model and *invitro* studies using SHYSY-5Y cells. The conversion of LC3I/II and formation of autophagosomes in the main feature of autophagy. We observed the increase levels of LC3II and Beclin1 following cadmium exposure. Further, decrease levels of p62 also compliment the activation of autophagy following cadmium exposure. We further observed the dose and time dependent increase in the LC3II lipidation and decrease in the p62 following cadmium exposure. Further, we found the increase expression of beclin1 and other Atg like protein (atg5, atg3, atg7 and atg12) both *in vivo* and *in vitro.*

We found the cadmium enhanced the formation of GFP-LC3II puncta in the cells reflecting the activation of autophagy. Studies carried out recently also compliment our changes where increase levels of LC3II following of cadmium exposure. We further, studied the mechanist aspect of autophagy where we tried to establish that whether the increase in autophagy due to cadmium exposure is due to increase in the autophagic flux or due to decrease in the proteolytic enzymes that lead to the decrease the fusion of autophagosome to lysosome that lead to the accumulation of autophgosomes in the cell. The accumulation of autophagosomes in the cells itself contribute to worsening the toxicity due to external stimuli in the cells. Report showed that environmental chemical has tendency to increase the autophagic flux. Results of the present study carried out exhibit that cadmium enhanced the levels of LC3II in presence of autophagy activator rapmaycin and related decrease in the p62 levels. Further, more interestingly in presence of autophagy inhibitor 3MA, the levels of LC3II get down regulated. The finding supports the facts that cadmium induces the autophagy that may be responsible for the cadmium induce neurotoxicity. Further, the cadmium induced ROS generation was found to be decrease in presence of rapmaycin and increases in presence of 3MA supporting the facts that autophagy appears to be a important mechanism in cadmium neurotoxicity. Studies are further in progress to understand whether this activation in autophagy is due

to accumulation of autophagosomes in the cell that lead to activation of excessive autophagy resulting in the breakdown to essential cell organelles including mitochondria that lead to the autophagy mediated cell death or it is due to decrease in the lysosomal activity that lead to diminish the activity of proteolytic enzyme. It has been found that cadmium induced neurotoxicity has been found to be modulated by several natural agents including flavonoids and others. In our studies we have found that protective potential of quercetin in cadmium induced cholinergic and dopaminergic modulations. Study carried out exhibits that quercetin have ability to combat the autophagy in chemical induced neurotoxicity (Abdalla et al., 2014; Chander et al., 2014). We also found that quercetin has down regulated the cadmium induced increase in the LC3II levels and other autophagy like proteins both *in vivo* and *in vitro*, shows that that quercetin have potential to combat the neurotoxicity of cadmium by alerting the autophagy process.

The results of the present study demonstrate that mitochondrial dysfunctions associated with enhanced oxidative stress and apoptosis significantly contribute in cadmium induced brain cholinergic and dopaminergic dysfunctions. The results also provide evidence that motor deficits on cadmium exposure are associated with alteration in DA-D2 receptors involving both canonical cAMP dependent PKA/DARRP32/PP1α and non-canonical βarrestin/AKT/GSK3β signaling in corpus striatum. Further, it is also evident that quercetin has the potential to protect cadmium induced brain cholinergic and dopaminergic dysfunctions. Interestingly, quercetin has also been found to ameliorate cadmium induced inflammation and autophagy by modulating the expression of targets proteins associated in the process. Although the protective efficacy of quercetin appears to be attributed to its antioxidant and metal chelating capacity, the data has relevance to implore its protective potential further in neurological and neurotoxicological disorders (Figure – 5.1, 5.2, 5.3).

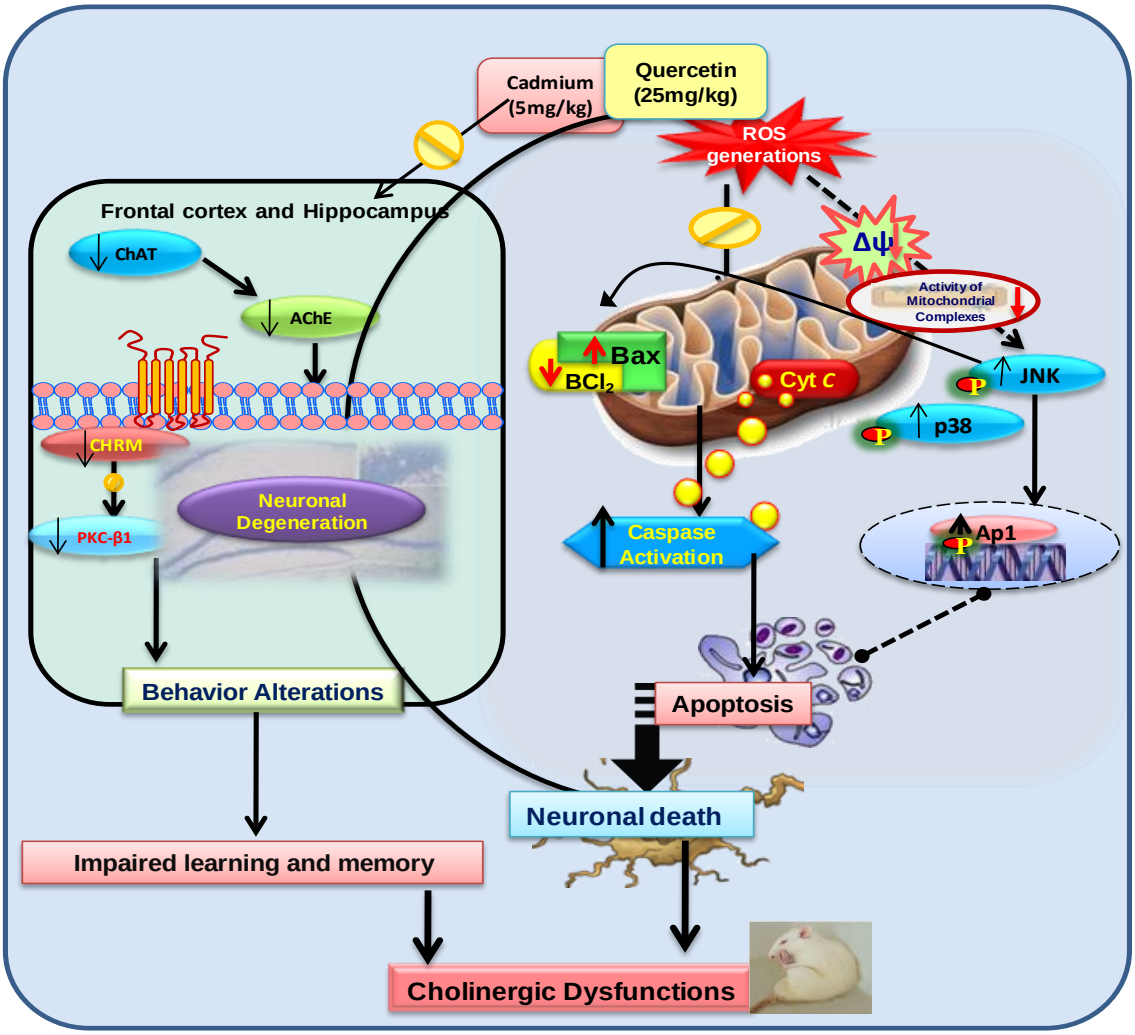


Figure – 5.1. Mechanism and targets associated with cadmium induced cholinergic alterations and protective potential of quercetin

Exposure to cadmium resulted to decrease the expression of ChAT and AChE and affect the integrity of cholinergic-muscarinic receptors associated with decrease in the expression of PKC-β1, a post-synaptic signaling protein. Brain cholinergic alterations on cadmium exposure may be linked to learning and memory deficits in rats. Cadmium exposure also caused mitochondrial dysfunctions associated with enhanced ROS generation and apoptosis involving activation of caspase cascade and MAPKinases. Disruption in the ultrastructures and loss of neurons both in frontal cortex and hippocampus were evident on cadmium exposure. Simultaneous treatment with quercetin in cadmium exposed rats reduced ROS generation and protected mitochondrial integrity by modulating proteins involved in apoptosis and MAPkinase signaling and protected cholinergic integrity both in frontal cortex and hippocampus.

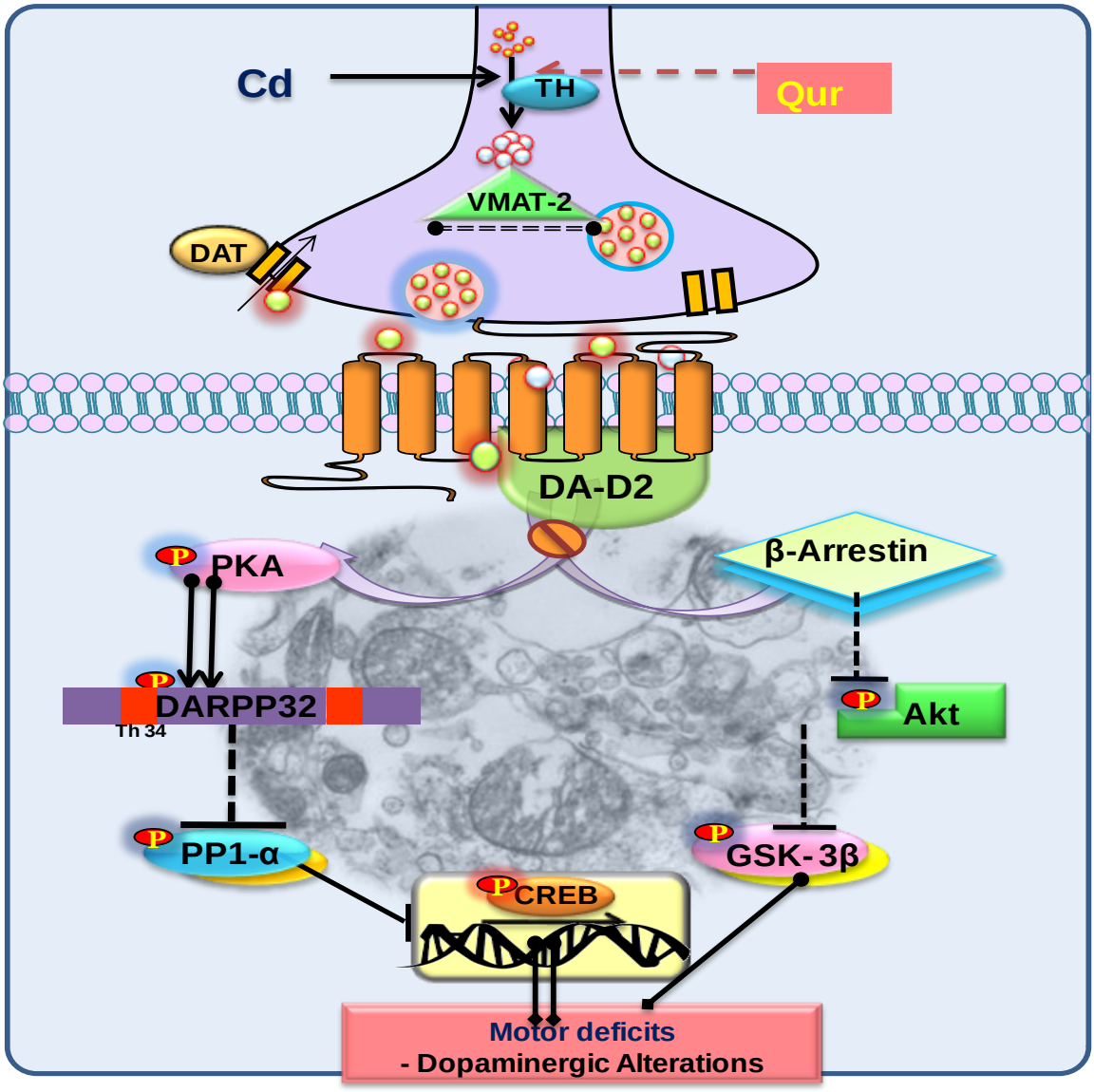


Figure –5.2. Mechanism and target associated with cadmium induced dopaminergic alterations and protective potential of quercetin in amolerating such changes.

Cadmium exposure causes alteration in dopaminergic signaling. A decrease in the Expression of TH, DAT and D2 receptor mediated PKA/DARPP32/PP1-α was observed. Further, β- Arrestin 2/ Akt/ GSK-3 β which is mediated by D2 receptor was also affected by cadmium which together alters behavioral response especially motor dysfunction and motor co-ordiantaion. Further, simultaneous exposure with quercetin in cadmium exposed rats protected such changes.

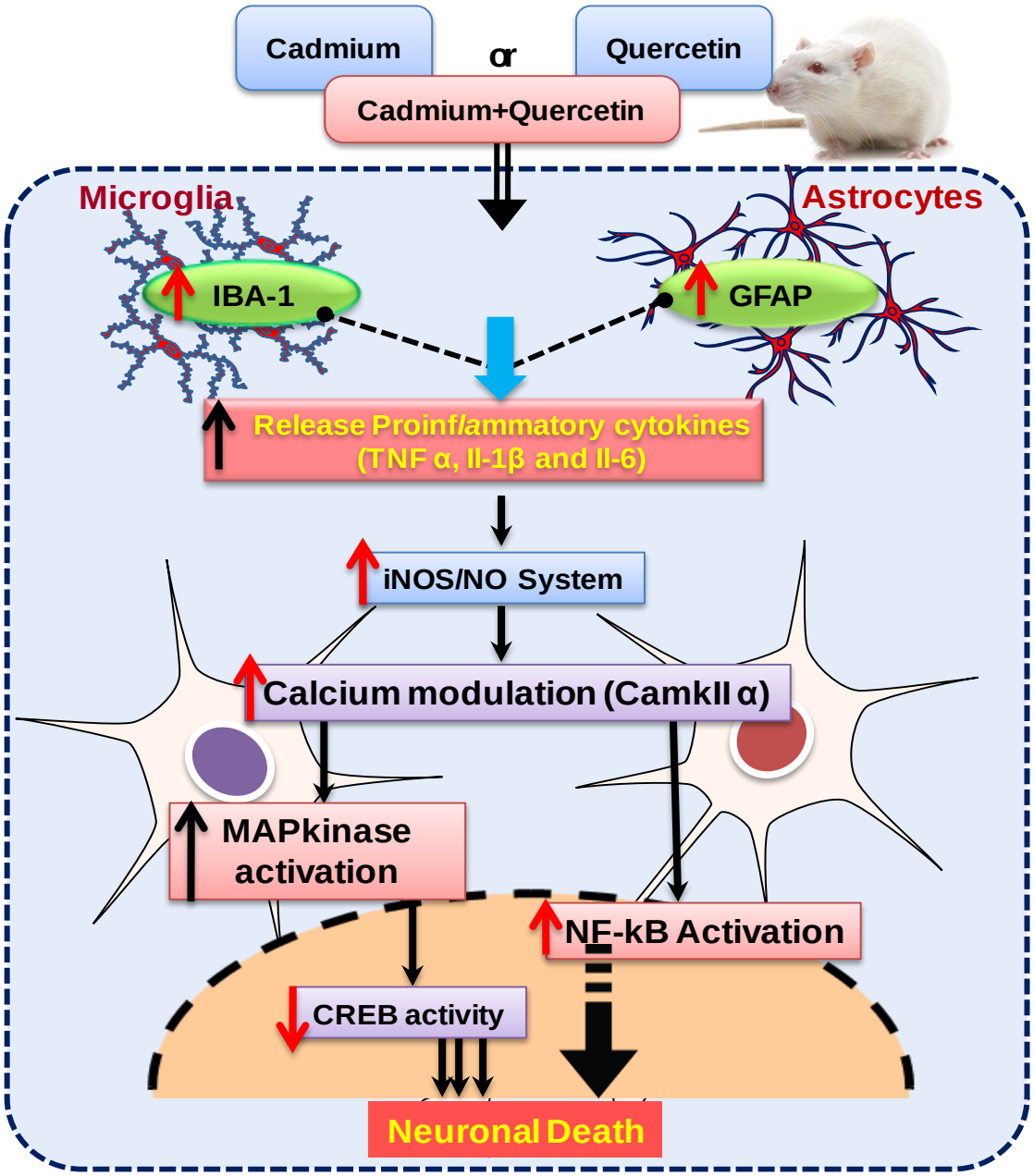


Figure –5.3. Mechanism and target associated with cadmium induced Neuroinflammation alterations and protective potential of quercetin in ameliorating such changes.

Cadmium exposure causes alteration in proinflammatory cytokines levels. A increase in the Expression of GFAP and IBA1 was observed in cadmium exposed rats. Further, iNOS/NO system get also activated following cadmium exposure. Further, simultaneous exposure with quercetin in cadmium exposed rats protected such changes.

Chapter VI

Summary & Conclusion

Cadmium, a heavy metal with wide occurrence in nature has extensive industrial and anthropogenic uses because of its non-corrosive nature and thus enhances the risk of human exposure. Presence of high cadmium levels in cigarette smoke and drinking water are potential sources of non-occupational exposure. Exposure to cadmium could also occur through ambient air in urban areas close to industrial settings. Due to high rate of transfer from soil to plants, cadmium has been found present in vegetables, fruits and cereals. General population therefore is exposed to cadmium through food chain while consuming contaminated dietary products. Due to poor elimination, the biological half life of cadmium is high. Therefore, cadmium is cumulative in nature and distributed in body organs.

Although cadmium has been found to be carcinogenic and ranked as Group IA carcinogen, functioning of lungs, liver and kidneys has been found to be affected in exposed individuals. There are convincing evidences exhibiting that cadmium readily crosses the blood brain barrier and reaches the brain and affects the functioning of nervous system. The severity of changes however is dependent on dose and duration of

exposure. Risk of Alzheimer's disease has been associated with cadmium exposure as high cadmium levels in plasma, brain and liver were detected in Alzheimer's patients. Acute exposure to cadmium has also been found to cause Parkinsonism. Increasing incidences of neurological and psychiatric disturbances associated with cognitive deficits on cadmium exposure in recent years is a cause of concern and reflect the vulnerability of brain. Role of enhanced oxidative stress and apoptosis has been suggested in the etiology of cadmium neurotoxicity. A number of studies have also suggested involvement of metallothionein in cadmium neurotoxicity. While cadmium has been found to affect the synaptic signaling by altering the levels of biogenic amines, the molecular targets involved in cadmium induced brain cholinergic and dopaminergic alterations which may be associated with functional deficits are not known. Further, increasing risk of cadmium induced neurotoxicity has aroused a concern if it could be protected.

Quercetin, a polyphenolic natural flavonoid has natural occurrence in vegetables and fruits. High levels of quercetin are present in onion, apple, berries and wine. Free radical scavenging and antioxidant activity of quercetin are well documented. Besides, quercetin has been found to have strong metal chelating effect. Because of these properties, quercetin has been found to have broad pharmacological spectrum exhibiting anti-inflammatory, antidiabetic, cardioprotective, anticancer and antiulcer activities. Protective effect of quercetin has also been reported in a number of neurodegenerative diseases. Studies carried out recently suggest that quercetin supplementation is effective in 3-nitropropionic acid induced Huntington's disease. Quercetin has also been found effective to protect neurobehavioral alterations in experimental models of Parkinsonism. Neurotoxicity associated with bis phenol and arsenic has also been found to be ameliorated by treatment with quercetin. Interestingly, promising effects of quercetin to modulate physiological functions in preclinical studies have strengthened its use in clinical situations in the management and treatment of many diseases including the neurodegenerative disorders. However, the mechanism behind largely focused on oxidative stress and relating signaling.

In view of this, the present study has been carried out to understand the cellular and molecular mechanism involved in cadmium induced neurobehavioral alteration and to

assess the potential of quercetin in combating such changes with the focus onto

I. unravel the molecular mechanisms associated with cadmium neurotoxicity focusing at the expression of the selected proteins involved in the process of synaptic transmission, oxidative stress and neuronal signaling and asses protective potential of quercetin, a flavonoid in rat brain
II. screen the prophylactic, protective and therapeutic effect of quercetin in cadmium induced neurotoxicity in PC12 cells
III. elucidate the bimolecular targets involving docking studies

Module 1 - Effect on Brain Cholinergic Modulations Following Exposure of Rats to Cadmium, Quercetin and their Co-Exposure for 28 Days

Exposure of rats to cadmium (5 mg/kg body weight, p.o.) for 28 days decreased the transfer latency time (TLT) in the retention trials as compared to the acquisition trial on monitoring the passive avoidance response by shuttle box. Further, significant impairment in alternation was observed in rats exposed to cadmium as compared to controls as assessed by Y-maze. Novelty seeking behavior on cadmium exposure was also found to be altered as % entries and % time spent in the novel arm and other arm was found to be similar in these rats exhibiting impairment in learning and memory.

A decrease in binding of cholinergic-muscarinic receptors and mRNA expression of cholinergic-receptor genes (M1, M2, M4) was evident in frontal cortex and hippocampus on exposure of rats to cadmium (5.0 mg/kg body weight, p.o.) for 28 days in comparison to controls. Cadmium exposure decreased the mRNA and protein expression of ChAT and AChE as compared to controls. Further, enhanced generation of ROS both in frontal cortex and hippocampus of cadmium exposed rats was distinct and associated with mitochondrial dysfunctions as evident by decrease in the activity of complex I, II-III and IV as compared to controls. Exposure to cadmium caused decrease in mitochondrial membrane potential both in frontal cortex and hippocampus. Enhanced apoptosis as evident by alterations in key proteins involved in pro- and anti-apoptotic pathway (Bax, Bcl2 and caspase-3) and MAPkinase signaling was evident on cadmium exposure. Cadmium exposure in rats resulted to disrupt the ultrastructures both in frontal cortex and

hippocampus. There was a marked deterioration in hippocampus and frontal cortex as visualized by the loss in cell organelles, vacuole formation in cytoplasm, mitochondrial damage, and swollen mitochondria with loss of cristae along with disruption in mitochondrial membrane in unmyelinated axons on cadmium exposure. Further, loss of myelin sheath was also clearly visible both in frontal cortex and in hippocampus on cadmium exposure as compared to control rats. Consistent with this, degeneration of neurons in frontal cortex and hippocampus in cadmium exposed rats was also visible as assessed by histological studies. Loss of synapse or the loss of neuron in cholinergic rich area is the major event of any cholinergic dysfunction and linked with functional changes.

Simultaneous treatment with quercetin (25 mg/kg body weight, p.o.) was found to protect cadmium induced alterations in cholinergic-muscarinic receptors, mRNA expression of genes (M1, M2 and M4) and expression of ChAT and AChE. The protective effect on brain cholinergic targets was attributed to antioxidant potential of quercetin which reduced ROS generation and protected mitochondrial integrity by modulating proteins involved in apoptosis and MAPkinase signaling.

The results exhibit that mitochondrial dysfunctions associated with enhanced oxidative stress and apoptosis significantly contribute in cadmium induced brain cholinergic dysfunctions. More interestingly, the data provide evidence that quercetin has the ability to modulate the molecular targets involved in brain cholinergic signaling and attenuate cadmium induced neurotoxicity.

Module II - Effect on Brain Dopaminergic Modulations Following Exposure to Cadmium, Quercetin and their Co-Exposure

While investigating the effect on dopaminergic system and associated signaling, exposure of rats to cadmium (5 mg/kg body weight, p.o.) for 28 days was found to cause selective decrease in mRNA and protein expression of DA-D2 receptors in the corpus striatum as compared to controls. Consistent with this, decrease in the binding of DA-D2 receptor, assessed by radioligand receptor binding assay, was also observed in cadmium exposed rats. Further, decrease in the binding of DA-D2 receptor was due to alteration in number

of receptor binding sites (Bmax) and no change in the binding affinity (kd) as revealed by Scatchard analysis. However, there was no change in the mRNA and protein expression of DA-D1 receptor in corpus striatum of cadmium exposed rats. Decrease in the expression of TH, a marker of dopaminergic neurons and DAT, a protein associated with dopamine transport in presynaptic dopaminergic neurons was clearly evident on cadmium exposure suggesting that cadmium may impair the expression of proteins associated with predopaminergic signaling. There was on change in the expression of VMAT2 as compared to controls.

Exposure to cadmium in rats resulted to decrease the expression of PKA, DARPP32 and CREB in corpus striatum as compared to controls. A significant increase in the expression of PP1α, a negative regulator of dopaminergic functions was observed in corpus striatum on cadmium exposure. Interestingly, cadmium induced decrease in DA-D2 receptors affected the post-synaptic PKA/DARPP32/PP1α and β-arrestin/Akt/Gsk-3β signaling suggesting that both canonical and non-canonical pathways are involved in cadmium induced dopaminergic dysfunctions.

Degeneration of neurons in corpus striatum as assessed by crysel violet staining in cadmium exposed rats was assessed on histological examination. Ultrastructural studies involving TEM have been found to complement the changes exhibiting synaptic loss in the corpus striatum of cadmium exposed rats. Decrease in total distance travelled, time moving, rearing and stereotypic counts were clearly evident in cadmium exposed rats as compared to controls. Time resting was however found to be increased in cadmium exposed rats. Further, exposure to cadmium impaired motor co-ordination assessed by Rotamex and forelimb grip strength monitored by grip strength meter as compared to controls. Simultaneous treatment of rats with quercetin (25 mg/kg body weight, p.o.) for 28 days was found to protect cadmium induced dopaminergic alterations in corpus striatum. Further, impairment in motor activity, motor coordination and grip strength in cadmium exposed rats were also found to be protected on simultaneous treatment with quercetin.

In vitro studies carried out using differentiated PC12 cells exhibited that treatment with cadmium (10 µM) for 24 hr resulted to decrease the expression of TH and DAT while

there was no significant change in the expression of VMAT2 as compared to the unexposed cells. Consistent with *in vivo* studies, treatment with cadmium caused a decrease in the expression of DA-D2 receptors while there was no change in the expression of DA-D1 receptors. Decrease in the expression of PKA, DARPP32 and CREB on cadmium treatment suggest that cadmium may affect the DA-D2 receptor mediated down streaming signaling. It was further interesting that expression of PP1α, a negative regulator of DARPP32 was increased on exposure to cadmium in PC12 cells. Cadmium treatment resulted to increase the phosphorylation of Akt associated with decreased expression of GSK-3β in PC12 cells. Pharmacological inhibition of PKA and Akt using specific pharmacological inhibitors exhibit that DA-D2 receptor mediated PKA/DARPP32/PP1α and β-arrestin/AKT/GSk3β signaling independently regulates the motor behavior. Simultaneous exposure with quercetin (100 µm) resulted to protect cadmium induced changes in the expression of these proteins associated with pre-and post dopaminergic signaling.

Molecular docking studies provided interesting evidence that decrease in DA-D2 receptors could be due to direct binding of cadmium at the competitive or non-competitive sites of dopamine on DA-D2 receptors. DFT studies suggest that quercetin has the tendency to form complex with cadmium and may be attributed to the metal chelating property of quercetin and thus reduced toxicity of cadmium. Interestingly, the data of *in silico* studies has been found to compliment the *in vivo* and *in vitro* findings.

The results provide interesting evidence that motor deficits on cadmium exposure are associated with alteration in DA-D2 receptors involving both canonical cAMP dependent PKA/DARRP32/PP1α and non-canonical β-arrestin/AKT/GSK3β signaling in corpus striatum. The results also exhibit that quercetin has the potential to protect cadmium induced dopaminergic dysfunctions.

Module III- Assessment of Biogenic Amines and Their Metabolites in Brain Regions

Involvement of biogenic amines considered to be important regulator of synaptic transmission and behavior. Metallothionein3, a cysteine protein linked with the toxicity of cadmium was assessed in hippocampus, frontal cortex and corpus striatum of rats on

cadmium exposure. Protective effect of quercetin in cadmium induced alterations in metallothionein and biogenic amines were also assessed.

Decrease in the levels of dopamine and its metabolites - DOPAC and HVA in frontal cortex, hippocampus and corpus striatum was evident on exposure of rats to cadmium (5 mg/kg body weight, p.o.) for 28 days as compared to controls. The dopamine turnover was found increased in cadmium exposed rats. Cadmium exposure caused a decrease in the levels of NE and 5HTin frontal cortex, hippocampus and corpus striatum while an increase in the levels of EPN as compared to controls. Treatment with quercetin (25 mg/kg body weight, p.o.) for 28 days alone had no significant change in the levels of biogenic amines or their metabolites in any of the brain region as compared to controls. Interestingly, simultaneous treatment with quercetin (25 mg/kg body weight, p.o.) for 28 days was found to protect cadmium induced alteration in the levels of neurotransmitter and their metabolites in the brain regions.

Exposure to cadmium (5 mg/kg body weight, p.o.) for 28 days increased the levels of metallothionein-3, a cysteine containing protein in frontal cortex, hippocampus and corpus striatum as compared to controls. Simultaneous exposure with quercetin (25 mg/kg body weight, p.o.) for 28 days in cadmium treated rats caused a decrease in the levels of metallothionein-3 in frontal cortex, corpus striatum and hippocampus in comparison to rats exposed to cadmium alone. No significant change in the expression of metallothionein-3 was observed in any of the brain regions of rats on treatment with quercetin alone as compared to controls.

High levels of cadmium were detected in all the three brain regions - frontal cortex, hippocampus and corpus striatum of rats on exposure to cadmium (5 mg/kg body weight, p.o.) for 28 days as compared to controls. Computational ADMET profiling of cadmium exhibits that cadmium easily crosses the blood brain barrier and this could be one of the possible reasons for enhanced cadmium levels in brain. Simultaneous exposure with quercetin (25 mg/kg body weight, p.o.) for 28 days in cadmium exposed rats was found

to reduce the levels of cadmium in all the brain regions - frontal cortex, hippocampus and corpus striatum in comparison to rats treated with cadmium alone.

Taken together, the results exhibit that quercetin has the potential to modulate cadmium induced changes in biogenic amines and metallothionein. The protective changes appear to be attributed to low cadmium levels in the brain and may be associate with antioxidant potential and chelating property of quercetin.

Module IV – Effect on the Neuroinflammation and Associated Signaling Following Exposure to Cadmium, Quercetin and their Co-Exposure

In view of convincing evidences demonstrating involvement of inflammatory processes in neurodegenerative disorders and chemical induced neurotoxicity, effect of cadmium was assessed on the levels of cytokines and associated signaling. Increased expression of IBA1 and GFAP in hippocampus, frontal cortex and corpus striatum was evident on exposure of rats to cadmium exhibiting activation of glial cells. Further, activation of pro-inflammatory cytokines (TNFα, IL1β and IL-6) associated decrease in the anti-inflammatory cytokines (IL-10) was found in frontal cortex, hippocampus and corpus striatum as compared to controls. A significant increase in the levels of NO and expression of both iNOS and nNOS was evident in all the brain regions - frontal cortex, hippocampus and corpus striatum regions was also evident on cadmium exposure. Increase level of STAT3 in the brain regions was also evident in cadmium exposed rats. Consequently, activation in the expression of MAPK and CamkIIα as observed on cadmium exposure may cause neuronal death. Interestingly, simultaneous treatment with quercetin was found to ameliorate cadmium induced changes both in pro-and anti-inflammatory cytokines.

These results also exhibit that cadmium induced inflammatory response are governed by glial mediated neuronal death and quercetin has the potential to protect these changes.

Module V – Effect on the Autophagy Process Following Exposure to Cadmium, Quercetin and their Co-Exposure

As autophagy is an important process for maintaining the cellular homeostasis and related metabolism, effect of cadmium on the expression of key proteins involved in the autophagic process was assessed in corpus striatum. Treatment of rats with cadmium (5 mg/kg body weight, p.o.) for 28 days increased basal levels of LC3II and decreased that of p62 as compared to controls. Increase in the protein expression of autophagy related genes - Atg5, 7 12, 3 and beclin1 was also evident in the corpus striatum of cadmium exposed rats. Expression of Lamp2a, a specific marker of lysosome was also found increased in the corpus striatum of rats exposed to cadmium. To further understand the mechanism involved in cadmium induced increase in autophagic flux, *in vitro* studies were carried out involving SHYSY-5Y cells. Treatment with cadmium increased the levels of LC3II and Beclin1 and decreased the expression of p62 in SHYSY-5Y cells cells in a dose and time dependent manner. Cadmium treatment in SHYSY-5Y cells also altered the Atg like proteins. Increase in GFP-LC3II puncta was evident in SHYSY-5Y cells on cadmium treatment. These observations exhibit that cadmium increases the autophagic flux in the cells and thus impart in its toxicity. Pre-exposed cells to rapmaycin, a known autophagy activator further enhanced cadmium induced increase in LC3II lipidation and decreased the p62 levels. Moreover, enhanced ROS generation in the cells following cadmium treatment was also found to be modulated by autophagy activator and inhibitor. Simultaneous treatment with quercetin was found to protect cadmium induced changes in autophagy both *in vivo* and *in vitro.*

The data indicate involvement of autophagy in cadmium induced neurotoxicity and further exhibit the protective potential of quercetin in ameliorating these changes.

Module VI - Assessment of Protective, Prophylactic and Therapeutic Approaches of Quercetin Vs Nanoquercetin

As nano formulations help in sustained drug delivery for a long period of time to maintain the drug concentration in therapeutic window, PLGA coated nanoparticles of quercetin were formed. The particles size of nano-quercetin was found to be 115nm

determined by Dynamic Light Scattering (DLS). Characterization of the nanoparticles was done by Transmission Electron Microsocpy and Scanning Electron Microscopy studies to ascertain that nanoparticles of quercetin were smooth, regular and round.

In vitro studies using differentiated PC12 cells revealed that nano-quercetin was able to maintain the cell viability till 72 hrs after treatment. The MTT assay was carried out to assess the viability of cells after treatment with both quercetin and nano-quercetin. Pre, post and co treatment with bulk and nano quercetin increased the viability of PC12 cells treated with cadmium. Interestingly, viability of cells in cadmium treated cells was more on treatment with nano-quercetin as compared to those treated with bulk quercetin. Further, coexposure with bulk quercetin and nano-quercetin showed high viability in comparison to pre and post treated groups.

Exposure to cadmium at a dose of 10µM decreased the protein expression of TH and DA-D2 receptors as compared to unexposed cells. Cadmium exposure in cells also decreased the phosphorylation of DARPP32 and CREB while increased the expression of PP1α, a negative regulator. Interestingly, exposure to bulk quercetin at a dose of 100µM was found to up regulate the expression of TH, DA-D2, DARPP32 and CREB associated with decrease in the expression of PP1α as compared to cadmium exposed cells. Nano - quercetin was found to be more effective to upregulate changes as compared to bulk quercetin and intensity was quite high in co and post exposed in comparison to group pre exposed with quercetin.

Treatment of cells with nano-quercetin also altered the levels of apoptotic proteins in cadmium induced toxicity. Treatment of cells with cadmium for 24 hr activated the apoptotic pathway as evident by increase in the expression of Bax and Caspase-3 and decrease in the expression of Bcl2. Further, treatment with bulk quercetin and nano - quercetin were found effective to alter cadmium induced changes. However, the intensity of changes was found to be more with nano-quercetin as compared to the bulk quercetin.

The results of the present study suggest the potential role nano-quercetin as compared to bulk quercetin since it retains more in the cells. It may possibly be due to its slow and continuous release of quercetin from nanoparticles. Further, co-exposure with bulk and

nano-quercetin was found to be more effective as compared to pre and post exposure. These results appear to be interesting and provide an interesting lead to use nano-quercetin after further validation.

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Paper Published

List of Publications

1. Richa Gupta, Rajendra K. Shukla, Lalit P. Chandravanshi, Pranay Srivastava, Yogesh K. Dhuriya, Jai Shanker, Manjul P. Singh, Aditya B. Pant, Vinay K. Khanna. Protective Role of Quercetin in Cadmium Induced Cholinergic Dysfunctions in Rat brain by Modulating Mitochondrial Integrity and MAPkinases Signalling. Mol Neurobiol 2016; 10.1007/s12035-016-9950-y.

2. Richa Gupta, Rajendra K. Shukla, Ankita Pandey, Tanuj Sharma, Yogesh Dhuriya, Pranay Srivastava, Manjul P. Singh, Mohammad Imran Siddiqi, AB Pant and Vinay K. Khanna. Involvement of PKA/DARPP32/PP1α and β-arrestin/Akt/Gsk-3β signaling in Cadmium Induced DA-D2 Receptor Mediated Motor dysfunctions: Protective Role of Quercetin. Scientific Reports (submitted after revision).

3. Rajendra K Shukla, Richa Gupta, Pranay Srivastava, Yogesh Dhuriya, Anshuman Singh, Lalit P Chandravanshi, Ajay Kumar, M. Haris Siddiqui, Devendra Parmar, Aditya B Pant, and Vinay K Khanna. Brain cholinergic alterations in rats subjected to repeated immobilization or forced swim stress on lambda-cyhalothrin exposure. Neurochemistry International 2016; 93: 51 – 36.

4. Pranay Srivastava, Yogesh Dhuriya, Richa Gupta, Rajendra K Shukla, Rajesh Singh Yadav, Hari Nath Dwivedi, Aditya B Pant and Vinay K Khanna. Protective Effect of Curcumin by Modulating BDNF/DARPP32/CREB in Arsenic-Induced Alterations in Dopaminergic Signaling in Rat Corpus Striatum. Mol Neurobiol. 2016; DOI 10.1007/s12035-016-0288-2.

5. Rajendra K Shukla, Yogesh Dhuriya, Lalit P Chandravanshi, Richa Gupta, Pranay Srivastava, Aditya B Pant, Ajay Kumar, M. Haris Siddiqui and Vinay K Khanna. Influence of immobilization and forced swim stress on the neurotoxicity of lambda-cyhalothrin in rats: Effect on brain biogenic amines and BBB permeability. Neurotoxicology 2016. doi: 10.1016/j.neuro.2016.07.002.

6. Rajendra K Shukla, Richa Gupta, Ajay Kumar, M. Haris Siddiqui. Influence of immobilization and forced swim stress on the neurotoxicity of lambda-cyhalothrin in rats: Effect on brain biogenic amines and BBB permeability. 2015 Toxicology International (in Press).

Paper Presented at Conference

Paper Presented at Conferences

1. **Richa Gupta**, Rajesh S. Yadav, Lalit P. Chandravanshi, and Vinay K. Khanna. Neuroprotective efficacy of Bacopa monnieri in monocrotophos induced neurotoxicity in rats
   Paper presented at International First BBDU Scholar conclave 2013 was held at Babu Banarasi Das University, Lucknow, India

2. **Richa Gupta,** Rajesh S. Yadav, Lalit P. Chandravanshi, and Vinay K. Khanna. Brain Cholinergic and Dopaminergic Alterations Following Monocrotophos Exposure Associated with Enhanced Oxidative Stress in Rats: Protective Efficacy of *Bacopa Monnieri*
   Paper presented at International Symposium on Emerging Trends and Challenges in Neuroscience & XXXI Annual Conference of Indian Academy of Neurosciences, was held at Vigyan Parishad & National Academy of Sciences, Allahabad, India from October 25th - 27th, 2013

3. **Richa Gupta**, Rajendra K. Shukla, Lalit P. Chandravanshi, Pranay Srivastava, Yogesh K. Dhuriya, Aditya B. Pant and Vinay K. Khanna. Neuroprotective potential of quercetin in cadmium induced cholinergic deficits in rats.
   Paper presented at the International Symposium on Translational Neuroscience & XXXII Annual Conference of Indian Academy of Neurosciences from November 01- 03, 2014 at Department of Neurophysiology, National Institute of Mental Health and Neuro Sciences, Bengaluru, India.

4. **Richa Gupta**, Rajendra K. Shukla, Lalit P. Chandravanshi, Pranay Srivastava, Yogesh K. Dhuriya, Aditya B. Pant and Vinay K. Khanna. Mitochondrial Dysfunctions in Cadmium Induced Brain Cholinergic Potential of Quercetin in Rats.Deficits and Protective
   Paper presented at the 47th Annual Conference of the Indian Pharmacological Society "IPSCON- Guwahati 2014" from 28th–30th December 2014at GMCH Convention Centre, Guwahati-32, Guwahati India.

5. **Richa Gupta**, Rajendra K. Shukla, Pranay Srivastava, Yogesh K. Dhuriya, Aditya B. Pant and Vinay K. Khanna. Mitochondrial Dysfunction in Cadmium induced Brain Dopaminergic Alteration and Protective Potential of Quercetin in rats
   Paper presented at the IBRO/APRC Chandigarh School from 26th–30th October 2016 at Punjab University, Chandigarh, India.

6. **Richa Gupta**, Rajendra K. Shukla, Pranay Srivastava, Yogesh K. Dhuriya, Aditya B. Pant and Vinay K. Khanna. Mitochondrial Dysfunction in Cadmium induced Brain Dopaminergic Alteration and Protective Potential of Quercetin in rats
   Paper presented at the XXXIII Annual Conference of the Indian Academy of neurosciencs from 31st October–2nd November 2015at Punjab University, Chandigarh India.

7. **Richa Gupta**, Rajendra K. Shukla, Lalit P. Chandrvanshi, Pranay Srivastava, Yogesh K. Dhuriya, Aditya B. Pant and Vinay K. Khanna. Deciphering the mechanism of cadmium induced brain dopaminergic dysfunctions and assess the protective potential of quercetin in rats.
   Paper presented at the IAPRD 2015 from 05th December– 09t^{h} November 2015 at Micomilano Congressi, Milan, Italy.

8. **Richa Gupta,** Rajendra K. Shukla, Pranay Srivastava, Yogesh K. Dhuriya, Rajeev Gupta, Aditya B. Pant and Vinay K. Khanna. Role of β - arrestin 2/Akt/GSK 3β Survival Pathway in Cadmium Induced Dopamine D2 receptor Mediated Function: Protective Efficacy of Quercetin
   Paper presented at the World Parkinsons Congress at Portland, Oregon, USA from September 20 – 23, 2016

9. **Richa Gupta**, Rajendra K. Shukla, Pranay Srivastava, Yogesh K. Dhuriya, Manjul P. Singh, Rajeev Gupta, Aditya B. Pant and Vinay K. Khanna. Role of iNOS/NO/ CamKII-α in cadmium mediated Neuroinflammation: Neuroprotective Potential of Quercetin,
   Paper presented at the XXXIIII Annual Conference of Indian Academy of Neurosciences being held at NBRC, Manesar from October 19 - 21, 2016

Honours & Awards

Honours and Awards

Research Fellowship (CSIR- GATE Fellowship) - Awarded by Council of Scientific & Industrial Research, New Delhi.

International Travel Fellowship - Awarded by International association of Parkinson and related disorders to present the research paper in XXI World Congress on Parkinson's disease and related Disorders, Milan, Italy from December 05-09, 2015.

IBRO-APRC Travel Grant - Awarded by IBRO to attend the IBRO/APRC Chandigarh School, Chandigarh, India from October 26- 30, 2015.

Academic Excellence Award - Awarded by Uttarakhand Technical University, Dehradun for academic excellence and securing Top Rank in M. Pharm Pharmacology in university in 2012.

Academic Excellence Award - Awarded by Vice Chancellor, Uttar Pradesh Technical University, Lucknow for academic excellence and securing first position in university in 2010 (B. Pharm with aggregate of 80%).

World Parkinson's Congress Travel Grant - Awarded by World Parkinson's Congress to present the research paper in 4th WPC2016, Portland, Oregon from September 20-24, 2016

Best Poster Presentation – Awarded by Indian Academy of Neurosciences to presented the research paper during the XXXIV Annual Conference of Indian Academy of Neurosciences being held at NBRC, Manesar from October 19 - 21, 2016.